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ORIGINAL ARTICLE

# Dissolving Chitin by Novel Deep Eutectic Solvents for Effectively Enzymatic Hydrolysis

**Qishun Liu[1,5] · Jia Che[1,2] · Yu Yu[1,3] · Deyu Chu[1,4] · Huiyan Zhang[1] · Fuyun Zhang[2] · Miao Zhao[3] · Heng Yin[1]**

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## Abstract

Chitin is the most productive nitrogen-containing polysaccharide in nature with immense potential for transforming into a range of chemicals. However, its dense crystal structure poses a challenge for depolymerization, limiting its applications. To overcome these challenges, a novel series of deep eutectic solvents (DESs) based on benzyltrimethylammonium chloride (TMBAC) as the hydrogen bond acceptor was developed. These TMBAC-based DESs, in combination with lactic acid, oxalic acid, and malic acid as the hydrogen bond donor demonstrated efficient chitin dissolution, achieving a solubility of up to 12% and an 88% recovery rate of regenerated chitin. The regenerated chitin was characterized using XRD, FT-IR, SEM, and $^{13}$C CP-MAS NMR, which indicated the preservation of chitin's chemical structure, a significant decrease in crystallinity, and a reduction in the molecular weight. Furthermore, the enzymatic hydrolysis efficiency of chitin was nearly doubled after treatment with TMBAC-based DESs, surpassing the effectiveness of untreated chitin. This approach holds promise for facilitating subsequent transformation and utilization of chitin.

**Keywords** Chitin dissolution · Benzyltrimethylammonium chloride · Deep eutectic solvents · Enzymatic hydrolysis

## Introduction

Fuels and chemicals serve as the fundamental building blocks for society. However, the extensive exploitation and utilization of fossil resources have led to resource depletion and consequent environmental problems. Consequently, there is an increasing drive towards the development and utilization of renewable resources, including natural polysaccharides [1, 2], with a particular focus on the conversion of biomass to bio-ethanol, lactic acid, alkanes, and other fuels and chemicals [3]. It is worth noting that most of the above processes are based on glucose-based biomass, which only contains C, H, and O [4]. The attention to other elements containing biomass, especially nitrogen-rich biomass, was relatively low [5]. Nitrogen-containing compounds were widely used in various fields, including medicine, agriculture, biodegradable materials, and so on [6, 7]. Typically, the nitrogen for organonitrogen was generally derived from the energy-intensive Haber–Bosch process.

Extended author information available on the last page of the article

In recent years, with increasingly strict $CO_2$ emission regulations, researches on the conversion of biomass into chemicals containing heteroatoms have gradually increased [6], with an aim to provide the innovatively scientific and engineering solutions for sustainable future of human beings and the nature.

Chitin is a polysaccharide consisting of N-acetylglucosamine linked by β-1,4 glycosidic bonds. The natural production of chitin is as high as $1.0\times10^{10}$ tons/year, which is second only to cellulose [8]. Chitin is mainly found in the shells of crustaceans (shrimp and crabs) and in the cartilage of squid and cuttlefish [9], as well as in the shells of insects and the cell walls of certain fungi and algae [10]. Globally, about 6–8 million tons of shrimp, crab, and lobster shells is produced each year, containing 15–40% chitin [11]. However, due to the lack of suitable methods, the majority of these chitin resources are only used for landfill incineration or as feed for breeding, resulting in environmental pollution and wasting resources. Therefore, the conversion of chitin biomass into high-value-added products was expected [6, 12, 13].

One of the hindrances to the utilization of chitin is its complex structure. Chitin consists of a quaternary structure, with the primary structure consisting of a sugar chain of N-acetylglucosamine linked by β-1,4 glycosidic bonds. The secondary structure is formed by hydrogen bonding between the hydroxyl group of the sugar chain and the N-acetylamino group. The tertiary structure is the aggregates formed by hydrogen bonds between sugar chains, and the conformation of the chitin main chain is related to it. The quaternary structure is the aggregate formed by the non-covalent bond between the long chains of chitin [9]. The dense structure with strong intermolecular hydrogen bonds makes chitin insoluble in water and most organic reagents, and further impedes the binding of chitin hydrolase [14], which catalyzes the hydrolysis of chitin into N,N'-diacetylchitobiose ($(GlcNAc)_2$) [15] and is crucial for the transformation and utilization of chitin [16]. To break the intermolecular and intramolecular hydrogen bonds, strong acids are often used in the pretreatment of chitin. However, strong acids cause various environmental problems, such as large amounts of acid release and water consumption [17, 18]. Green solvents, such as ionic liquids and deep eutectic solvents (DESs), provide an alternative approach to the pretreatment of chitin [19]. Although regular ionic liquids can dissolve chitin, it takes a long time and the dissolution efficiency of natural chitin is low [20]. Additionally, the environmental safety of regular ionic liquids has always been a concern [21].

DESs are a set of homogeneous and stable liquids formed by the intermolecular hydrogen bonds of the hydrogen bond donor (HBD) and the hydrogen bond acceptor (HBA). DESs have several characteristics such as adjustable structure, more hydrogen bonding sites, and lower vapor pressure [22]. They also have good solubility, high thermal stability, and excellent electrical conductivity [23]. In comparison to regular ionic liquids, DESs are less expensive and toxic, with a simple preparation process. Since being discovered by Abbot et al. in 2003 [24], they have been widely used in biomedicine, nanomaterials, electrochemistry, extraction and separation, and the pretreatment of biomass resources, including lignin and chitin for sustainable purposes [23, 25, 26].

Chitin is a polysaccharide with acetamido groups, which results in stronger intermolecular hydrogen bonding than cellulose, making it more difficult to depolymerize and dissolve. Within two decades, only a few trials on the dissolution of chitin by DESs based on choline chloride and betaine as hydrogen bond acceptors have been reported. Sharma et al. [27] discovered that DESs formed by choline chloride as HBA with urea and thiourea as HBD were good solvents for dissolving chitin. The maximum solubility of chitin can reach (9.0% w/w), and microwave heating and ultrasonic-assisted heating lowered the dissolution temperature and reduced the dissolution time. Vicente et al. [28] reported that three

different eutectic solvents (choline-lactic acid, choline chloride-urea, and betaine-glycerol) can dissolve chitin. In addition, some other reports have also explored choline chloride-based DESs for chitin dissolution and downstream processing [29, 30].

