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Direct extraction of chitosan from snail shells by natural deep eutectic solvent

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A B S T R A C T

The processes involved to extract chitosan biopolymers from natural resources often employ hazardous chemicals and long processing time. This work provides a sustainable direct extraction method of chitosan from snail shells. Previous attempts using acetogenin in graviola extracts succeeded in the extraction of chitosan from marine shells. However, the slow reaction has prompted the addition of hydrogen bond acceptor solution into the graviola extract. Choline chloride is an excellent hydrogen bond acceptor mixed with acetogenin as hydrogen bond donor to form natural deep eutectic solvent (NADES) for the direct extraction of chitosan. Chitosan obtained from this method has a degree of deacetylation of 91% and a molecular weight of 481 kDa with fiber-like morphology. The direct extraction of chitosan from NADES consisting of choline chloride and acetogenin has proven to extract chitosan with comparable properties to commercial chitosan.

1. Introduction

Cerithidea obtuse is a snail used as a food source which the shells are later discarded. Based on Conus inscriptus sea snail shell, 21.65% of its dry weight basis consisted of chitin[1] which is comparable to other chitin marine sources.[2] In addition to possessing high chitin content, the use of sea snail shells provides a way of utilizing a major source of waste in the seafood industry. Chitin is made up of linear chain of acetyl glucosamine groups. Chitin's derivative, chitosan is obtained by removing enough acetyl groups, leaving behind amino group in addition to hydroxyl group which are both active sites in many chemical reactions.[3] Due to the compact structure of solid state of chitin, it remains insoluble in most solvents and dilute acids. This then usually leads to carrying out a chemical deacetylation of chitin to solve the problem of insolubility and produce chitosan which is the most common derivative of chitin.[5]

Chitosan can be obtained by deproteination, demineralization and deacetylation process using acid and base which causes serious environmental problems.[5] The high susceptibility of chitosan to processing conditions, such as heating can impose stress on its structure and cause polymer degradation. Natural deep eutectic solvent (NADES) consists of hydrogen bonding formation between natural metabolites capable of acting as hydrogen bond acceptor (HBA) or hydrogen bond donor (HBD)[5] has been successfully used in the extraction of chitin with comparable properties as conventional method from marine sources.[2] The NADES system employed in this study consist of choline chloride, a biodegradable and non-toxic quaternary ammonium chloride salt that can be extracted from biomass as the HBA.[6] The HBD used in this NADES system is acetogenin, a secondary metabolite found in the graviola leaves extract.[7] The minerals in the form of calcium carbonate in chitin are removed by the hydrogen ions released from NADES which causes decrease linkages between protein and chitin. Proteins in chitin is removed by the intermolecular hydrogen bonds formed with NADES, which damaged the intramolecular hydrogen bond formed in protein-chitin fibers.[5] As a result, chitin is dissolved in NADES and separated from the proteins.[8] The hydroxyl and carboxyl groups of proteins acted as HBD to compete for the chloride anion

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electrostatic interactions by inducing hydrogen ions. Thus, the hydrogen bonds between NADES are broken and new bonds are formed between NADES and protein.[8]

Although NADES is capable of extracting chitin, the ability of NADES to deacetylate in the extraction of chitosan in a one pot process has yet been reported. A recent study has found that deacetylation reaction of chitin to chitosan is facilitated by the nucleophilic attack of hydroxyl groups from acetogenin on the carbonyl group of chitin.[7] However, solely using acetogenin as extraction solvent was time consuming. From this preliminary study, the acetogenin as HBD as a direct extracting solvent by forming NADES with HBA to reduce the time taken for direct extraction of chitosan is conducted. The interactions of hydrogen bond and solubility between NADES and biopolymer matrix in the shells strongly affect the efficiency of chitosan extraction. The interactions can be observed in the density, viscosity and bonding interactions.

Subsequently, the structure and physicochemical properties of chitosan resulting from NADES treatment is characterized based on the purity, molecular weight and degree of deacetylation values of extracted chitosan.

