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A new green technology for direct production of low molecular weight chitosan

Weiping Wang [a,b], Yumin Du [a,*], Yanlin Qiu [b], Xiaoying Wang [a], Ying Hu [b], Jianhong Yang [a], Jun Cai [b], John F. Kennedy [c]

[a] College of Resource and Environmental Science, Wuhan University, Wuhan 430072, PR China
[b] Hubei Provincial Key Laboratory of Industrial Microbiology, Hubei University of Technology, Wuhan 430068, PR China
[c] Birmingham Carbohydrate and Protein Technology Group, Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham B15 2SQ, UK

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Abstract

Low molecular weight chitosan (LMW-chitosan) in the molecular range 5–10 kDa was firstly prepared directly from the *Absidia coerulea* mycelia. To improve the LMW-chitosan production, the solid-state fermentation media were optimized to investigate the influence of substrate and supplemental medium components on LMW-chitosan production. The LMW-chitosan was obtained after treatments with 2% NaOH and 10% acetic acid. Maximal LMW-chitosan production was 6.12 g/kg substrate. Gel permeation chromatography combined with laser light scattering gave a M_w of 6.4 kDa with M_w/M_n of 1.09. FT-IR, X-ray diffraction and ^{13}C NMR spectra of the product showed typical peak distributions the same as those of standard chitosan which confirmed the extracted product to be chitosan-like. The method provided a new, simple and green technology to produce LMW-chitosan directly.

Keywords: *Absidia coerulea*; Green technology; Low molecular weight chitosan; Solid-state fermentation; Media optimization

1. Introduction

Chitosan, the deacetylated derivative of chitin, is a linear copolymer of 1,4-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) units. It is polycationic, nontoxic, biodegradable as well as antimicrobial and has been reported to have numerous applications especially in food, pharmaceutics and cosmetics (Dodane & Vilivalam, 1998; Shahidi, Arachchi, & Jeon, 1999; Kumar, 2000). However, the suitability of chitosan for such applications depends upon the molecular weight and its degree of deacetylation (McGahren, Perkinson, Growich, Leese, & Ellestad, 1984). Low molecular weight chitosan (LMW-chitosan) is attracting increasing attention, since this group of polymers has potentially important biological activities, such as induction of chemotactic migration of polymorphonuclear cells and applications in gene therapy, fat-binding, antithrombotic activity, antitumor activity, antimicrobial activity or stimulating plant growth. Notably, the LMW-chitosans with M_w in the range of 5–10 kDa, are known to possess strong bactericidal and superior biological activities (Kittur, Vishu Kumar, & Tharanathan, 2003), antitumour activity (Qin, Du, & Xiao, 2002), and have potential in DNA delivery systems as DNA carrier (Richardson-Simon, Kolbe-Hanno, & Duncan, 1999).

Hitherto LMW-chitosan has been prepared by degradation of chitosans with a high M_w. This depolymerization could be carried out either with a chemical method (Chang, Tai, & Cheng, 2001), or bio-chemically with enzymes (Zhang & Neua, 2002). The chemical approach has several disadvantages including harsh conditions of hydrolysis,

* Corresponding author. Tel./fax: +86 27 68778501.
E-mail address: duyumin@whu.edu.cn (Y. Du).

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low yields of the product, chemical modifications of glucose ring and others (Qin & Du, 2002), and the enzymatic approach is limited due to prohibitive cost and limited availability (Yalpani & Pantaleone, 1994). Moreover, commercially available chitosan is obtained by chemically deacetylating crustacean chitin with strong alkali, which appears to have limited potential for industrial acceptance because of seasonal and limited supply, difficulties in processing particularly with the large amount of waste of concentrated alkaline solution causing environmental pollution problems. So these current LMW-chitosan production methods have high costs and bring great environmental pollution. New green technology is needed to produce LMW-chitosan directly.

