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Environmentally friendly pretreatment of chitin using relatively low concentration KOH/urea mixture for enhanced nanofiber preparation --Manuscript Draft--

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Corresponding Author:	Fuyuan Ding Jiangsu University Zhenjiang, CHINA		
First Author:	Siman Long		
Order of Authors:	Siman Long		
	Fuyuan Ding		
	Xiaowei Huang		
	Jiyong Shi		
	Megan Povey		
	Xiaobo Zou		
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Suggested Reviewers:	Lingyun Chen lingyun.chen@ualberta.ca Yixiang Wang vixiang.wang@mcgill.ca		
	Xiaoying Wang xyw@scut.edu.cn		
Opposed Reviewers:			

Dear Editor

I am writing to submit our manuscript entitled " Environmentally friendly pretreatment of chitin using relatively low concentration KOH/urea mixture for enhanced nanofiber preparation" for consideration as a Research Article in International Journal of Biological Macromolecules.

In this work, we present a novel approach to prepare chitin nanofibers using an environmentally friendly process. The method utilizes low-temperature freeze pretreatment with a relatively low concentration of KOH/urea mixture, which can reduce the crystallinity of β -chitin. This strategy bypasses the need for harsh chemicals or high-energy inputs which are needed to purify the chitin in traditional methods. Our study demonstrates that pretreatment with a 7 wt% KOH/1 wt% urea mixture at -30°C reduces β -chitin crystallinity from about 85% to 67.1%. After pretreatment, TEMPO-mediated oxidation was employed to prepare nanofibers. By adjusting the concentration of KOH in the pretreatment, we successfully controlled the morphology of the nanofibers, achieving rod-like structures of approximately 150 nm in length. Furthermore, we observed an increase in carboxyl content to 0.43 mmol/g in nanofibers prepared from chitin treated with a 3 wt% KOH/1 wt% urea mixture, compared to 0.25 mmol/g in solely freeze-treated chitin. In addition, our pretreatment method can be applied to α -chitin and offers solvent reusability, making it a sustainable option. This technique has the potential to be extended to other biomass sources for nanofiber production, which can broad its applicability.

Sincerely yours Fuyuan Ding

Traditional methods to prepare chitin nanofibers often require circular chemical pretreatment and the morphology of the prepared nanofibers is commonly rod-like one. This study introduces an approach utilizing low-temperature freeze pretreatment of chitin in a relatively low concentration KOH/urea mixture. The crystallinity of β -chitin reduced as the concentration of KOH increased in the pretreatment solvent. The crystallinity of β -chitin reduced to 67.1% after pretreating with 7 wt% KOH/ 1 wt% urea mixture at -30 °C. The concentration of urea had little effect on the structure of chitin. The pretreated chitin was then oxidized in a 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)/NaBr/NaClO system to prepare nanofibers. The morphologies of the nanofibers can be regulated by adjusting the concentration of KOH. The nanofibers obtained by oxidizing chitin pretreated by 3 wt% KOH/ 1 wt% urea mixture showed rod-like morphologies with length about 150 nm. The carboxyl content of nanofibers prepared with 3 wt% KOH/ 1 wt% urea mixture treated chitin increased to 0.43 mmol/g compared to that of solely freeze treated chitin nanofibers (0.25 mmol/g). In addition, the pretreatment solvent can be used to treat α -chitin and can be reused. The freeze KOH/urea mixture pretreatment methods can be extended to treat other biomasses for nanofiberization.

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8 9 10	4	Siman Long ^a , Fuyuan Ding ^{*,a} , Xiaowei Huang ^a , Jiyong Shi ^a , Megan Povey ^b , Xiaobo Zou ^a
11 12 13	5	
14 15 16	6	^a International Joint Research Laboratory of Intelligent Agriculture and Agri-products Processing,
17 18	7	Joint Laboratory of China-UK on Food Nondestructive Sensing, School of Food and Biological
20 21	8	Engineering, Jiangsu University, Zhenjiang, 212013, China
22 23 24	9	^b School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK
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28 29 30	11	*Corresponding author: <u>dingfuyuan@ujs.edu.cn</u> (F. Ding)
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Recent advances in the preparation of chitin nanofibers have explored the use of milder, more environmentally benign solvents and conditions to maintain the structural integrity and functional properties of the resultant nanofibers [6, 12-15]. For instance, glycerol swelling and low-temperature freezing have been applied to pretreatment of chitin [16]. Then, the TEMPO-mediated oxidation method was applied to oxidize the pretreated chitin in order to obtain chitin nanofibers. The results indicated that low-temperature freezing and swelling pretreatment can enhance the preparation of nanofibers. Alkali/urea mixtures have emerged as promising solvents for the processing of chitin [10, 17-20], with different kinds of chitin-based biomaterials fabricated in this way at low temperature [21, 22]. Crystallinity and hydrogen bonding in the chitin structure were disrupted in alkali/urea mixtures at low temperature. We hypothesize that the pretreatment of dried chitin in alkali/urea mixtures at low temperature facilitates the preparation of chitin nanofibers.