2. Results and Discussion

2.1 Physicochemical Properties of NADES

The density of NADES at room temperature was determined to be 1.06 g/mL. The result agrees with other choline chloride-based density at 1.05 g/mL.[9] These densities were reported to be higher compared to the density of distilled water at 0.99 g/mL. This is attributable to the different degrees of hydrogen bonding in NADES. The molecules in NADES stay connected in smaller spaces of their structures, resulting in an increased density.[9]

The viscosity value of NADES prepared is 6.45 mPa.s. The viscosity of distilled water used in the preparation of NADES was found to be 1.2 mPa.s. These results indicate that the NADES system has higher viscosity compared to water as a solvent. The higher viscosity influences the mass transfer, solute solubility, dispersion and stability.[9]

2.2 Physicochemical Properties of Chitosan

Ash is an indication of oxides of metals and minerals component contained in the raw material chitin and chitosan. The volume of ash in the chitosan quantification is a significant predictor for the demineralization process in removing calcium carbonate.[10] The ash content in the extracted chitosan is 39% which is greater than the commercial chitosan at 0.11%. It is possible that the minerals contained in chitosan were not fully removed under mild conditions of NADES.

The moisture content of the extracted chitosan was observed to be 0.4% which is significantly lower compared to commercial chitosan of 9.6%. It was found that the moisture content was also lower compared to chitosan obtained from shrimp which was 9.34%.[10] The difference in moisture content for the extracted chitosan as compared to commercial chitosan is due to the difference in the extraction method which may reduce the moisture content in the extracted chitosan. The study performed by Szymanska and Winnicka (2015) also suggested that the moisture content of chitosan must be low which ranges from 6-10%,[11] so that it has a greater capability to form hydrogen bond. Furthermore, the higher the water content in the chitosan structure, the faster the damage of the polymer via hydrolysis reactions.[12]

The FT-IR spectra for snail shells, commercial chitosan and extracted chitosan are shown in **Fig. 1.** The characteristic bands in the spectrum of extracted chitosan (**Fig. 1(a)**) are in conformity with the pattern exhibited by the commercial chitosan (**Fig. 1(b)**). The characterization of chitosan with FT-IR yielded similar results to those obtained in the studies conducted earlier.[3,13] The FT-IR spectrum of the extracted chitosan exhibited a broad band of multiple peaks between 3400-3000 cm^{-1}, which is attributed to the stretching of –OH overlapping with $–NH_2$ groups and intermolecular and intramolecular hydrogen bonds. This broad band is also observed in commercial chitosan. Uptake between 3000-2800 cm^{-1} shows the presence of alkyl C-H stretching associated with $-CH_2$ groups, which in the figure are shown by peaks of 2918 cm^{-1} and 2851 cm^{-1}. The peaks at 1651 cm^{-1} and 1615 cm^{-1} are related to the absorption band of the carbonyl (C=O) stretching of secondary amide from the residual N-acetyl group and the N-H bending vibrations of the primary amide ($-NH_2$). The absorption band at 1149 cm^{-1} can be attributed to be asymmetric stretching of the C-O-C bridge. In addition, the stretching band of C–O in chitosan spectrum was observed at 1030 cm^{-1}. The presence of peak at 857 cm^{-1} showed that the extracted chitosan has some carbonate groups present despite lower intensity compared to the snail shells. For the snail shells in **Fig. 1(c)**, the main bands of absorption observed at 1455 cm^{-1} and 857 cm^{-1} is assigned to C-O vibrations of carbonate group.[14]

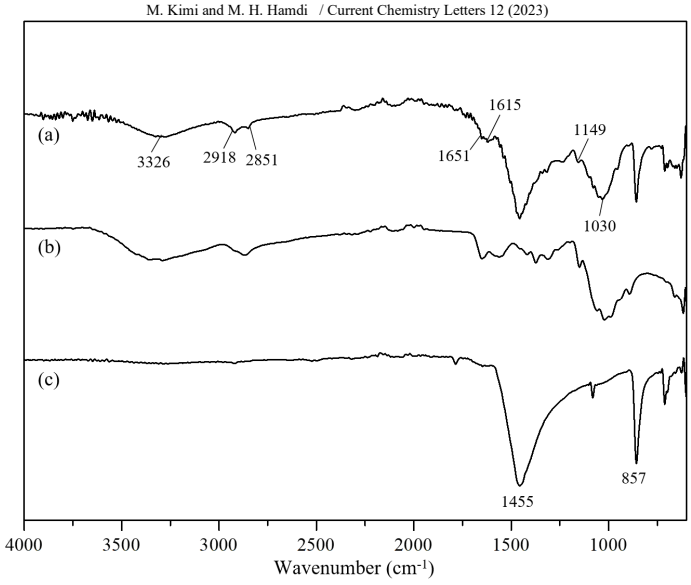

Fig. 1. FT-IR spectra of (a) extracted chitosan, (b) commercial chitosan and (c) snail shells

