Production of chitin from shrimp shell powders using *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 8014 successive two-step fermentation

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ABSTRACT

Shrimp shell powders (SSP) were fermented by successive two-step fermentation of *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 8014 to extract chitin. Taguchi experimental design with orthogonal array was employed to investigate the most contributing factors on each of the one-step fermentation first. The identified optimal fermentation conditions for extracting chitin from SSP using *S. marcescens* B742 were 2% SSP, 2 h of sonication time, 10% incubation level and 4 d of culture time, while that of using *L. plantarum* ATCC 8014 fermentation was 2% SSP, 15% glucose, 10% incubation level and 2 d of culture time. Successive two-step fermentation using identified optimal fermentation conditions resulted in chitin yield of 18.9% with the final deproteinization (DP) and demineralization (DM) rate of 94.5% and 93.0%, respectively. The obtained chitin was compared with the commercial chitin from SSP using scanning electron microscopy (SEM), Fourier transform infrared spectrometer (FT-IR) and X-ray diffraction (XRD). Results showed that the chitin prepared by the successive two-step fermentation exhibited similar physicochemical and structural properties to those of the commercial one, while significantly less use of chemical reagents.

Key words: Chitin, Shrimp shell powders, *Lactobacillus plantarum* ATCC 8014, *Serratia marcescens* B742, Successive two-step fermentation

1. Introduction

Chitin, one of the most abundant renewable biopolymer on earth, is a linear chain molecule composed of several hundred units of (1→4)-2-acetamido-2-deoxy-β-D-glucan. Based on the different orientations of its microfibrils, chitin can be classified into three forms including α, β and γ. Among them, α-chitin is the most widely used and can be prepared from a variety of natural sources.[1] Chitosan, the most important derivative of chitin, is prepared by deacetylization of chitin. Chitin and chitosan have many unique functional properties including biocompatibility, biodegradability, and non-toxicity, and have been widely applied in the field of food, agriculture, medicine, and materials.[2-7] Up to now, chemical,[8,9] enzymatic,[10,11] and microbiological methods[12-15] have been used for preparing chitin from shrimp shell powders (SSP). The chemical method involves deproteinization (DP) and demineralization (DM) using strong acids and/or alkaline. However, the use of these chemicals can seriously pollute the ecological environment, produce abundant waste, and are harmful to human health. In addition, the application of acid and alkali can hydrolyze the polymer, resulted in inconsistent physiological properties of the final product. Along with increased demands on environment-friendly society and the development of fermentation technology, more eco-friendly processes using enzymatic and microbiological methods for producing chitin have attracted great interests. The enzymatic method includes the use of trypsase, papain, and pepsase.[10] However, the high cost of enzymes and the low extraction efficacy are some of the pitfalls of this method.

Lately, there are increased interests in applying protease, chitinase and lactic acid produced by microbial fermentation to extract chitin because this method is relatively simple and less expensive, thus overcame the shortcomings of the chemical and enzymatic treatments. Moreover, extraction of chitin using microbial fermentation prevents the uneven deacetylation and relevant

molecular weight reduction caused by strong acid and alkali. Furthermore, the remaining fermentation waste contains abundant protein hydrolysate (amino acid and polypeptide), which can be collected as culture medium of other microbes for decreasing the cost of wastewater treatment.[16] Therefore, microbial fermentation is a promising method for the preparation of chitin.[17-18]

Serratia marcescens strains are well known for producing enzymes including protease, chitinase and chitosanase, and have been widely used to extract the bioactive molecules by decomposing proteins from shrimp shell biowaste.[16,19,20] The proteins from shrimp shell powders (SSP) can be broken down to water-soluble protein hydrolysates by means of enzymes produced during *S. marcescens* fermentation.[16] Meanwhile, *Lactobacillus* strains produce lactic acid and may be employed to remove Ca ion from shrimp biowaste.[21-23] Therefore, these two bacteria strains were employed to extract chitin from SSP in this study. In addition, applying high-intensity ultrasound during chitin preparation from shrimp biowaste showed significant reductions in time and solvent requirement,[24,25] thus sonication time was considered as a contributing factor during *S. marcescens* B742 fermentation in this study.