This study investigated the efficacy of pretreating chitin with a relatively low concentration KOH/urea mixture at low temperature (Scheme 1). Then, the pretreated chitin was oxidized by the TEMPO/NaBr/NaClO system to prepare chitin nanofibers. The focus on low-temperature conditions is due to the dissolution of chitin in KOH/urea mixture being an enthalpy change process [23]. By optimizing the conditions of the KOH/urea pretreatment, this research aims to develop a scalable and environmentally friendly methodology that can enhance the preparation of chitin nanofibers. The innovations herein are as follows: (1) The use of KOH/urea mixture combined with low-temperature freeze pretreatment can effectively disrupt the structure of chitin, enhancing the efficiency of nanofiber extraction. (2) The morphological and physicochemical properties of chitin nanofibers can be adjusted by regulating the concentration of KOH during the

- pretreatment process. (3) The KOH/urea mixture used to pretreat the chitin can be reused, which offers environmental and economic benefits. We anticipate that this pretreatment method can be
- applied to treat other biomass, which can enhance the preparation of nanofibers.



Scheme 1 Pretreatment of chitin in KOH/urea mixture and preparation of chitin nanofibers

2. Materials and methods

2.1 Materials

 β -chitin and α -chitin were purchased from Golden-Shell Biochemical Co. Ltd. (Zhejiang, China). The characterization of chitin was performed according to the reported method [12, 24]. The viscosity average molecular weight (M_n) of β -chitin was 6.3×10^5 g·mol⁻¹ by viscometry and the degree of deacetylation (D.D) was about 5%. The molecular weight (M_n) of α -chitin was 10.5 \times 10⁴ g·mol⁻¹ and its degree of deacetylation was 3%. TEMPO was purchased from Sigma-Aldrich Co., Ltd (USA). Sodium hypochlorite solution (5.5~6.5%) was obtained from Shanghai Macklin Biochemical Co., Ltd (China). KOH, NaOH, HCl, NaBr and urea were supplied by Sinopharm

84 2.2 Pretreatments of chitin and characterization

The β -chitin was pretreated by KOH/urea mixed solution [12, 25]. In brief, 2 g β -chitin was dispersed in a given concentration of KOH/urea solution (100 mL) and then frozen at -30 °C for several hours. Subsequently, the mixture was thawed at room temperature, and washed thoroughly with deionized water until approximately neutral following filtration. Finally, the pretreated chitin was freeze-dried for further characterization. The chitin pretreated with KOH/urea of 0 wt%-0 wt%, 0 wt%-1 wt%, 3 wt%-1 wt%, 7 wt%-1 wt%, 3 wt%-0 wt%, and 3 wt%-4 wt% were denoted as PCN00, PCN01, PCN31, PCN71, PCN30 and PCN34. The morphologies of the original and pretreated chitin were observed using a scanning electron microscope (SEM) (Hitachi S-3400, Hitachi, Japan). Before tests, all samples were freeze-dried for 48 hours and coated with gold to enhance the conductivity. Fourier Transform infrared spectroscopy (FT-IR) of the original and pretreated chitin were conducted with an attenuated total reflectance (ATR) accessory (Nicolet is50, USA). All spectra were obtained in the range of 500 to 4000 cm⁻¹.

97 X-ray diffraction analysis was performed on an X-ray diffractometer (D8 ADVANCE, 98 Germany), ranging from 5° to 60° at a speed of 5 °C/min. The degree of crystallinity was 99 calculated from the peak intensity I_{110} and the baseline intensity $I_{\text{amorphous}}$ by using the following 100 equation.

101 Crystallinity =
$$(I_{110} - I_{amorphous}) / I_{110}$$
 (1)

where I_{110} denotes the maximum intensity at $2\theta = 19-20^{\circ}$ and $I_{\text{amorphous}}$ is the intensity of amorphous diffraction from chitin at $2\theta = 12-14^{\circ}$.

 $D = K\lambda/\beta_{1/2}\cos\theta$ (2)

107 where λ is the wavelength of Cu-K α radiation ($\lambda = 1.5418$ Å), θ is the scattering angle, *K* is a 108 shape factor (0.9), and $\beta_{1/2}$ is the FWHM.

2.3 TEMPO-mediated oxidized chitin

TEMPO-mediated oxidation of chitins was conducted according to the reported method [26, 27]. In the process of TEMPO/NaBr/NaClO oxidation, 2 g pretreated β-chitin was firstly suspended in 200 mL distilled water, which contained sodium bromide (0.2 g, 1 mmol) and TEMPO (0.032 g, 0.1 mmol). A 5% NaClO solution was adjusted to pH 10 by the addition of 0.1 M HCl. The TEMPO-mediated oxidation was initiated by adding a desired amount of NaClO (1-10 mmol of NaClO per gram of chitin) in solution, and the mixture was stirring continuously at room temperature. Meanwhile, 0.5 M NaOH was added gradually in the solution to maintain the pH at 10. The reaction was stopped when no consumption of NaOH was observed. After that, the oxidized chitin was dialyzed with deionized water for three days. The slurry of oxidized chitin was then sonicated for 30 min by an ultrasonic processor (JY92-IIN, 20-25 KHz) at 650 W. After ultrasonic dispersion and centrifugation (8000 rpm, 20 min), a uniform supernatant was obtained. Finally, the suspension was freeze-dried for later use. The oxidized PCN00, PCN01, PCN31, and PCN71 were denoted as OPCN00, OPCN01, OPCN31, and OPCN71, respectively.