Degree of deacetylation is an important quality parameter that is mostly used in the characterization of chitosan. This shows the percentage of acetyl groups that were successfully removed during the preparation of chitosan and the substitution of reactive amino groups.[3] Compared to commercial chitosan, extracted chitosan exhibited the higher deacetylation extent at 91%. This value is higher than the commercial chitosan at 76% from shrimp waste. It can be concluded that using the prepared NADES is efficient in deacetylating chitin. DDA is taken into consideration for chitosan as it influences the physicochemical and biological properties of chitosan. The temperature employed during the chitosan extraction using NADES had significant impact on the DDA.

The crystalline structures of chitosan were investigated via XRD in the 2θ range of 5-25° as shown in **Fig. 2**. The extracted chitosan demonstrates the characteristic broad diffraction peaks at 2θ values of about 10.0° and 21.5° that corresponds to crystalline reflections of (020) and (110), respectively.[15] These peaks have low intensity when the chitosan has higher degree of deacetylation. These peaks are typical fingerprints of semi crystalline chitosan. This result was consistent with the α-crystalline structures of chitosan prepared from swimming crab.[15] This result suggested that high deacetylation temperatures interrupted the hydrogen bonds within the structure of chitin, leading to lower crystallinity.

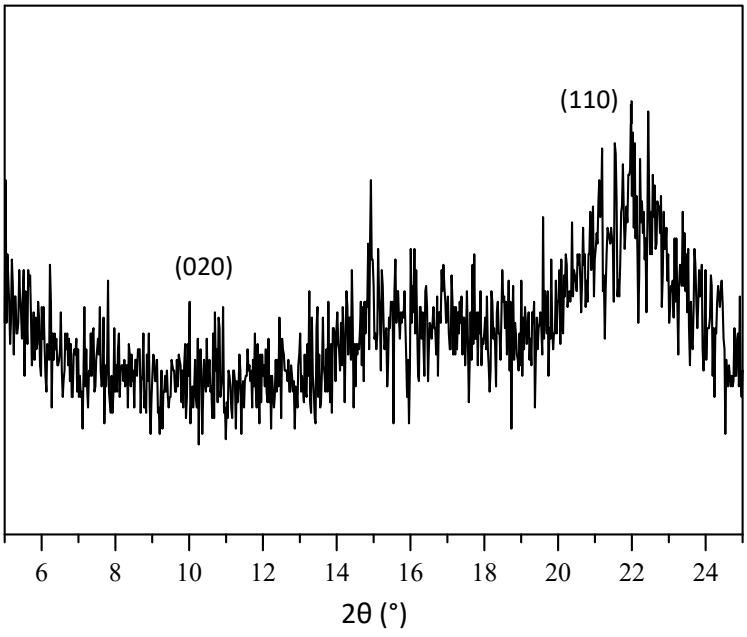

Fig. 2. XRD pattern of extracted chitosan

The SEM micrographs illustrated the morphological features of extracted chitosan and commercial chitosan as depicted in **Fig. 3**. Both the extracted chitosan and commercial chitosan showed that they had similar surface morphologies. They exhibited non-homogeneous, non-porous structure and some randomly oriented grains or fiber-liked shape similar to the observation recorded for chitosan from horn snail.[16] The commercial chitosan gives higher bulk density than that of the extracted chitosan. This might be due to a different source of chitosan used for the commercial sample.