It can be found from the existing reports that DESs based on choline chloride and betaine have low solubility for chitin, both of which are less than 10% (w/w), and require a long dissolution time. Extra heating methods, such as microwave heating [27], are needed to improve efficiency. Currently, there is a need for novel DESs that can achieve a higher chitin solubility and facilitate further enzyme-catalyzed hydrolysis conversion processes, thereby reducing costs and improving the feasibility of technical routes such as preparation of chemicals from chitin.

Benzyltrimethylammonium chloride (TMBAC) is an environment-friendly surfactant that is widely used in medicine, oil extraction, and pesticides [31, 32]. Its unique quaternary ammonium salt structure offers unique advantages for the destruction of hydrogen bonds. TMBAC has been reported as HBA to prepare DESs and has been used in biomass pretreatment [33] for the removal of lignin and hemicellulose from lignocellulose, without dissolving cellulose [34, 35].

At present, choline and betaine-based DESs are frequently employed for the dissolution of chitin. However, their solubilities are restricted to below 10%, which poses a challenge to the further conversion and utilization of chitin. To overcome this limitation, we have developed TMBAC-based DESs that can dissolve chitin with a solubility of 12% within 2 h. This represents a significant improvement in efficiency and solubility compared to previous reports. Furthermore, the regenerated chitin exhibited reduced crystallinity, which led to a nearly onefold increase in the hydrolysis efficiency of chitin to $(GlcNAc)_2$ by exonuclease. These findings hold the potential for promoting the conversion and utilization of chitin.

## Materials and Methods

### Materials and Equipment

#### Materials

Chitin from shrimp shell (practical grade) was purchased from Sigma Aldrich (Merck KGaA, Germany). Benzyltrimethylammonium chloride (98%) was purchased from Aladdin Biochemical Technology Co., Ltd. (China). N,N'-Diacetylchitobiose ($(GlcNAc)_2$) was purchased from J&K Scientific Ltd. (USA). Lactic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Oxalic acid, malic acid, citric acid, ethylene glycol, glycerol, and LiCl were purchased from Tianjin Damao Chemical Reagent Factory (China). Urea was purchased from Dalian Meilun Biotechnology Co., Ltd. (China). N,N-Dimethylacetamide was purchased from Guangdong Guanghua Sci-tech Co., Ltd. (China). Unless otherwise specified, all reagents were of analytical grade.

#### Equipment

Fourier transform infrared spectroscopy (Nicolet iS50, Thermo Fisher Scientific Co., Ltd., USA); X-ray powder diffractometer (X' pert Pro, Panalytical Co., Ltd., Netherlands); field emission scanning electron microscope (JSM-7800F, JEOL Ltd., Japan); electron

microscope equipped with polarizing module (Olympus IX73, Olympus Corporation, Japan); nuclear magnetic resonance spectrometer (Bruker AVANCE III HD 400 MHz (WB), Bruker Corporation, Switzerland); high-performance liquid chromatography equipped UV detector (Waters e2095, Waters Corporation, USA); Ubbelohde capillary viscometer ($\Phi = 0.5$ mm, Hefei Shenyi Glass Products Co., Ltd., China).

### Preparation of Deep Eutectic Solvents

To prepare the TMBAC-based DESs, 1.00 g TMBAC (5.39 mmol) was placed into the round bottom flask. Subsequently, predetermined molar ratios of organic acids (lactic acid (LA), oxalic acid (OA), malic acid (MA), citric acid (CA)), polyols (ethylene glycol, glycerol), or urea, as listed in Table 1, were added and mixed well. The resulting mixture was stirred in an oil bath at a pre-set temperature until a clear liquid was formed.

### Dissolution of Chitin in TMBAC-Based DESs

After the DESs solution became completely transparent, chitin was gradually added in a proportion of 1% (w/w) each time until it was unable to dissolve. Chitin with predetermined mass fractions was added to the prepared evaluation mixture. Following mixing, the samples were heated at 80 °C, 100 °C, and 140 °C for 2 h, respectively, until the mixture appears homogeneous. Samples were photographed before and after dissolution on microscope.

### Regeneration of Chitin from the Dissolution of DESs

To precipitate the dissolved chitin, three times the mass of deionized water was added to the DESs solution of chitin and stirred at room temperature for 10 min. The resulting

**Table 1** Preparation of TMBAC-based DESs with different HBD

| HBD | HBD:HBA molar ratio | Temperature (°C) | Appearance of the mixture |
|---|---|---|---|
| Lactic acid | 1:2 | 100 | Homogeneous |
| Oxalic acid | 1:1 | 80/100 | Homogeneous |
| | 1:2 | | |
| Malic acid | 1:1 | 80/100 | Homogeneous |
| | 1:2 | | Heterogeneous/homogeneous |
| Citric acid | 1:1 | 80/100 | Heterogeneous, solid |
| | 1:2 | | Heterogeneous, solid |
| Ethylene glycol | 1:1 | 80/100 | Homogeneous |
| | 1:2 | | Homogeneous |
| Glycerol | 1:1 | 80/100 | Homogeneous |
| | 1:2 | | Homogeneous |
| Urea | 1:1 | 80/100 | Heterogeneous |
| | 1:2 | | Heterogeneous/homogeneous |
| | 1:3 | | Homogeneous |

mixture was then centrifuged at 3500 rpm/min for 15 min to obtain chitin precipitate. The precipitate was stirred with 50 mL of water for 10 min, then centrifuged at 3500 rpm/min for 15 min, and the process was repeated three times to remove DESs. The regenerated chitin was subsequently dried in a freeze dryer to obtain a dried chitin powder. The recovery rate of chitin from DESs solution is calculated according to Eq. (1).

$$Recovery\ rate = \frac{Mass\ of\ regenerated\ chitin}{Mass\ of\ dissolved\ chitin\ in\ DESs} \times 100\% \tag{1}$$

## Characterization of Regenerated Chitin

The X-ray powder diffraction (XRD) patterns of the samples were performed on an X-ray diffractometer with Cu Kα radiation ($\lambda = 1.54178$ Å). The operation voltage and current were maintained at 40 kV and 40 mA, respectively. The scanning range was set to $2\theta = 5 \sim 60°$ for 7 min.