2.4 Characterization of TEMPO-mediated oxidized chitin

The morphology of the TEMPO-oxidized chitins was observed by transmission electron microscopy (TEM, HT7800). Prior to imaging, a drop of 0.03 wt% suspension was deposited on a copper grid with a carbon-reinforced film and maintained on the grid for a specific time to dry naturally. After that, it was stained with phosphotungstic acid and blotted with filter paper. FT-IR spectra of the TEMPO-oxidized chitins were recorded on Fourier Transform infrared spectroscopy (Nicolet is50, USA), with an attenuated total reflectance (ATR) accessory. Zeta potential measurements of the TEMPO-oxidized chitins were made using a Malvern Zeta-sizer. The tests were carried out at 25°C using 1 mL of diluted suspensions, and the pH value changed during measurement from 7 to 9. XRD patterns of the TEMPO-oxidized chitins were performed by X-ray diffractometer (D8 ADVANCE, Germany), ranging from 5° to 60° at a speed of 5 °C/min. The carboxylate content (CC) of the TEMPO-oxidized chitins was determined using an electrical conductivity titration method, according to the cited method [28]. Briefly, dried samples (0.1 g) were added to 30 mL water (pH 9.0) and the mixture was stirred for 30 min to obtain a

well-dispersed mixture. Then, the pH was set in the range 2.5~3.0 by adding 0.1 M HCl to the
mixture. Finally, 0.05 M NaOH was added to adjust the pH to 11, and the consumption of NaOH
was recorded. The content of carboxyl groups can be calculated by the following equation.

142
$$CC = \frac{c \times (V_2 - V_1)}{M}$$
(3)

where *c* is the concentration of NaOH solution (mol L^{-1}), V_I is the volume of NaOH solution (mL) at the first titration jump, V_2 is the volume of NaOH solution at the second titration jump, *M* is the sample weight (g).

147 2.5 Preparation of Pickering emulsions and 3D printing

The TEMPO-oxidized chitin nanofibers stabilized high internal phase Pickering emulsions (HIPPE) were prepared as follows. Briefly, white beeswax was melted into sunflower oil at 70°C to obtain a beeswax-oil mixture (containing 10 wt% beeswax) as the oil phase. Next, 1 wt% TEMPO-oxidized chitin suspension was treated by high-pressure homogenization (Ouhor A25-digital, China) at 10000 rpm for 2 min for further use. Then, nanofiber suspension was mixed with the beeswax-oil mixture in a 1:1 w/w ratio by high-speed shearing at 15000 rpm for another 2 min. The Pickering emulsions with a phase volume ϕ of 0.5 were achieved through the above procedures, and ϕ was adjusted to 0.9 by continuously adding oil mixture whilst blending at 15000 rpm. After 5 min, the final high internal phase Pickering emulsion (HIPPE) was acquired. The emulsions stabilized by chitin nanofibers were observed using an optical microscope (Nikon, China) equipped with a digital camera. A drop of sample was dropped onto a microscope slide and covered with a glass coverslip.

The HIPPE was used as an ink via a syringe-type extrusion-based 3D Food printer (Shiyin
Technology, Hangzhou, China) 50 °C. Before printing, 30 mL emulsion was placed into a 60 mL
syringe, with a nozzle diameter of 0.1 mm, and a print speed of 0.5 mm/s.

3. Results and discussion

3.1 Effects of KOH concentration on the structure of β-chitin

Alkali/urea mixtures have been widely applied to the dissolution and processing of biomasses
such as cellulose and chitin [29]. Many kinds of biomaterials have been fabricated using this
solvent. KOH is a commonly used alkali in this case. A high concentration of KOH (14 wt%) /
urea (4 wt%) combined with low-temperature freeze can quickly dissolve β-chitin [12] and its

170 concentration has large effects on the dissolution behaviors of chitin. We hypothesis that the low 171 concentration of KOH in this solvent system can loosen the structure and facilitate the subsequent 172 nanofibrillation. Chitin pretreated with 0 wt% KOH/1 wt% urea, 3 wt% KOH/1 wt% urea and 7 173 wt% KOH/1 wt% urea solvents were denoted as PCN01, PCN31 and PCN71 respectively. Chitin 174 pretreated only by freeze methods was denoted as PCN00 and was used as control.

The change of β -chitin in the solution after pretreating with various KOH/urea mixtures was firstly characterized by digital images. As shown in Fig. 1 (A), the PCN00 solution stratified after thawing at room temperature, which demonstrated that freeze treatment had little effect without KOH. When the solution was added with 1 wt% urea, the PCN01 solution also stratified. This was because a low concentration of urea does not change the structure of chitin. Further increasing the KOH concentration to 3 wt%, the chitin suspension transformed to a semi-soluble state. As shown in Fig. 1 (A), there were some white aggregates in the PCN31 solution due to the assembly of partial chitin molecule. When the concentration of KOH increased to 7 wt%, the solution became transparent indicating the dissolution of chitin in the high alkali solution. During the pretreatment process, the KOH and chitin formed a clathrate by hydrogen bonding, and then surrounded by urea [30]. Subsequently, the self-assembled clathrate compound was dissolved in water owing to its strong hydrophilicity.