The objectives of this work were to first determine the optimal fermentation conditions of SSP using *S. marcescens* B742 and *L. plantarum* ATCC 8014 one-step fermentation by considering their efficacy of DP and DM, respectively, and then to investigate the successive two-step fermentation using identified optimal single fermentation conditions. Physicochemical and structural properties of extracted chitin were evaluated by scanning electron microscopy (SEM), Fourier transform infrared spectrometer (FT-IR), and X-ray diffraction (XRD) assays. Base on our best knowledge, no study has reported the preparation of chitin from SSP using successive two-step fermentation, where all contributing factors were statistically considered.

2. Materials and methods

2.1. Materials

Shrimp shells of headless *Penaeus vannamei* were collected from Nantong Xingcheng Biological Products Factory (Nantong, China) and stored under dried conditions till further usage. The dried samples were pulverized with Waring blender (Shanghai Shibang Machinery Co., Ltd. China) and passed through a 0.75 mm-sieve to prepare shrimp shell powders (SSP). *L. plantarum* ATCC 8014 was obtained from American Type Culture Collection (ATCC, USA), and *S. marcescens* B742 from Shanghai Institute of Industrial Microbiology (Shanghai, China). The Mann-Rogosa Sharpe (MRS) broth culture and Luria Bertani (LB) culture broth were purchased from Shanghai Yayan Biotechnology Co., Ltd. (Shanghai, China). Other chemical reagents were all obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of inoculum

The two bacteria strains preserved in ampoule tube were broken up and the cells were transferred into 100 mL of sterile MRS broth for *L. plantarum* ATCC 8014 and LB broth for *S. marcescens* B742 and incubated with shaking (160 r/min) at 37 ℃ and 30 ℃ for 2 days, respectively. To prepare an inoculum for strain fermentation, 4.0 mL of the starter culture was transferred into 100 mL of sterile MRS broth (2% inoculation) or LB culture broth, and incubated with shaking (160 r/min) at 37 ℃ and 30 ℃ for 2 days, respectively. The inoculum yielded a cell concentration of approximately 10^9 and 10^8 colony-forming units (CFU)/mL, respectively.

2.3. Optimization of fermentation conditions using *L. plantarum* ATCC 8014 and *S.*

***marcescens* B742**

To investigate the most contributing factors and the suitable level of each contributing factor in the one-step fermentation using *L. plantarum* ATCC 8014 and *S. marcescens* B742, Taguchi experimental design with orthogonal array was employed.[26] Each experiment had nine treatment trials as shown in Table 1. The orthogonal array offered a simple and systematic approach to optimize the fermentation conditions and significantly reduced the numbers of treatment combinations when multiple factors were considered.[26]

Four independent factors were considered for the optimization of deprotenization using *S. marcescens* B742, including the amount of SSP (A, % w/w), sonication time (B, h), incubation level (C, % v/w) and culture time (D, day). The resultant media with same volume were aerobically cultured at 30 ℃ for 1-5 d on a rotary shaker (160 r/min) (Shanghai Jing Hong Laboratory Instrument Co., Ltd, Shanghai, China), the supernatants were collected for the measurement of protease and chitinase. For the optimization of demineralization using *L. plantarum* ATCC 8014 fermentation, four tested treatment factors were the amount of SSP (A, % w/w), glucose concentration (B, % v/w), incubation level (C, % v/w) and culture time (D, day) (Table 1). The resultant media with the same volume were anaerobically cultured at 37 ℃ for 1-5 d on a rotary shaker (160 r/min), and the supernatants were collected for the measurement of pH and total titratable acidity (TTA).

2.4. Analysis of chemical properties of samples

The pH and TTA of the supernatant during *L. plantarum* ATCC 8014 fermentation were determined using a potentiometer (pH 210 HANNA, Italy) and by titration with 0.1 N NaOH to a final pH of 8.0, respectively.

The total nitrogen content was measured by Kjeldahl 2300 (FOSS, Danmark) in an automated apparatus.[27] Corrected protein contents were calculated by the subtraction of the chitin nitrogen to the total nitrogen content and multiplied by 6.25. The dried samples were placed in an oven at 550 ℃ for 12 h to quantify ashes. The ashes were collected by centrifuge tube and detected by Flame Atomic Absorption Spectrometry (Varian AA, USA). DP% was calculated as:

$$DP(\%) = \frac{\left[\left(P_O \times S_O\right) - \left(P_R \times S_R\right)\right] \times 100}{P_O \times S_O}$$

where P_O and P_R were the protein or ash content in raw and fermented samples, respectively; S_O and S_R were the weight of raw and fermented samples (g), respectively. DM (%) was calculated using the same equation, but replacing P_O and P_R with A_O and A_R in which represented ash content in raw and fermented samples, respectively. All experiments were conducted in triplicate and the values were reported as mean ± standard deviation.