The crystallization index (CrI) of chitin was calculated according to Eq. (2) [36].

$$CrI_{110} = \frac{I_{110} - I_{am}}{I_{110}} \times 100\% \tag{2}$$

$I_{110}$ was the maximum diffraction intensity of $2\theta \cong 19.2°$, and $I_{am}$ was the amorphous diffraction intensity of $2\theta \cong 16°$.

The morphology of the chitin was observed by scanning electron microscopy (SEM). Fourier-transform infrared (FT-IR) spectra were conducted with samples embedded in KBr pellets, and the spectra were recorded in the range 4000–400 $cm^{-1}$.

Solid-state $^{13}$C-CP/MAS NMR signals were detected at a Bruker AVANCE III HD 400 MHz (WB).

The viscosity average molar weight was measured by using the Ubbelohde capillary viscometer ($\Phi = 0.5$ mm) at 30 °C. The obtained viscosity was applied to the Mark–Houwink–Sakurada equation and a viscosity law, and the viscosity average molecular weights of chitin were calculated by Eq. (3) [37].

$$[\eta] = KM_{\omega}^{\alpha} \tag{3}$$

The parameters employed were $\alpha = 0.95$ and $K = 7.6 \times 10^{-5}$ dL•$g^{-1}$ for the chitin solution prepared in a 5% LiCl/DMAc solution.

## Enzymatic Hydrolysis of Regenerated Chitin from TMBAC-Based DESs

The exochitinase hydrolysis of chitin to $(GlcNAc)_2$ was carried out in 20 mM PBS buffer (pH 6.0) containing 5 mg•$mL^{-1}$ chitin, 1 μM *Sm*ChiB (an exo-type chitinase from *Serratia marcescens*, which was expressed in *E. coli* and purified as described previously [38]), and 1 mM ascorbic acid mixed in 1 mL of reaction. The mixtures were incubated at 30 °C for 4, 8, 12, 24, 36, 48, 60, and 72 h with 800 rpm shaking. After separating the products from the reaction mixture by filtration through a 0.22-μm membrane, an equal volume of acetonitrile was added to the product solution.

For the synergetic enzymatic hydrolysis of chitin, the experiments were carried out in 20 mM PBS buffer (pH 6.0) containing 5 mg•$mL^{-1}$ chitin, 1 μM *Sm*ChiB, 1 μM *Bt*LPMO10A (a lytic polysaccharide monooxygenase (LPMO) from *Bacillus thuringiensis*,

which was expressed in *Escherichia coli* and purified as described previously [39]), and 1 mM ascorbic acid mixed in 1 mL reaction. The mixtures were incubated at 30 °C for 4, 8, 12, 24, 36, 48, 60, and 72 h with 800 rpm shaking. After separating the products from the reaction mixture by filtration through a 0.22-μm membrane, an equal volume of acetonitrile was added into the product solution.

The $(GlcNAc)_2$ released from the reactions was analyzed using high-performance liquid chromatography (HPLC) equipped with an X-Amide column (5 μm, 100 A, 4.6×250 mm, Acchrom, China) and a UV detector at 195 nm. The mobile phase consisted of 70% acetonitrile and 30% water at the rate of 1 mL•min$^{-1}$. The concentration of $(GlcNAc)_2$ in samples was calculated using commercial $(GlcNAc)_2$ as a standard. All experiments were performed in triplicate.

## Results and Discussion

### Preparation of DESs

Although the first DESs were formed with choline chloride and urea in 2003 by Abbot et al. [24], there are only few reports on HBA of DESs, including quaternary ammonium salts, quaternary phosphate salts, and amino acids [40]. Here, new DESs based on TMBAC were explored, for their lower raw material costs, and its special chemical structure suitable as an HBA for DESs.

Initially, TMBAC was combined with organic acids (LA, OA, MA, and CA), polyols (ethylene glycol, glycerol), and urea to explore the possibility of preparing DESs. The tested combinations of ratio and temperature for different HBDs are summarized in Table 1.

TMBAC and several HBDs are solid or immiscible liquids at room temperature. However, when mixed in a certain proportion and heated, TMBAC and all the aforementioned HBDs, except CA, formed transparent liquids. This is due to the formation of hydrogen bonds between the HBDs and TMBAC, which reduces the electrostatic attraction between the anion and the cation. The ionic volume difference between cations and anions increased, and the ions moved freely, resulting in the formation of transparent and uniform liquids [41, 42]. While at a relatively low temperature (80 °C), the TMBAC-MA and TMBAC-urea system at a molar ratio of 1:2 formed an opaque state with undissolved solids. However, when the temperature was raised to 100 °C, the liquid appeared transparent, indicating that a low temperature was not conducive to the formation of these two DESs. When the molar ratio increased to 1:3, 80 °C was enough to form DESs, whereas at a molar ratio of 1:2, 100 °C was required to form DESs.

In addition to the ratio of HBD and HBA, the reaction temperature was also an important factor. An increase in the temperature accelerates the molecular motion, which benefits lowering the freezing point of DESs, thereby forming DESs [43, 44]. Meanwhile, as the temperature rose, the preparation times for DESs were shortened.

The optimal conditions for the preparation of TMBAC-based DESs are listed in Table 2. Furthermore, the stability of DESs formed by TAMBC and different HBDs was further investigated. The freezing point of DESs is an important indicator of its stability, which is related to the molecular structure, charge size, and interaction force of HBD and HBA [42]. The greater the interaction force between the HBD and HBA, the lower the freezing point. It is observed that the DESs prepared by TMBAC and LA, OA, MA, ethylene

**Table 2** Optimal conditions for preparation of TMBAC-based DESs

| HBD | HBD:HBA molar ratio | Temperature (°C) | Stability at room temperature |
|---|---|---|---|
| Lactic acid | 1:2 | 100 | Stable |
| Oxalic acid | 1:1 | 80 | Stable |
| Malic acid | 1:2 | 100 | Stable |
| Ethylene glycol | 1:2 | 80 | Stable |
| Glycerol | 1:2 | 80 | Stable |
| Urea | 1:3 | 80 | Unstable |

glycol, and glycerol at room temperature can maintain a transparent and uniform morphology, forming stable DESs. However, despite multiple attempts at different ratios and temperature, TMBAC-CA failed to form DESs and maintained the independent phases of each component. Although TMBAC-urea successfully formed DESs, the system was found to be unstable and prone to crystallization at room temperature, which is not conducive to subsequent experiments. Therefore, TMBAC and HBDs, including LA, OA, MA, ethylene glycol, and glycerol, were selected for subsequent chitin dissolution experiments.