To further investigate the effect of KOH on the pretreatment of chitin, the pretreated chitin was characterized by FT-IR spectra. As shown in Fig. 1 (B), in the spectrum of original β-chitin, the intensity of the O-H located at 3432 cm⁻¹, the weak shoulder peak at 3270 cm⁻¹ correspond to N-H stretching, and the two characteristic peaks at 1633 cm⁻¹ and 1556 cm⁻¹ correspond to amide I and II band stretching respectively [31, 32]. Besides, the sharp characteristic peaks at 1064 cm⁻¹

and 1029 cm⁻¹ correspond to symmetrical and asymmetric tensile vibrations of C-O-C and C-O on the sugar ring [33, 34]. The broad peak of 1633 cm⁻¹ indicated the lack of inter-sheet hydrogen bonds in the β-chitin crystal structure. When no KOH was added, the spectrum of PCN00 and PCN01 were similar to the β -chitin. While the KOH concentration reached 3 wt%, two characteristic peaks at 1655 cm⁻¹ and 1621 cm⁻¹ were observed. Moreover, the intensity of peaks slightly increased with higher KOH concentration. The double peaks were associated with the intra chain C=O...H-N hydrogen bond interaction and inter chain C=O...H-O hydrogen bond interaction of the amide I band, respectively [19]. The addition of KOH played a role, facilitating the formation of intra- and intermolecular hydrogen bonding network during pretreatment. In addition, the spectra of the pretreated chitin were similar to the original chitin, which demonstrated that the pretreatment was mild and did not change the chemical groups in the chitin molecule.

X-ray diffraction patterns of various pretreated chitins are displayed in Fig. 1 (C). Obviously, the low-temperature frozen chitin without KOH (PCN00 and PCN01) showed two distinct crystalline diffraction peaks at 8.3° and 19.7°, corresponding to the (010) reflection, and a superposition of (100), (110), and (110) reflections of typical β -chitin, respectively [35]. As the concentration of KOH increased to 3 wt%, the two broad crystal diffractions of β -chitin at 8.3° and 19.7° shifted to 9.1° and 19.2° after pretreatment. In addition, the peak intensity reduced compared with the PCN00 and PCN01. Further increase of KOH to 7 wt% (PCN71) reduced peak intensity at 9.1° and a weak diffraction peak appeared at 26.4° corresponding to a diffraction peak of α -chitin (013) compared to PCN31, implying the slight transformation from β -chitin to α -chitin [36, 37]. More importantly, the crystallinity of various pretreated chitin is shown in Fig. 1 (D).

Obviously, the crystallinity of different pretreated chitin presented a declining trend as KOH concentration increased. This may be due to KOH/urea swelling involved the change of hydrogen bonding and chitin crystalline structure [12]. The crystal size of the pretreated chitin was also obtained. The crystal size of the KOH/urea treated chitin was slightly larger than solely freeze treated samples. This might be because the alkali partially solubilized chitin and some amorphous regions in chitin broke down. Larger crystals might then form due to the remaining crystalline regions growing and aggregating into larger crystals following regeneration from KOH/urea solution.

The pretreatment process was further monitored by SEM. As shown in Fig. 1 (E), the freeze treated chitin (PCN00 and PCN01) was a compact structure with large sheets stacked layer by layer, displaying smooth surface regions, which is a typical structure of β -chitin. When the concentration of KOH increased to 3 wt%, the area of the lamellae greatly decreased and split into many coarse ribbon fiber bundles. More importantly, the smooth surface became rougher with increased fibrous protrusions. A small amount of nanofibers were observed in the looser structure, and the fibrillation became more obvious. Here, in comparison to original chitin, folds appeared in the structure, forming an uneven surface like a ravine, accompanied by irregular round holes and cracks. This may be due to the semi-dissolved state of chitin in a relative high concentration of KOH, thus changing the microstructure greatly after swelling at low temperature. When the KOH concentration reached to 7 wt%, the regenerated chitin showed a dramatic loose lamellar structure, and the overall fibers was more visible. More chitin was dissolved by higher amount of alkali, facilitating the expansion of chitin fiber bundles both on the interior and exterior surface. These results indicated that KOH/urea pretreatment altered the microstructure of chitin, producing more

fragmented and looser lamellar structure on one hand. On the other hand, a portion of fibers were effectively converted to nanoscale, which was beneficial for subsequent preparation of chitin

nanofibers.



Fig. 1. (A) Appearance of β -chitin (PCN00, PCN01, PCN31, and PCN71) solution after freeze pretreatment in different KOH/urea mixture. (B) FT-IR spectra of the original and different KOH/urea pretreated β-chitin. (C) X-ray diffraction patterns of pretreated β-chitin. (D) Changes of crystallinity and crystal size of the pretreated β -chitin. (E) SEM images of different KOH/urea pretreated β-chitin.

3.2 Effects of urea concentration on the structure of β-chitin

Besides the effects of KOH concentration of the structure of β -chitin, the effects of urea concentration on the structure were also studied. It's reported that the urea concentration played an auxiliary role during dissolution of cellulose and chitin. The chitin pretreated with 3 wt% KOH/0 wt% urea, 3 wt% KOH/1 wt% urea and 3 wt% KOH/4 wt% urea solvent was denoted as PCN30, PCN31 and PCN34 respectively. Chitin pretreated solely by freezing was denoted as PCN00 and was used as control. As shown in Fig. 2 (A), the PCN00 solution stratified after thawing at room temperature, while the chitin suspensions (PCN30, PCN31 and PCN34) appeared to be in a

semi-soluble state. The appearance of solutions with urea concentration of 0 wt%, 1 wt% and 4 wt% were similar, preliminarily demonstrating that the concentration of urea had little effect on the structure of β -chitin.