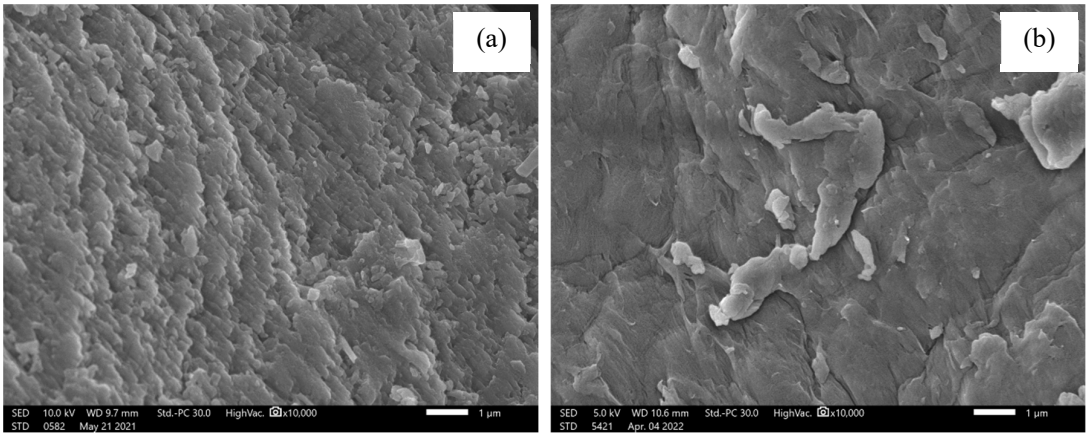

Fig. 3. SEM micrograph of (a) commercial chitosan and (b) extracted chitosan

The molecular weight of chitosan extracted from the snail shells was calculated to be 481 kDa, while the commercial chitosan was found to be 412 kDa. These results are similar to the result obtained from chitosan extracted from crabs in the range of 483 – 526 kDa in previous studies.[17] The molecular weight of chitosan varies with respect to the difference in DDA and the source of chitosan. The extraction solvent used in mild conditions also affected the molecular weight of chitosan.

3. Conclusions

The current approach using newly synthesized NADES component of choline chloride and acetogenin for the extraction of chitosan from snail shell waste in a single step is considered a facile and sustainable process. This study has demonstrated that NADES can be used to isolate chitosan as an alternative method compared with the existing methods replacing the use of strong acid and base. The extracted chitosan showed comparable physicochemical properties as the commercial chitosan. The optimization of processing methods is further required for designing chitosan for desired application.

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4. Experimental

4.1. Materials and Methods

All reagents were purchased from commercial sources and were used without further purification. Fouriertransform infrared spectroscopy (FT-IR) spectra of the samples were recorded on Agilent Cary 630 spectrometer to identify chemical bonding and functional groups. X-ray Diffraction (XRD) patterns of the samples were obtained by D8 Advance (BRUKER AXS, Germany) diffractometer using Cu Kα radiation (λ= 1.54056 Å) from 5° to 25° to investigate the structural properties. Scanning Electron Microscope (SEM) images were taken using JSM-6390 (JEOL, USA) to identify the morphology and estimate the particle size of the samples.

4.2. Synthesis of Natural Deep Eutectic Solvent (NADES)

NADES was prepared according to direct heating method previously reported.[18] Both the choline chloride as hydrogen bond acceptor and acetogenin as hydrogen bond donor was mixed in a mole ratio of 2:1 as this ratio provide appropriate viscosity for extraction. The mixture was heated in a round bottom flask at 80 °C in an oil bath under the magnetic stirring for 2 hours until a homogeneous solution is formed as shown in Fig. 4. Afterwards, the solution was cooled to room temperature and stored in a desiccator to reduce moisture absorption.