## Efficient Dissolution of Chitin in TMBAC-Based DESs

The TMBAC-based DESs have been found to efficiently dissolve chitin, with the solubility of chitin reaching 12.0% in DESs of TMBAC-LA (Table 3, Fig. 1), which was much higher than reported (Table 3). Furthermore, the dissolution of chitin in the DESs of TMBAC with OA, MA, ethylene glycol, and glycerol was also explored (Fig. 1).

Although DESs of TMBAC-MA can dissolve chitin, the freezing point of the system was low, and it was easy to form a solid after being placed at room temperature for a period of time. Granular chitin was observed in the DESs prepared from TMBAC with glycerol and ethylene glycol, indicating incomplete dissolution of chitin (result not shown). After allowing the chitin solution dissolved by DESs to stand for 12 h, chitin precipitation appeared in the dissolution system of TMBAC with glycerol and ethylene glycol, demonstrating these two DESs were unable to effectively dissolve chitin.

In the DESs created by TMBAC with MA and OA, 1%, 2%, 5%, 10%, and 12% mass fractions of chitin were added and treated at 100 °C and 140 °C for 2 h, and the result was similar to chitin dissolution in TMBAC-LA. However, the chitin treated at 140 °C had a darker color than that at 100 °C, which may be caused by some side reactions such as hydrolysis and dehydration at high temperatures [46, 47]. The solubility of chitin in DESs of both TMBAC-OA (Table 3, entry 10) and TMBAC-MA (Table 3, entry 12) reaches 12.0% at 140 °C. It can be concluded from Table 3 that only TMBAC-based DESs composed of acidic HBDs can dissolve chitin. This may be because the acidic HBDs can break part of the hydrogen bonds of the chitin, allowing the solvent to penetrate and cause the chitin to dissolve. However, natural chitin is not 100% acetylated, so in the acidic DES-dissolved chitin system, there may still be a small amount of ionic bonding between DES and chitin. Additionally, due to the presence of benzene rings in TMBAC, there may be also a small amount of hydrophobic interaction during the dissolution process.

Microscopic observations were performed before and after dissolution in DESs of TMBAC-LA (Fig. 2). It was observed that chitin was obviously suspended in DESs in the

**Table 3** Dissolution of chitin in different DESs

| Entry | HBA | HBD | Heating method | Temperature (°c) | Time (h) | Solubility (%, w/w) | Reference |
|---|---|---|---|---|---|---|---|
| 1 | Choline chloride | Urea | Oil bath | 100 | 10 | 6.0 | [27] |
| 2 | | | Microwave radiation | 80 | 2 | 7.0 | [27] |
| 3 | | Thiourea | Oil bath | 100 | 6 | 9.0 | [27] |
| 4 | | | Microwave radiation | 80 | 2 | 8.0 | [27] |
| 5 | Trimethylglycine | Urea | Oil bath | 100 | 10 | 5.0 | [27] |
| 6 | | | Oil bath[a] | 80 | 1 | 5.0 | [27] |
| 7 | | Iron (III) chloride hexahydrate | Oil bath | 100 | 1 ~ 4 | —[b] | [45] |
| 8 | | Glycerol | Oil bath | 80 | 2 | —[b] | [28] |
| 9 | TMBAC | Lactic acid | Oil bath | 100 | 2 | 12.0 | This work |
| 10 | | Oxalic acid | Oil bath | 100 | 2 | 12.0 | This work |
| 11 | | Malic acid | Oil bath | 100 | 2 | 10.0 | This work |
| 12 | | | | 140 | 2 | 12.0 | This work |

[a]Ultrasonic-assisted oil bath heating

[b]No specific data listed

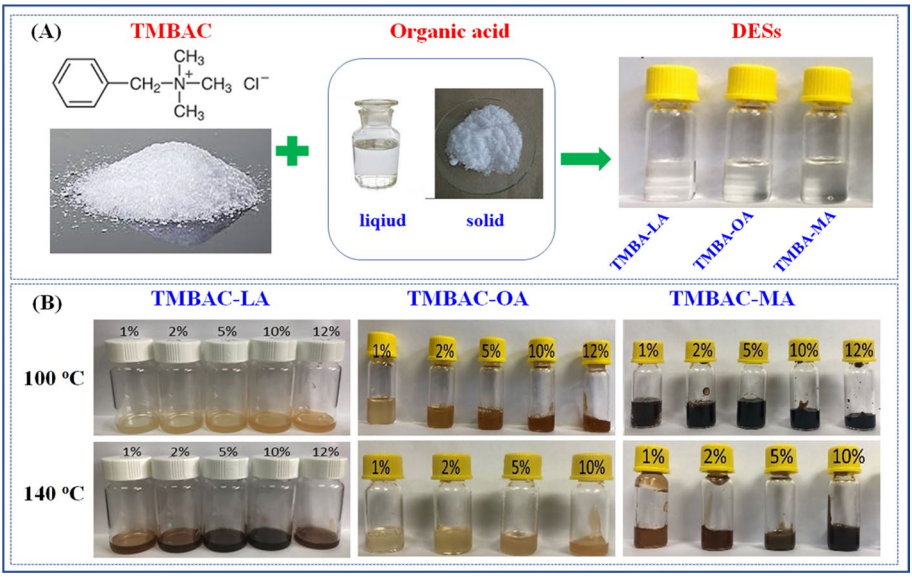


**Fig. 1** The TMBAC-based DESs and dissolution of chitin. **A** Preparation of TMBAC-based DESs. **B** Dissolution of chitin with different combinations of ratios of temperatures

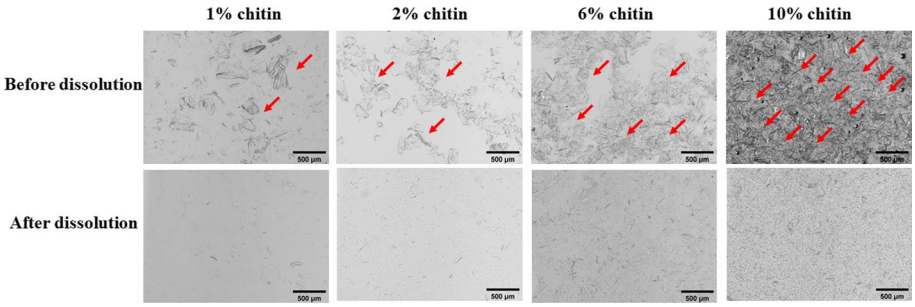


**Fig. 2** Micrograph of chitin dissolved in TMBAC-LA DESs. Dissolving conditions: molar ratio of TMBAC:LA was 1:2, 2 h at 100 °C. Red arrows indicate chitin crystals in DESs which disappeared then

granular form before dissolution. After dissolving, the entire system became uniform and transparent, and even 10% of the chitin was dissolved.