The fermented supernatant was filtered, collected and then detected for the protease and chitinase activity. For measuring protease activity, 0.2 mL diluted enzyme solution (the supernatant after *S. marcescens* B742 fermentation) was mixed with 1.25 mL of 1.25% casein in phosphate buffer (pH 7.0±0.2) and incubated at 37 ℃ for 30 min. The reaction was stopped by adding 5 mL of 0.19 M trichloroacetic acid (TCA). The mixture was then centrifuged, and the soluble peptide in the supernatant fraction was measured with tyrosine as the reference.[28] One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per min. Chitinase activity was measured by incubating 0.2 mL of the enzyme solution (the supernatant after *S. marcescens* B742 fermentation) with 1 mL of 0.3% (w/v) water soluble chitosan in 50 mM phosphate buffer (pH 7.0±0.2) at 37 ℃ for 30 min. The reaction was stopped by heating above solution at 100 ℃ for 15 min. The amount of reducing sugar produced was

measured with glucosamine as reference. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmoL of reducing sugars per min.[29]

Bacterial growth of *S. marcescens* B742 and *L. plantarum* ATCC 8014 strains was determined by measuring the optical density (OD) of the cell suspension at a wavelength of 660 nm and 600 nm, respectively.

2.5. Scanning electron microscopy (SEM) analysis

SEM (FEI SIRION-200, USA) was used to clarify the superficial characteristics of the samples at 2000 × magnification. The samples were fixed on a sample holder, dried by a critical point dryer (LADD 28000, USA), and coated with a thin gold layer of 3 mm by a sputter coater (JBS E5150, USA) for conductivity.

2.6. Fourier transform infrared spectroscopy (FT-IR) analysis

A Nexus 670 FT-IR (ThermoNicolet Co., Mountain View, CA) was used to record infrared spectra of samples between 4000 and 500 cm^{-1}. The degree of acetylation (DA) were determined using the following equation.[30]

$$DA(\%) = 115 \times \left(\frac{A_{1655}}{A_{3450}} \right)$$

where A_{1655} and A_{3450} were the absorbance of samples at wave number of 1655 and 3450 cm^{-1}, respectively. All samples were scanned for three times and DA values were reported as mean ± standard deviation.

2.7. X-ray diffraction

The wide-angle X-ray diffraction (WAXD) analysis was applied to detect the crystallinity of chitin prepared by single and successive two-step fermentation and their patterns were recorded using a Rigaku III diffractometer (Rigaku Co., Japan). 2θ was scanned from 5 to 50 ° at a coating time of 2 s with an angle step width of 0.05°. The crystallinity index (Cr_{Ipeak}) was calculated as [31]

$$Cr_{Ipeak} = \frac{(I_{110} - I_{am})}{I_{110}}$$

where I_{110} was the maximum intensity (arbitrary units) of the (110) lattice diffraction pattern at 2θ = 20° and I_{am} was the intensity of amorphous diffraction in the same units at 2θ = 16°.

2.8. Experimental design and data analysis

The Taguchi design with orthogonal array was employed in this study to identify the most contributing factors during *S. marcescens* B742 and *L. plantarum* ATCC 8014 fermentation. [26] Similar methods as described by Jung and Zhao were applied for the data analysis. [26] In brief, one-way ANOVA was carried out to determine the significant differences among different treatment factors and their levels, and the LSD test was done for multiple comparisons in the Taguchi design method ($P<0.05$) using the SPSS program (SPSS 17.0, IBM SPSS institute, Inc., USA). All experimental data were observed in triplicate, and means ± standard deviations were reported.