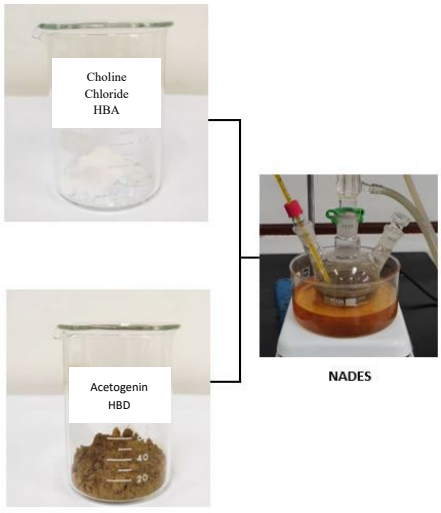

Fig. 4. Synthesis of natural deep eutectic solvent

4.3. NADES-based Extraction of Chitosan from Snail Shell Powder

The sea snail (Cerithidea obtuse) shell was utilized as a precursor for the extraction of chitosan. The shells were collected from Kuching, Sarawak, Malaysia. The shells were washed with distilled water, dried in the oven at 100 °C and ground into fine powder using mixing blender. The extraction of chitosan by NADES was performed as reported for extraction of chitin with modifications.[2] Snail shell powder and NADES solvent in the ratio of 1:10 (w/v) was mixed in a round bottom flask with magnetic stirring at room temperature until supernatant is observed. The supernatant was centrifuged and the precipitate was collected, washed with distilled water until neutral pH. The precipitate was oven dried at 40 °C until constant weight.

4.4. Characterizations of Samples

4.4.1. Characterization of NADES

The density of NADES was calculated using the formula ρ = m/v, where m and v are the mass and volume of the NADES, respectively. The mass was measured using a calibrated electronic analytical balance (HR-250AZ, A&D, Japan) with a sensitivity of 0.1 mg. A 10 mL vial was placed on the scale and tared to zero. Using a calibrated 1 mL pipette, exactly 1 mL of NADES was carefully and slowly transferred to the bottom of the vial. The mass of the 1 mL NADES was recorded and the density was calculated. The measurement was performed in triplicates at room temperature. The viscosity of NADES was measured on a digital rotary viscometer (NDJ-5S) using No.1 spindle at a rotational spindle velocity of 60 rpm at room temperature. Briefly, 30 mL NADES was poured into a beaker and the viscosity tests were performed in triplicates.

4.4.2. Characterization of Chitosan

The ash content of extracted chitosan and commercial chitosan was determined gravimetrically by incinerating 1.0 g of sample until a constant weight in a muffle furnace at 600 °C as previously reported.[2] The sample was then cooled in desiccator. The ash content was measured by the weight of the crucible residue and the results were given as percentage. By employing the gravimetric method, the moisture content of chitosan was determined[2] wherein the sample was dehydrated in a hot air oven for 2 hours to constant weight and the variation in the weights of wet and oven-dried sample was calculated as percentage moisture. Fourier transform infrared spectroscopy (FT-IR) spectra of the samples were recorded on Agilent Cary 630 spectrometer to identify chemical bonding and functional groups. The FT-IR sample technique used was attenuated total reflection (ATR). All samples are in powder form which was placed onto the ATR crystal and pressed down using the swivel press to ensure optimal contact between sample and crystal. Transmission spectra were measured in the range of 4000 to 400 cm^{-1}. The degree of deacetylation (DDA) can be determined from the spectrum of FT-IR spectrophotometer according to the method used by Brugnerotta *et al.*[19] Comparison of wavenumbers between the absorption band of amide (1320 cm^{-1}) with amine absorption band (1420 cm^{-1}) was used according to **Eq. (1).**

$$\% DA = [(A_{1320} / A_{1420}) - 0.3822] / 0.0313, \quad \textbf{(1)}$$
$$\% DDA = 100 - \% DA,$$

where DDA is the degree of deacetylation (%) and DA is the degree of acetylation (%).

X-ray Diffraction (XRD) patterns of the samples were obtained by D8 Advance (BRUKER AXS, Germany) diffractometer using Cu Kα radiation (λ= 1.54056 Å) from 5° to 25° to investigate the structural properties. Scanning Electron Microscope (SEM) images were taken using JSM-6390 (JEOL, USA) to identify the morphology of the samples. The molecular weight of chitosan was determined using viscometer at room temperature. Chitosan solutions of different concentrations in 0.25 M acetic acid and 0.25 M sodium acetate were prepared. During preparation, all the solutions were magnetically stirred for 1 hour to ensure proper dissolution of chitosan and were filtered using filter paper. The molecular weight was then calculated by using Mark-Houwink equation in **Eq. (2).**[20]

$$[\eta] = k[MV]^{\alpha}, \quad \textbf{(2)}$$

where MV is the viscosity average molecular weight of polymer, α and k are constants (α = 0.83 and $k = 1.4 \times 10^{-4}$ for 0.25 M acetic acid and 0.25 M sodium acetate solvent system) and $[\eta]$ is the intrinsic viscosity.

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