Previous reports on the dissolution of chitin by DESs were primarily based on the choline chloride and betaine, such as choline chloride and urea, and betaine and urea. This work investigated some new combinations, and the reported dissolution of chitin in DESs is listed in Table 3 for comparison. TMBAC-based DESs can dissolve 12% of chitin after being heated in a regular oil bath for 2 h. On the other hand, the DESs formed by choline chloride and urea, and betaine and urea take a longer time (6–10 h), or require special heating methods, such as microwave heating or ultrasonic treatment, and the solubility was less than 10% (Table 3). TMBAC-based DESs show advantages in dissolving chitin. They can dissolve chitin in a shorter time (2 h), and achieve efficient dissolution of chitin (12%) under regular heating methods, which is the highest reported ratio in the DESs system, making TMBAC-based DESs viable for industrial applications [33].

The recovery rates of regenerated chitin from DESs, with different HBDs, including MA, OA, and LA, were approximately 85% when the dissolved amount of chitin was 2% under 100 °C. And the HBD of MA was slightly lower, which was 83% (Fig. 3A). When

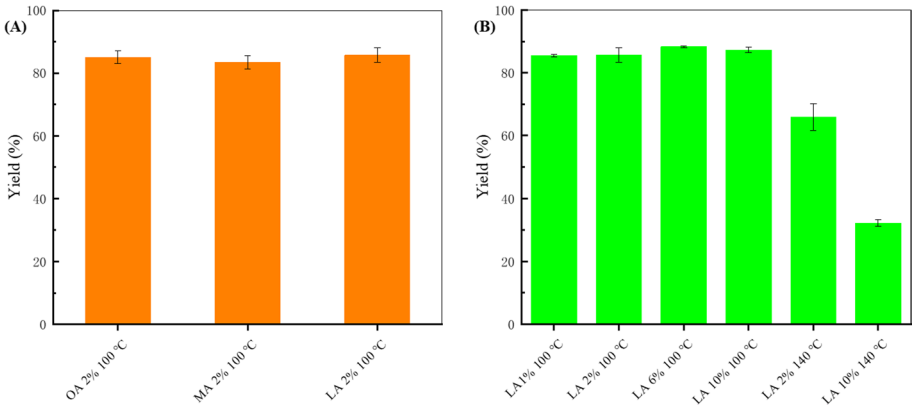


**Fig. 3** Recovery of chitin from DESs. **A** Recovery of 2% chitin dissolved in TMBAC-based DESs with different HBDs at 100 °C. **B** Recovery of MBAC-based DESs with LA as HBD at different chitin solubility and different temperatures

the HBD was LA, the recoveries of regenerated chitin were all over 85%, even when the amount of dissolved chitin was increased from 1 to 10%. When the dissolved amount was high, the recovery rate of regenerated chitin increased instead. However, when the dissolution temperature increased to 140 °C, the recovery rate of recovered chitin dropped sharply, decreasing to 65% when the dissolved amount of chitin was 2%, and even to 32% when the dissolved amount was 10% (Fig. 3B). This may be caused by the degradation of chitin, which led to a decrease in molecular weight and becomes water-soluble, making it impossible to precipitate.

## Characterization of Regenerated Chitin

The FT-IR spectra of chitin regenerated from different DESs treatments are presented in Fig. 4. The peaks observed at 3472 $cm^{-1}$ and 3265 $cm^{-1}$ correspond to the symmetric stretching vibrations of N–H and -OH groups, respectively [48, 49]. Furthermore, the stretching peaks of symmetric $CH_3$ and asymmetric $CH_2$ are observed at 2891 $cm^{-1}$. The presence of intermolecular and intramolecular hydrogen bonds of chitin results in the appearance of two amide I peaks at 1659 $cm^{-1}$ and 1624 $cm^{-1}$. Additionally, amide II and amide III bands can be observed at peaks at 1556 $cm^{-1}$ and 1312 $cm^{-1}$, respectively [50, 51]. These peaks are the characteristic absorption peaks of chitin. Moreover, a small absorption peak is observed at 1726 $cm^{-1}$ in FT-IR spectra of chitin treated with DESs, indicating a small amount of O-acylation [29, 52]. Overall, the FT-IR spectra of four samples in Fig. 4A are similar, whether the chitin regenerated after DESs treatment or untreated chitin.

The FT-IR spectra (Fig. 4B) of the regenerated chitin from DESs composed of TMBAC-LA with varying concentrations (2% and 10%) or processed at different temperatures (100 °C and 140 °C) revealed that the structure remained unchanged compared to the untreated of chitin. This observation suggests that the treatment temperature and concentration have any significant effect on the structure of chitin.

To further characterize the configuration of chitin, we employed $^{13}C$ CP-MAS NMR to characterize the configuration of chitin (Fig. 5). The characteristic peaks of untreated chitin were observed at 174 ppm, which was assigned to C = O bond, and at 104 ppm, 83 ppm, 76 ppm,73 ppm, 61 ppm, and 55 ppm were assigned to C1, C4, C3, C6, and C2,