3. Results and discussions

3.1. Optimal conditions for deproteinization using *S. marcescens* B742 fermentation

DP (%) and DM (%) and the rank of each contribution factor on these two parameters are reported in Table 2. For DP, R_i value for culture time was the lowest among all contributing factors, while SSP, sonication time and incubation level were ranked first, second and third,

202 respectively. SSP provides the rich carbon and nitrogen source for the growth of *S. marcescens*
203 B742, thus was considered as an important contributing factor. Therefore, 2% SSP, 2 h of
204 sonication time, 10% of incubation level and 4 d of culture time were identified as the optimal
205 treatment conditions for achieving high efficacy of deprotenization. Note that the reason of
206 selecting 4 d for culture time was because the enzyme activity increased with culture time and
207 reached the maximum at 4 d (Fig. 1).

208 Previous study reported that ultrasonic treatment improved the efficacy of DP when using
209 chemical extraction method.[24] However, no study had reported the effect of ultrasonic treatment
210 on the deproteinization using microbial fermentation. In this study, sonication for 1, 1.5 and 2 h
211 with sonication frequency 40 kHz and power 300 W (Ultrasonic processor, model KH-600E,
212 Hechuang, Shanghai) was evaluated since our preliminary results showed that DP was not
213 significantly improved when sonication time was longer than 2 h (data not shown). It might be
214 explained that the high-intensity ultrasonic treatment caused swelling of the microfibers of chitin
215 and resulted in a loose structure, thereby made the reagent easily permeate to improve
216 deprotenization efficacy. For DM, the incubation level was ranked the first and the sonication
217 time ranked the lowest among all contributing factors. Therefore, ultrasonic treatment did not
218 improve the efficacy of DM (Table 2). [24]

219 The cell growth, protease and chitinase activity of *S. marcescens* B742 using optimal
220 fermentation conditions are exhibited in Fig. 1. The maximum protease and chitinase activity
221 was displayed as 160 and 24.5 mU/ml at 4 d, respectively. Previous studies also found that the
222 maximum protease and chitinase activity was 160 and 22 mU/mL, respectively when using 2%
223 squid pen powders as C/N source.[20] However, the maximal DP only reached 30.88% among all
224 treatments. Therefore, the efficacy of DP was not satisfactory using *S. marcescens* B742

fermentation alone. This might be explained that the protein and chitin in the skeletal tissue are tightly combined to form a protein-chitin matrix, which made it difficult to remove protein from SSP by a single fermentation treatment.[24]

3.2. Optimal treatment conditions for demineralization using *L. plantarum* ATCC 8014 fermentation

The key factors determining the fermentation efficacy of *L. plantarum* strains might include the amount of sugar, the inoculation level, and the culture time.[32] As stated above, SSP provided C/N source for the growth of strains, thus the level of SSP was also considered as a contributing factor. Ca, DM, pH and TTA from *L. plantarum* ATCC 8014 fermentation are reported in Table 3. R_i value for SSP and incubation level on Ca, pH and TTA were ranked first and second, respectively. Because the aim of *L. plantarum* ATCC 8014 fermentation was to remove $CaCO_3$, the concentration of Ca ion was considered as an indicator for the optimization of the fermentation conditions. Based on the statistical results from the Taguchi design, SSP significantly affected Ca, DM, pH and TTA, but both culture time and glucose concentration had no significant effect on Ca. However, the culture time was the second most contributing factor on DM. This may be because DM closely related to the production of lactic acid produced by *L. plantarum* ATCC 8014. Therefore, the optimal conditions for *L. plantarum* ATCC 8014 fermentation were 2% SSP, 15% glucose, 10% incubation and 2 d of culture time. Because the culture time had no significant effect on Ca, 2 d was selected as optimal culture time. It should also note that the growth of *L. plantarum* ATCC 8014 was slightly inhabited when the pH decreased to ~3.2 at 2 d (Fig. 2).