The effect of urea concentration on the structure of β -chitin was studied by FT-IR spectra. As shown in Fig. 2 (B), the peak at 3270 cm⁻¹ corresponded to N-H stretching, and the two peaks at 1633 cm⁻¹ and 1556 cm⁻¹ corresponded to characteristic bands of amide I and II. The peaks at 1064 cm⁻¹ and 1029 cm⁻¹ corresponded to symmetrical and asymmetric tensile vibrations of C-O-C and C-O, respectively. The characteristic peaks of the pretreated chitin (PCN30, PCN31 and PCN34) were similar, which further suggested that the concentration of urea had little effect on the structure of chitin [23, 30].

X-ray diffraction patterns of various pretreated chitins were further obtained. As shown in Fig. 2 (C), two characteristic peaks at 8.3° and 19.7° were observed, corresponding to a (010) reflection, and a superposition of (100), (110), and (110) reflections respectively, typical of β -chitin. These two peaks shifted to 9.1° and 19.2° when the chitin was treated with 3 wt% KOH/ 0 wt% urea. The shift of peaks indicated the slight transformation from β -chitin to α -chitin [36, 37]. In addition, the peak intensity of the two peaks decreased which demonstrated that the intra and inter-molecular hydrogen bonds of chitin were broken during pretreatment process, leading to the reduction of crystallinity [38]. Further increase of the urea concentration to 1 wt% and 4 wt%, made little difference compared to PCN30. The crystallinity of PCN31 (72.5%) and PCN34 (68.5%) was close to PCN30 (72.7%), as shown in Fig. 2 (D). The crystal size of pretreated chitin (PCN34) was larger than PCN30 and PCN31, which might be due to the high concentration of urea increasing the solubility of chitin and subsequently the remaining crystalline material

aggregated into larger crystals.

The effect of urea concentration on the structure of chitin was further characterized by SEM. As shown in Fig. 2 (E), the morphology of pretreated chitin (PCN00) was typical of β -chitin showing a compact surface. When the chitin was treated with 3 wt% KOH, the PCN30 showed a sheet structure with many holes on the surface. This was ascribed to partial dissolution of chitin 3 wt% KOH solution and subsequent regeneration after the KOH was removed. Further increase of the urea concentration to 1 wt% caused the surface of the chitin to become rougher and some nanofibers were observed. When the concentration of urea reached to 4 wt%, chitin showed a large sheet morphology with small lamellae on the surface. This might be due to increasing dissolution of chitin in the higher urea concentration solution and the regeneration after removal of the solvent. No loose structures or nanofibers were observed at the higher concentration of added urea, which suggested that the concentration of urea had little effect on the structure of chitin.



Fig. 2. (A) Appearance of β-chitin (PCN00, PCN30, PCN31, and PCN34) solution after freeze
pretreated with 3 wt% KOH and different concentration of urea. (B) FT-IR spectra of β-chitin
pretreated with different concentration of urea. (C) X-ray diffraction patterns of pretreated β-chitin.
(D) Changes of crystallinity and crystal size of theβ-chitin pretreated different concentration of
urea. (E) SEM images of different KOH/urea pretreated β-chitin.

β-chitin obtained with various concentrations of KOH/urea pretreatment was oxidized by

3.3 TEMPO-mediated oxidation of pretreated β-chitin to prepare nanofibers

TEMPO/NaBr/NaClO system with 5 mmol/g NaClO. FT-IR spectra of original and TEMPO oxidized β -chitin pretreated in different KOH/urea solutions are presented in Fig. 3 (A). The characteristic peak at around 3270 cm⁻¹ is the O-H stretching of chitin [39-41]. The peak intensity reduced in OPCN71, suggesting the conversion of some hydroxyl groups to carboxyl groups. The peak at 1555 cm⁻¹ corresponded to N-H bending and C-N stretching. This peak intensity reduced in the oxidized chitins due to the hydrogen bonding network in chitin being disrupted by the carboxyl groups. A significant peak appeared around 1404 cm⁻¹ in the oxidized samples, especially in OPCN31 and OPCN71. This peak is characteristic of the asymmetric stretching of carboxylate anions (-COO⁻) [27]. The increasing intensity of this peak indicated the formation of more carboxylate groups. The peak around 1066 cm⁻¹, which corresponds to the C-O-C stretching of glycosidic linkages, disappeared in the oxidized chitins. This might be due to molecular partial glycosidic linkages breaking during oxidation.

 β -chitin and oxidized β -chitin pretreated by KOH/urea were further studied by X-ray diffraction. The results are shown in Fig. 3 (B). The original β -chitin showed two strong peaks at 8.3° and 19.7°, corresponding to the (010) and (110) peaks of chitin [42, 43]. The peak intensity at 8.3° and 19.7° reduced in the oxidized chitin. This might be due to the selective conversion of the primary hydroxyl groups at the C6 position of the glucose units in β -chitin into carboxyl groups by TEMPO oxidation. This introduction of carboxyl groups increases the negative charge density on the chitin chains, leading to electrostatic repulsion between the chains. In addition, the native

structure of β-chitin is stabilized by extensive hydrogen bonding between the hydroxyl groups of
adjacent chains. The oxidation process disrupts these hydrogen bonds because the carboxyl groups
formed are less capable of participating in hydrogen bonding compared to hydroxyl groups [24].
This disruption weakens the interchain interactions.