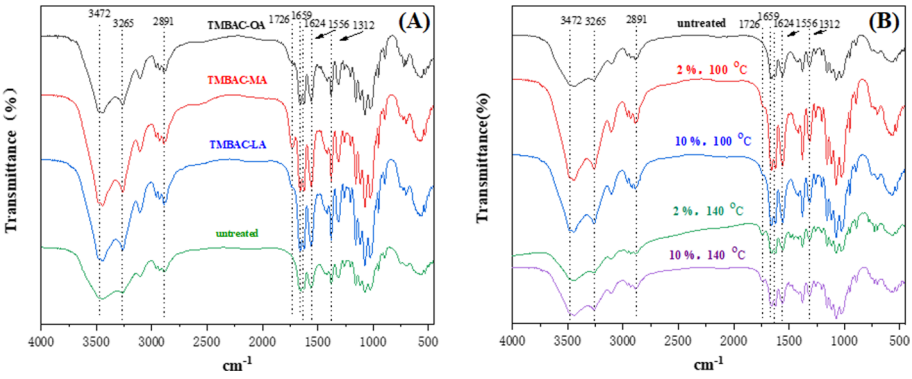


**Fig. 4** FT-IR spectra of regenerated chitins. **A** From different HBDs of TMBAC-based DESs, and **B** from different conditions in TMBAC-LA

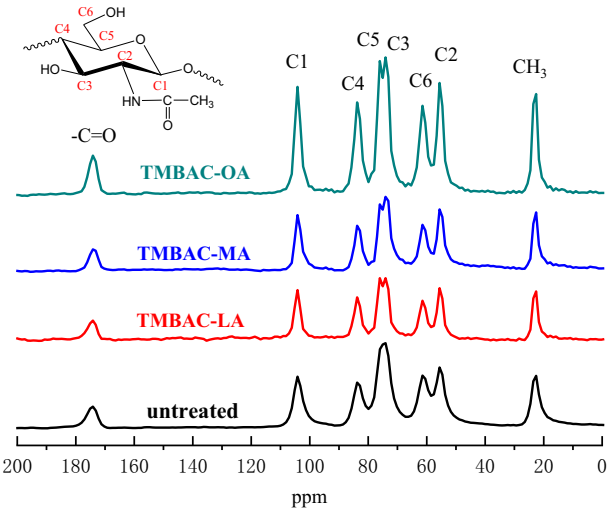


**Fig. 5** Solid-state CP-MAS $^{13}C$ NMR spectrum of untreated and regenerated chitin. C1 ~ 6 indicates the carbons in the sugar molecule, which is shown on the top left

respectively. Additionally, the peak at 23 ppm was assigned to the carbon atom of -$CH_3$ in acetylamino group, which was consistent with the literature reports [50, 53]. The $^{13}C$ CP-MAS NMR spectra of the DESs-treated chitins and the untreated chitin were essentially identical, indicating that all chitin samples were of the α-chitin type [51].

The XRD analysis was conducted to examine the crystallinity of chitin regenerated from TMBAC-LA. As shown in Fig. 6A, the untreated chitin exhibited characteristic peaks at 2θ of 9.2°, 19.2° and 28°, which correspond to the (020), (110), and (013) crystal planes of chitin crystals, respectively [27]. The crystal structure of the chitin treated with different temperatures and different mass fractions with DESs also displayed the same typical crystal diffraction peaks as untreated chitin, but the peaks were weakened. The degree of crystallinity was calculated using formula (1) and is presented in Table 4. The crystallinity of untreated chitin was found to be 85.0%. On the other hand, the crystallinities of regenerated chitin with the mass fractions of chitin 2% and 10% treated at 100 °C were 57.5% and 83.8%, respectively, while the

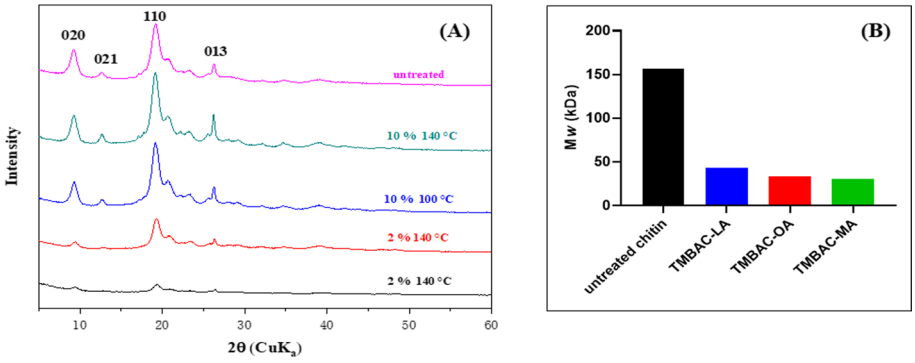


**Fig. 6** Crystallization and average molecular weight of chitin regenerated from DESs. **A** X-ray diffraction pattern of regenerated chitin from TMBAC-LA, and **B** viscosity average molar weight of chitin regenerated from TMBAC-based DESs

**Table 4** Crystallinity of regenerated chitin from TMBAC-LA

| Chitin concentration (w/w, %) | Temperature (°C) | Treated time (h) | Crystallinity (%) |
|---|---|---|---|
| 2 | 100 | 2 | 57.5 |
| 2 | 140 | 2 | 79.9 |
| 10 | 100 | 2 | 83.8 |
| 10 | 140 | 2 | 84.7 |
| Untreated chitin | - | - | 85.0 |

mass fractions dissolved were 2% and 10% treated at 140 °C were 79.9% and 84.7%, respectively.

The treatment of chitin with DESs resulted in a reduction in the crystallinity, leading to a looser structure that is beneficial to the further transformation and utilization of chitin, such as accelerating the efficiency of enzymatic hydrolysis and increasing the yield of products. Chen et al. [36] studied several pre-treatment methods, including wet ball milling, dry ball milling, steam explosion, acid–base immersion, and ionic liquid treatment, to reduce the crystallinity of chitin. They found that ball milling pretreatment reduced the crystallinity the most, with the final degree of crystallinity of 28%, but the process needed to be milled by a ball mill at 650 rpm for more than 4 h, which was time-consuming and had higher machine requirements. The crystallinity of chitin after treatment with wet ball milling, steam explosion method, acid–base immersion method, and ionic liquid treatment showed no obvious change or little change from the untreated chitin crystallinity. In comparison, DESs treatment was found to be more effective.

The viscosity average molecular weights of chitin regenerated from TMBAC-based DESs all decreased, from 156 kDa to tens of kDa (Fig. 6B). This may be attributed to the destruction of intermolecular hydrogen bonds of chitin when dissolved in DESs, and the acidity of LA, MA, and OA as HBD in DESs, which were more conducive to the partial hydrolysis of glycosidic bonds. This finding was consistent with that of other DESs, such as betaine and choline-based DESs, for dissolving chitin [46, 47].