It was found that SSP could not provide enough nutrition for the growth of *L. plantarum*

ATCC 8014 and the type of sugar and its initial concentration had significant effect on the production of lactic acid.[33] Glucose, lactose, maltose, rice and manioc might be added into the culture broth for providing nutritional substances.[33] In this study, it was found that the concentration of Ca ion is the lowest when adding 15% glucose. Although the high concentration of glucose could greatly shorten the fermentation cycle, the fermentation could be inhibited and pH value could increase when the concentration of glucose surpassed 15%.[34]

The highest DM obtained was only 65.9% among nine experimental runs, thus not satisfactory for using *L. plantarum* ATCC 8014 fermentation alone. Fortunately, the protein on SSP was partially removed using *L. plantarum* ATCC 8014 fermentation (data not shown), which might be because SSP provided C/N source for the bacterial growth to remove the protein in the skeletal tissue of SSP, thereby the partially soluble protein was dissolved in fermentation medium and washed. Because the deproteinization efficacy was not high enough, DP (%) values were not reported in Table 3. Based on the results from this study, SSP was closely related to the efficacy of DM. This result was consistent with previous studies, in which the satisfactory fermentation could be obtained when using glucose as C/N source.[32,35]

The changes of cell growth, pH and TTA during *L. plantarum* ATCC 8014 fermentation using the optimal fermentation conditions are illustrated in Fig. 2. The abundant lactic acid produced could dissolve $CaCO_3$ to obtain water-soluble calcium lactate, hence the minerals could be separated from chitin.[36] The pH change was closely related to the production of lactic acid and gradually decreased along with the extended culture time.[36] The pH 4.0 was a critical point for the growth of *L. plantarum* ATCC 8014 at 2 d of incubation time. This result was similar to the previous report that the satisfactory chitin recovery was obtained at optimized fermentation conditions of pH= 4.3, 5% inoculum level, 15% glucose and 72 h of incubation time at 37±1

271 °C.[35] By considering the unsatisfactory DP and DM using *S. marcescens* B742 and *L. plantarum*
272 ATCC 8014 fermentation alone, successive two-step fermentation was thus employed.
273
274 **3.3. Successive fermentation**
275 Identified optimal DP conditions using *S. marascens* B742 fermentation was further applied
276 on SSP, and obtained Ca, DM and DP at 4 d of culture time were 1363.77 ± 9.79 mg/kg, 51.83%
277 and 83.37%, respectively (Table 4). DP at 4 d was not significantly different from that at 5 d,
278 thus 4 d of fermentation using *S. marascens* B742 was chosen for the successive two-step
279 fermentation study. Similarly, Jo et al. prepared the chitin from crab shells using *S. marcescens*
280 fermentation, and reported that DM and DP were 41.2% and 66.8% after 5 d of fermentation,
281 respectively.[13] After applying the optimal DM treatment conditions using *L. plantarum* ATCC
282 8014 fermentation, obtained DM and DP were 85.3% and 60.9%, respectively after 2 d of
283 fermentation, and no further increase after that (Table 4). Hence, 2 d was selected as optimal
284 fermentation time for DM using *L. Plantarum* ATCC 8014 fermentation.
285 After successive two-step fermentation (*S. marcescens* B742 fermentation first followed by *L.*
286 *Plantarum* ATCC 8014 fermentation, Fig. 3), the chitin yield reached 18.9%. It was found that
287 the successive two-step fermentation gave the best result in co-removal of protein and Ca from
288 SSP. As it has been well known that the chitin-protein complex combined in the SSP skeletal
289 tissue is not easy to be removed completely.[24] The reason to implement *S. marcescens* B742
290 fermentation first was because the produced protease could significantly remove the minerals
291 and further loose the structure of swelling chitin microfibers when soaking in fermented medium,
292 thus greatly improved the DP efficacy during *L. Plantarum* ATCC 8014 fermentation. As shown
293 in Table 4, DM and DP were ~94.5% and 93.0%, respectively at the end of two-step fermentation.

These results were similar to the previous findings, in which the yield of chitin from shrimp shell waste was only 7.2% when using chemical method, but increased to 13% when using *L. plantarum* 541 fermentation.[33] It might be explained that when chitin was broken down by strong acid and alkali reagent, it also lead to the undesired deacetylation and depolymerization. Microbial fermentation could avoid these problems, thus promising for the extraction of chitin from shrimp biowaste.