The zeta potential and particle size of the oxidized β -chitin were obtained and shown in Fig. 3 (C). The absolute potential values of all samples were greater than 20 mV, indicating that the solution had good colloidal stability in both neutral (pH 7.0) and basic (pH 9.0) conditions. Obviously, the absolute value of the potential at pH 9.0 was larger than at pH 7.0, suggesting that the nanofibers were more likely to disperse in an alkaline environment. This was probably due to the substitution of the primary hydroxyl group at the C_6 position of the nanofibers with the COOH group after TEMPO-mediated oxidation, resulting in an anionic charge on the surface of -COO⁻. When the concentration of KOH in the pretreatment solution increased to o 3 wt%, the absolute value of the potential gradually improved, which may be ascribed to the increased number of free C₆ carboxyl groups. In other words, pretreatment enhanced the oxidation efficiency and added more carboxyl groups to the chitin molecule. However, the zeta potential became less negative when the pretreatment KOH concentration increased to 7 wt%. This might be due to the aggregation of the water insoluble part of OPCN71 and subsequent reduction in the effective surface charge. The particle size decreased from OPCN00 to OPCN31 which indicated that the oxidation process, along with moderate KOH treatment, resulted in smaller particles. The particle size of OPCN71 increased again which might be due to that the aggregation of OPCN71 molecules.

The water-insoluble fraction and carboxyl content of the oxidized β-chitin were also obtained

and shown in Fig. 3 (D). The water-insoluble fraction consistently decreased with increasing KOH concentration from OPCN00 to OPCN71. This was because the high concentration of KOH lowers the crystallinity of the pretreated chitin (PCN71). The swelling degree of chitin was higher in higher concentrations of KOH solution. These facilitate the TEMPO oxidation process transforming more β -chitin into water-soluble molecules. The carboxyl content increased from OPCN00 to OPCN31, indicating more effective oxidation and the introduction of carboxyl groups in the chitin molecule after KOH/urea pretreatment. The carboxyl content of OPCN71 slightly decreased compared to OPCN31 which might be due to part of the water-insoluble oxidized chitin (OPCN71) aggregating and reducing surface charge. Transmission electron microscope (TEM) images of the insoluble part of chitin suspension were obtained. As shown in Fig. 3 (E), part of OPCN00 to OPCN01 nanofibers intertwined with

each other, forming a close network. This was due to the retention by β-chitin of its native structure with minimal dispersion in the absence of significant KOH treatment. The nanofibers were not well separated, which indicated that oxidation was limited. A large portion of individual nanofibers were observed when the chitin was pretreated with 3 wt% KOH. The fibers appeared more evenly spread out compared to OPCN00 and OPCN01, due to the pretreatment and oxidation leading to better dispersion and increased surface area. Moderate KOH concentration (3 wt%) pretreatment appears to be optimal for TEMPO oxidation, resulting in significant structural breakdown of the chitin fibers into individual fibrils. However, when the pretreatment concentration of KOH increased to 7 wt%, the morphology showed a highly disrupted structure with fewer visible fibers. The chitin nanofibers were more amorphous, and tended to aggregate. High KOH concentration might lead to excessive pretreatment, resulting in significant structural

disruption, such as a large reduction in crystallinity [44]. After oxidation, some fibers depolymerized into small chitin molecules and formed an amorphous mass, which could be the reason for the aggregation and lack of distinct fibrillar structure. Thus, KOH/urea pretreatments contributed to easier fibrillation of chitin bundles and reduced length of chitin fibers during TEMPO-mediated oxidation. These results suggest the possibility of high efficiency preparation of individualized nanofibers via KOH/urea pretreatments.



Fig. 3. (A) FT-IR spectra of original and TEMPO oxidized β -chitin pretreated with different KOH/urea mixtures. (B) X-ray diffraction patterns of TEMPO oxidized β -chitin pretreated with different KOH/urea mixtures. (C) Zeta potential and particle size of various TEMPO oxidized β -chitin. (D) Water-insoluble fraction and carboxyl content of the TEMPO oxidized β -chitin pretreated with different KOH/urea mixtures. (E) TEM images of TEMPO oxidized β -chitin pretreated by diverse KOH/urea solutions.

The concentration of NaClO also had influence on the structure of the oxidized chitin nanofibers. Here, the concentration of NaClO was set to be 5 and 10 mmol/g and the pretreated chitin PCN31 was used. The oxidized chitin was firstly characterized by TEM. As shown in Fig. 4 (A), the chitin showed well-dispersed nanofibers morphology with little clustering, which suggested that 5 mmol/g NaClO is effective in breaking down the β -chitin into nanofibers. In the case of a further increase in the concentration of NaClO to 10 mmol/g, the resultant chitin nanofibers appeared more disrupted, shorter and with an amorphous structure compared to the nanofibers oxidized with 5 mmol/g NaClO, as shown in Fig. 4 (B). This was ascribed to higher concentration of NaClO possibly leading to over-oxidation which can result in partial degradation of the β -chitin [27].