Generally, the surface morphology of chitin can be classified into five different types, including rough and hard, without fibers and pores; only composed of nanofibers, without pores; a combination of fibers and pores; only composed of pores without nanofibers; and fibers with pores [54, 55]. The surface morphologies of untreated chitin and chitin treated with DESs (TMBAC with LA, OA, and MA) were observed by SEM at 20,000 magnification (Fig. 7).

Regarding the surface macrostructure, there was significant difference between untreated chitin and chitin regenerated from DESs. The surface morphology of untreated chitin appears rough and dense, and it is difficult to observe the fiber structure. On the other hand, chitin regenerated from the DESs of TMBAC-LA and TMBAC-OA exhibits a more regular texture, and more obvious fibers in chitin can be observed [27]. Chen et al. [36] found that the chitin treated by ball milling appears to have pores. Similarly, the chitin regenerated from DESs of TMBAC with LA, OA, and MA also displays pore structures. Furthermore, the chitin regenerated from DESs of TMBAC with LA has the most wrinkles, which is more conducive to degradation and transformation.

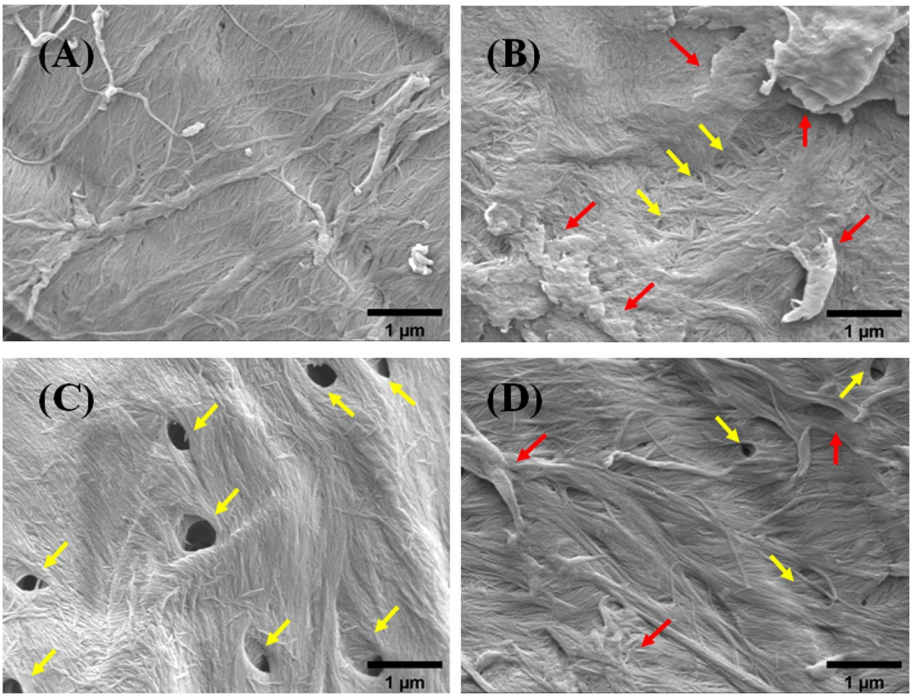


**Fig. 7** Scanning electron microscopy observations of the morphology of regenerated chitin from DESs before and after dissolution. **A** Untreated chitin, **B** regenerated chitin from dissolved in TMBAC-LA, **C** regenerated chitin from dissolved in TMBAC-OA, **D** regenerated chitin from dissolved in TMBAC-MA. Red arrows indicate wrinkles, and yellow arrows indicate pores

## Enzymatic Hydrolysis of Regenerated Chitin from TMBAC-Based DESs

Furthermore, the impact of treatment with DESs on chitin was evaluated by analyzing the rate of exochitinase *Sm*ChiB and synergetic enzyme of exochitinase *Sm*ChiB with Lytic *Bt*LPMO10A hydrolysis of chitin to $(GlcNAc)_2$. The crystallinity of regenerated chitin was significantly reduced compared to untreated chitin, and this decrease in crystallinity promoted the binding and hydrolysis activity of enzymes [56, 57], ultimately leading to an improved conversion efficiency into $(GlcNAc)_2$. The wrinkle structure and pore structure, as confirmed by TEM, also increased the specific surface area and improve the accessibility of enzymes, further enhancing the conversion efficiency [58, 59]. In addition, the molecular weight of the regenerated chitin was relatively lower, which may also have contribution to the hydrolysis of chitin. As a result, the hydrolysis rate of regenerated chitin was higher than that of untreated chitin. Finally, the hydrolysis of chitin pretreated with DESs of TABAC-LA to $(GlcNAc)_2$ had the highest efficiency, nearly twice that of the untreated (Fig. 8A). The enhanced enzymatic hydrolysis efficiency of chitin pretreated by TMBCA-based DESs was even higher than that of ionic liquid pretreatment, which was 24% [60]. Therefore, TMBCA-based DESs pretreatment of chitin was an effective method for chitin treatment.

However, in the case of the synergistic catalytic hydrolysis of chitin with *Sm*ChiB and *Bt*LPMO10A, the difference in hydrolysis efficiency between treated and untreated chitin

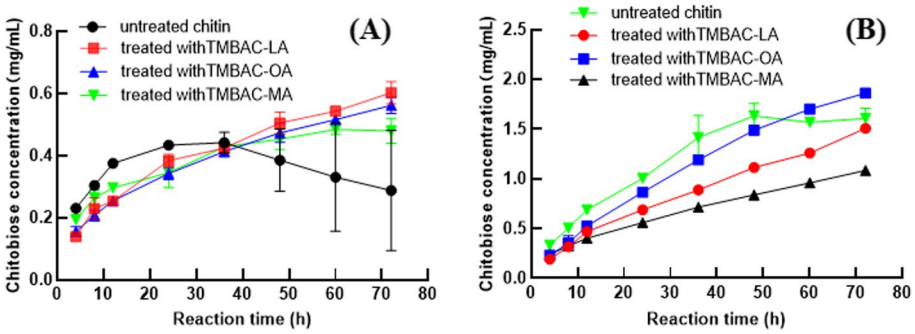


**Fig. 8** Effects of DESs treatment of chitins on the enzymatic activities. **A** Exochitinase *Sm*ChiB hydrolysis, and **B** synergetic enzyme of exochitinase *Sm*ChiB with *Bt*LPMO10A hydrolysis regenerated chitin from TMBAC-based DESs to generate $(GlcNAc)_2$