3.4. Analysis of superficial characteristics by scanning electron microscopy (SEM)

SEM images of SSP, SSP after DP by *S. marascens* B742 fermentation, SSP after DM using *L. plantarum* ATCC 8014 fermentation and successive two-step fermentation are shown in Fig. 4. The smashed SSP displayed smooth microfibrillar crystalline structure and left layer structure largely intact (Fig. 4A), while the SEM images of SSP after DM showed more slightly fracture (Fig. 4B) than that of SSP after DP with sonication treatment (Fig. 4C). The graph of DP under the optimal conditions using *S. marascens* B742 fermentation left the stacked layers and showed the signs of perforation (Fig. 4C). These results confirmed the previously stated hypothesis that high-intensity ultrasonic treatment may improve the accessibility of the solvent, thus improved the DP efficacy.[24] The SSP processed by *S. marascens B742* fermentation and successive two-step fermentation (Fig. 4D) was morphologically similar. However, the shrimp shell fragments became highly fractured and spongy after successive two-step fermentation and the chitin sheets became shattered (Fig. 4D). It was apparent that the successive two-step fermentation improved DP and DM efficacy.[37]

3.5. FT-IR and X-ray diffraction analysis

317 The differences in the FT-IR scans among all samples are displayed in Fig. 5. The spectra
318 were characterized by three bands at 1577, 1654, and 2932 cm^{-1}, which corresponded to the
319 vibrations of -NH, -C-O and -CO-CH_3 group, respectively.[37,38] The bands between 890 and 1156
320 cm^{-1} represented polysaccharide structures. It was notable that the band at 2932 and 1577 cm^{-1}
321 for other samples were more intense than that of commercial chitosan (Fig. 5A), which
322 confirmed the existence of chitin.[3] Compared to the commercial chitin, the band observed at
323 2932 cm^{-1} demonstrated an intensification of the peak (Figs. 5C-5E), and suggested the
324 occurrence of deacetylation. DA of the samples after *S. marascen*s B742 fermentation, *L.*
325 *plantarum* ATCC 8014 fermentation and successive two-step fermentation were 25.3%, 83.09%,
326 and 80.17%, respectively (Table 5). It was found that ultrasonic treatment significantly reduced
327 DA of chitin prepared by *S. marascen*s B742 fermentation alone. This may be because ultrasonic
328 treatment destroyed the skeletal structure of chitin and improved the accessibility of acetyle
329 group to reagent during ultrasonic treatment. This effect needs to be addressed in the future
330 studies when incorporating ultrasonic treatment in the extraction of chitin.
331 The crystallinity indexes of commercial chitin and chitin extracted by one-step and
332 successive two-step fermentation were determined from the scattering intensity at two angles,
333 one at 2θ=9-10° and another at 2θ=19-20° (Fig. 6). The results were consistent to the literature,
334 in which the purified chitin had wide-angle X-ray diffraction pattern and showed two crystalline
335 peaks at 2 θ=9.3° and 19.1°.[39] Similarly, Yen and Mau reported that fungal chitin displayed two
336 crystalline reflections at 5.4-5.6° and 19.3-19.6°.[40] The crystallinity index of commercial chitin,
337 chitin obtained from *S. marascens* B742 fermentation, from *L. plantarum* ATCC 8014
338 fermentation and from the successive two-step fermentation were 98.6%, 80.0%, 92.2% and
339 79.2%, respectively (the baseline at 2 θ =16°). Overall, it was found that the application of

ultrasonic treatment reduced the crystallinity of chitin, from 92.15% in *L. plantarum* ATCC 8014 fermentation to 80.04% in *S. marascens* B742 fermentation (Table 5). A lower crystallinity of polysaccharides indicates disruption of intra- and inter-molecular hydrogen bonds, in turn provides the possibility for more efficient chemical modifications in subsequent processing steps.[41] The X-ray diffractograms of chitin powder obtained from the successive two-step fermentation showed narrowed peak areas than the commercial chitin, confirmed that further purification is necessary to obtain satisfactory chitin extractive.

4. Conclusions

The study found that fermentation using *S. marascens* B742 or *L. plantarum* ATCC 8014 alone could not obtain chitin with satisfactory DM and DP efficacy, but the successive two-step fermentation using these two bacteria improved the extraction efficacy with chitin yield of 18.9%, and the resultant chitin has similar physicochemical and structural properties to commercial chitin. The microbial fermentation is a relatively simple and environment-friendly alternative to the chemical method. However, it should note that this study at shaking flask level under laboratory conditions may not be suitable on large scale operations. Hence, modifications and adjustments on the specific treatment conditions and processing procedures might be necessary. Moreover, the application of ultrasonic treatment appeared to change the morphology and crystallinity index of chitin, thus should be considered in the future studies.

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