The FT-IR spectra of TEMPO-oxidized chitin prepared with various NaClO addition levels are displayed in Fig. 4 (C). In comparison to chitin pretreated without NaClO, the absorption peak at 3427 cm⁻¹ disappeared following the addition of NaClO, correspond to O-H stretching, and the intensity of peaks at 3263 cm⁻¹, 3099 cm⁻¹, 2877 cm⁻¹ were weakened, correspond to N-H stretching and CH₂ symmetric and asymmetric stretching, respectively. Most importantly, a new peak appeared at 1730 cm⁻¹ and became larger at higher amounts of NaClO, confirming the presence of carboxyl groups in oxidized chitins. The new peak at 1404 cm⁻¹ corresponded to asymmetric stretching of carboxylate anions (-COO⁻), which also demonstrated that the chitin had been oxidized [45]. Furthermore, the XRD profile of oxidized chitin was maintained in the absence of NaClO, exhibiting two distinctive crystalline reflections at 8.9° and 19.0°, corresponding to (110) and (010) reflections of β -chitin, as shown in Fig. 4 (D). When 5 mmol/g NaClO is applied to chitin, the intensity of the two reflections was dramatically reduced, as a result of oxidation. When the amount of added NaClO was increased to 10 mmol/g, the intensity of (110) and (010) reflections became weaker due to excess NaClO.



Fig. 4. (A) TEM image of the TEMPO oxidized β-chitin pretreated with 3 wt% KOH/ 1 wt% urea
(5 mmol/g NaClO). (B) TEM image of the TEMPO oxidized β-chitin pretreated with 3 wt% KOH/
1 wt% urea (10 mmol/g NaClO). (C) FT-IR spectra of TEMPO oxidized β-chitin pretreated with 3
wt% KOH/ 1 wt% urea. (D) XRD diffraction patterns of TEMPO oxidized β-chitin pretreated with 3
3 wt% KOH/ 1 wt% urea.

3.4 TEMPO oxidized pretreated α-chitin to prepare nanofibers

The pretreatment solvent can not only to treat β -chitin but also can be used to pretreat α-chitin. The microstructure of TEMPO oxidized original and KOH/urea (3 wt%, 1 wt%) pretreated α-chitin was observed by transmission electron microscope (TEM). As displayed in Fig. 5 (A), the TEMPO oxidized α -chitin showed nanofiber structure with some aggregation. The image in the insert shows that the solution was a cloudy suspension with less clarity, which indicated that the chitin nanofibers aggregated into larger bundles. Compared to the original α -chitin, the TEMPO oxidized α -chitin pretreated with 3 wt% KOH/1 wt% urea showed a well-dispersed nanofibers morphology with 250-450 nm in length and 8-25 nm in cross-sectional diameter, as shown in Fig. 5 (B). The inserted image shows a light blue solution indicating that the 415 chitin nanofibers were stable and well-dispersed in the solution, scattering light at shorter 416 wavelength indicates particle size of the order of 100 nm. This was due to the looser structure of 417 chitin following pretreatment with KOH/urea mixture and facilitating the subsequent TEMPO 418 oxidation process.

FT-IR characterization was measured to investigate the changes in chemical structure of α -chitin after TEMPO-mediated oxidation. As displayed in Fig. 5 (C), in the spectrum of α -chitin, the broad absorption at 3435 cm⁻¹ corresponded to O-H stretching, and peaks at 3259 cm⁻¹ and 3099 cm⁻¹ corresponded to N-H stretching. The absorption peaks at 1656 cm⁻¹ and 1614 cm⁻¹ associated with amide I region, are the characteristic peaks of α -chitin [46]. The peak associated with amide II presented at 1557 cm⁻¹. The peak at around 1060 cm⁻¹ is the C-O-C stretching from glycosidic linkages [47]. In the spectrum of TEMPO oxidized α -chitin, the amide I region merged into a peak at 1618 cm⁻¹ and a new peak at 1404 cm⁻¹ appeared, which demonstrated the chitin had been oxidized in the presence of carboxylate anions (-COO⁻) [26]. In the spectrum of TEMPO oxidized α -chitin pretreated with KOH/urea, the peaks at 3435 cm⁻¹ and 1060 cm⁻¹ weakened, was due to the conversion of -OH groups and breakage of molecules during oxidation. In addition, a new peak was observed at 1404 cm⁻¹ with intensity greater than the TEMPO oxidized original α-chitin, which demonstrated that the pretreatment process facilitated the oxidation process and that more carboxylate anions (-COO⁻) were present in the oxidized chitin [48]. The zeta potential of TEMPO oxidized pretreated α -chitin was -49.91 mV, which was higher than that of the TEMPO oxidized original α -chitin (-41.23 mV).

435 The α-chitin and TEMPO oxidized original or pretreated α-chitin were further studied by
436 X-ray diffraction spectrum. The results are shown in Fig. 5 (D). The original α-chitin showed two

437 strong peaks at 9.0° and 19.0°[49]. The peak intensity at 9.0° and 19.0° reduced in the oxidized 438 α -chitin without KOH pretreatment, which indicated a partial loss of crystallinity due to TEMPO 439 oxidation. The peak intensity further decreased in the XRD of oxidized α -chitin pretreated with 440 KOH/urea, which was due to the pretreatment enhanced oxidation process. More carboxyl groups 441 formed in the oxidized α -chitin pretreated with KOH, which would result in a more significant 442 disruption of the crystalline structure to the chitin.