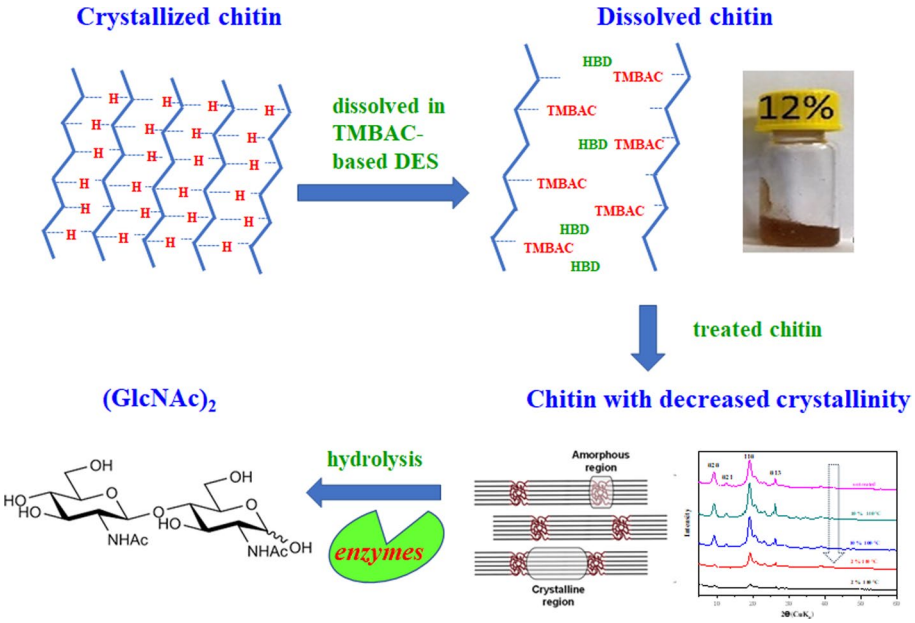


**Fig. 9** Schematic diagram of a novel TMBAC-based DESs treatment of chitin and enhance enzymatic hydrolysis to $(GlcNAc)_2$

was not as significant as that of exonuclease hydrolysis (Fig. 8B). LPMO was known to be an enzyme capable of hydrolyzing crystalline chitin [39] and, when used in synergy with exochitinase, improves the hydrolysis efficiency of crystalline chitin. Since the DESs-treated chitin still had crystalline regions, it was reasonable that there was no difference in the efficiency of this synergistic enzymatic hydrolysis with untreated chitin. This observation was also consistent with the result of exochitinase-catalyzed hydrolysis of chitin.

In summary, this study developed a series of TMBAC-based DESs that can break the hydrogen bonds of chitin, resulting in efficient dissolving of chitin up to 12% (w/w), and the recovery rate of regenerated chitin can reach up to 88%. The chemical structure of chitin regenerated

from these DESs was not destroyed, but the chitin surface had a structure of pores and layers, and the crystallinity was significantly reduced. These factors promoted twice efficiency of enzymatic hydrolysis of chitin to $(GlcNAc)_2$ (Fig. 9). Therefore, this study provided a green, cost-effective, and efficient chitin pretreatment method using DESs based on TMBAC, which can promote the conversion and refining of chitin. Based on the results of the efficient dissolution of chitin by acidic TMBAC-based DESs found in this work, future research can focus on the extraction and pretreatment of chitin from chitin biomass (lobster, crab, etc.).

## Conclusion

In conclusion, this study successfully prepared DESs based on TMBAC and HBDs, including LA, OA, MA, ethylene glycol, and glycerol. The new TMBAC-based DESs with LA, OA, and MA were found to dissolve 12% of chitin, which is nearly 20% higher than previously reported in DESs (less than 10%) and with a shorter processing time by the regular oil bath heating method. The regenerated chitin from TMBAC-based DESs was characterized, and it was observed that the primary structure of chitin remained intact. The crystallinities of chitin were all reduced, and the efficiency of enzymatic hydrolysis of chitin to (GlcNAc)2 was significantly improved nearly onefold after treatment with TMBAC-based DESs. These findings are advantageous for subsequent transformation and utilization of chitin. Overall, this study provides a green, cost-effective, and efficient chitin pretreatment method using DESs based on TMBAC. The improved efficiency of enzymatic hydrolysis of chitin after treatment with TMBAC-based DESs also increases the potential for the conversion and refining of chitin.

**Author Contribution** Qishun Liu: conceptualization, investigation, writing—original draft preparation, funding acquisition. Jia Che: investigation, visualization. Yu Yu: investigation, visualization. Deyu Chu: investigation, visualization. Zhang Huiyan: investigation, visualization. Fuyun Zhang: writing—reviewing and editing. Miao Zhao: writing—reviewing and editing. Heng Yin: writing—reviewing and editing, funding acquisition, supervision.

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**Data Availability** The data and materials are available from the corresponding author upon reasonable request.

## Declarations

**Ethics Approval** Ethics approval was not required for this study.

**Competing Interests** The authors declare no competing interests.

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## Authors and Affiliations

**Qishun Liu[1,5] · Jia Che[1,2] · Yu Yu[1,3] · Deyu Chu[1,4] · Huiyan Zhang[1] · Fuyun Zhang[2] · Miao Zhao[3] · Heng Yin[1]**

✉ Qishun Liu
liuqishuen@dicp.ac.cn

✉ Heng Yin
yinheng@dicp.ac.cn

1 Group of Natural Products and Glyco-Biotechnology, Liaoning Provincial Key Laboratory of Carbohydrates, Dalian Technology Innovation Center for Green Agriculture, Dalian Engineering Research Center for Carbohydrate Agricultural Preparations, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, Liaoning, China

2 College of Food Science and Engineering, Dalian Ocean University, Dalian 116023, Liaoning, China

3 School of Textile and Materials Engineering, Dalian Polytechnic University, Dalian 116034, Liaoning, China

4 School of Environment and Chemical Engineering, Dalian Jiaotong University, Dalian 116028, Liaoning, China

5 Key Laboratory of Se-Enriched Products Development and Quality Control, Ministry of Agriculture and Rural Affairs, National-Local Joint Engineering Laboratory of Se-Enriched Food Development, Ankang 725000, Shaanxi, China