Fig. 5. (A) TEM image of TEMPO oxidized original α-chitin. (B) TEM image of TEMPO
oxidized α-chitin pretreated with 3 wt% KOH/ 1 wt% urea. (C) FT-IR spectra of α-chitin and
TEMPO oxidized α-chitin pretreated with 3 wt% KOH/ 1 wt% urea. (D) XRD diffraction patterns
of α-chitin and TEMPO oxidized α-chitin pretreated without or with 3 wt% KOH/ 1 wt% urea.

3.5 Reuse of the KOH/urea mixture

450 The KOH/urea solution could be reused as a pretreatment solvent for β -chitin. The KOH/urea 451 was separated by filtration, as shown in Fig. 6 (A). The effects of recycled 3 wt% KOH/ 1 wt% 452 urea mixture on the structure of β -chitin was firstly studied by FT-IR. As shown in Fig. 6 (B), the

FT-IR spectrums of the chitin pretreated with recycled KOH/urea mixture were similar, which demonstrated that the KOH/urea mixture was mild and would not change the chemical properties of the chitin. The pretreated chitin was further characterized by SEM technology. As observed in Fig. 6 (C-F), in comparison to the original β -chitin as mentioned, the smooth surface become wrinkled, and the dense stacks became fragmented. Furthermore, a large portion of the generated surface regions were rough, induced by the swelling effect of KOH pretreatment. In addition, more visible fibrillated edges in a looser microstructure could be observed, confirming the enhanced fibrillation of chitin due to KOH/urea pretreatment. This result provided a better proof of the efficient fabrication of individualized chitin nanofibers via KOH/urea pretreatment. Meanwhile, it was important to note that the microstructure of chitin was similar, even though the KOH/urea mixture was used four times. Recycling the KOH/urea system facilitates environmental protection, endowing the system with the unique feature of a high-efficiency pretreatment.



Fig. 6. (A) Schematic illustration of reusable process of KOH/urea mixture. (B) FT-IR spectra of
β-chitin after each 3 wt% KOH/ 1 wt% urea pretreatment. (C-F) SEM images of β-chitin after
being pretreated by recycled 3 wt% KOH/ 1 wt% urea mixture.

3.6 3D printing of the chitin nanofibers stabilized Pickering emulsions

The chitin nanofibers prepared by TEMPO oxidation method can be used to stabilize Pickering emulsions. The prepared high internal phase Pickering emulsion can be applied in 3D printing. As shown in Fig. 7, the chitin nanofiber stabilized Pickering emulsion was stable and gel-like in the bottle which demonstrated that the emulsion is homogeneous and suitable for 3D printing. The digital images show uniform distribution of droplets within the emulsion, suggesting effective stabilization by chitin nanofibers. The printed figures, including "1" "2" and "7" were clear and well-defined, indicating the emulsion can be applied in 3D printing. In addition, a variety of shapes and letters can also be printed, including "U" "J" "S", a paw, and a tree, further indicating fine details can be achieved. The successful formation of these complex shapes demonstrated the potential of chitin nanofiber stabilized Pickering emulsions as stable inks for 3D printing applications.

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Fig. 7. 3D printing of the chitin nanofibers stabilized high internal phase Pickering emulsion

485 4. Conclusions

In this study, we have demonstrated an enhanced method for preparing chitin nanofibers bypretreating the chitin in a low-temperature frozen KOH/urea mixture. The concentration of KOH

in the pretreatment solvent has significant effects on the structure of β -chitin. The crystallinity of β-chitin decreased with increasing concentrations of KOH in the pretreatment solvent. The concentration of urea showed minimal effects on the β-chitin structure. The pretreatment facilitated the subsequent TEMPO oxidized process to prepare β-chitin nanofibers. The morphologies of the prepared β -chitin nanofibers from rod-like to amorphous-like can be regulated by adjusting the concentration of KOH during pretreatment. The carboxyl content and zeta potential of nanofibers prepared from TEMPO oxidized β-chitin pretreated with KOH/urea mixture were enhanced compared to the original β -chitin. In addition, the KOH/urea mixture can be used to treat α -chitin. The pretreatment solvent can be reused to treat chitin, which demonstrated that the sustainability and economic advantages of this solvent. This freeze KOH/urea mixture pretreatment method also showed promise for the treatment of other biomass materials for nanofiberization. Future work may explore the extension of this method to other biomasses and prepare nanofibers with designed morphologies and properties.

502 CRediT authorship contribution statement

Fuyuan Ding: Conceptualization, Writing-original draft, Funding acquisition, Formal
analysis, Visualization. Siman Long: Investigation, Methodology, Writing-original draft. Xiaowei
Huang: Review & editing. Jiyong Shi: Review & editing. Megan Povey: Review & editing.
Xiaobo Zou: Supervision, Review & editing.

508 Declaration of competing interest

- 509 The authors declared that they have no conflicts of interest to this work.

511 Data availability

512 Data will be made available on request.

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- Low-temperature KOH/urea mixture freeze pretreatment reduces β-chitin crystallinity
- Nanofibrillation of chitin by TEMPO method enhanced after pretreatment
- KOH concentration can control the nanofiber morphology
- KOH/urea pretreatment method is reusable and applicable to other biomasses

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Fuyuan Ding

School of Food and Biological Engineering, International Joint Research Laboratory of Intelligent Agriculture and Agri-products Processing, Joint Laboratory of China-UK on Food Nondestructive Sensing, Jiangsu University, Zhenjiang, 212013, China