Chitosan prepared from fungi has received much attention, especially from the *Zygomycetes* species, which are known to contain chitosan as natural components of their cell wall (Bartniki-Garcia, 1968). Among them, the genus *Absidia* has been reported to produce chitosan feasible for commercial development (Gao, Katsumoto, & Onodera, 1995; Shimahara, Takiguchi, Kobayashi, Uda, & Sannan, 1989). The fungal approach has advantage of easy handling, harvesting and control to produce high quality chitosan, and it is possible to produce different chitosans by different strains under different environmental and nutritional conditions. The physicochemical characteristics of the resultant chitosans, such as molecular weight and degree of deacetylation (Tan, Tan, Wong, & Khor, 1996) can be varied and controlled by screening strains and controlling the environmental and nutritional conditions.

However, it is normally reported to produce chitosan with a high M_w, the same as from crab shells (Benjakul & Sophanodora, 1993). There is only one article which reports a method for direct preparation of LMW-chitosan (Niederhofer & Muller, 2004), but the productivity was low, the chitosan polymers were inhomogeneous in size (M_w/M_n, 2.63), and the M_w was still relatively disadvantageously high (45 kDa).

Therefore, this work set out to investigate whether *Absidia coerulea,* by solid-state fermentation with conditions variation by one-factor-at-a-time and by orthogonal array design methods using conventional characterization methods including GPC, FT-IR, X-ray diffraction and ^{13}C NMR could be made to yield a successful product.

2. Materials and methods

2.1. Strain and materials

Absidia coerulea was purchased from China Center for Type Culture Collection at Wuhan University, China (CTCC AF 93105). The strain was maintained on 10% potato dextrose agar slants at 4 °C. Shrimp chitosan (M_w 210 kDa, DD 92%) was obtained from Yuhuan Biochemical Co. (Zhejiang, China). Concentrated NaOH solution was commercial grade; other chemicals were analytical grade. Potato, soybean residue and corn steep liquor was collected from a local market. Corn residue was provided by Huangshi Citric Acid Production Plant, Hubei, China.

2.2. Media optimization methods

2.2.1. Selection of basal substrates by one-factor-at-a-time method

In order to extract LMW-chitosan from mycelia, the first difficulty was to separate hyphal body from solid mycelial cultures. Various cheap substances such as potato pieces, cotton seed hulls, soybean residue and corn residue (shown in Table 1) were compared by solid-state fermentation in 500 ml flasks for the purpose of selecting one which produced a good yield of LMW-chitosan and for which the hyphal body and basal substrates were easily separable.

2.2.2. Supplementary carbon and nitrogen sources by one-factor-at-a-time method

The various supplementary carbon and nitrogen sources that influence LMW-chitosan production during SSF were optimized over a wide range. The strategy adopted for standardization was to evaluate the effect of an individual source by one-factor-at-a-time method before standardizing the next one. Based on the components of conventional growth media, water (20 ml) containing various carbon sources (e.g., maltose, glucose, sucrose, fructose, molasses, 2% w/v), nitrogen sources (e.g., urea, $NaNO_3$, $(NH_4)_2SO_4$, yeast extract and corn steep liquor, 1% w/v) (shown in Table 2) were used to supplement the basal substrate to study their effects on LMW-chitosan production independently. The basal substrate without additional sources was used as control.

2.2.3. Orthogonal experiment of medium

The effect of different concentrations of various supplements supplied with the basal medium were studied on LMW-chitosan production, which were optimized through

Table 1
Selection of substrate for the production of LMW-chitosan

Substrate	Cotton seed hulls	Corn residue	Soybean residue	2–4 × 2–5 mm potato pieces	1–1.5 × 4–7 cm potato pieces
LMW-chitosan (g/kg substrate)	1.62	1.95	1.84	2.19	2.34

Table 2
Effects of supplementation of carbon and nitrogen sources to the substrate on the production of LMW-chitosan

Carbon source	LMW-chitosan (g/kg substrate)	Nitrogen source	LMW-chitosan (g/kg substrate)
Maltose	2.54	Urea	5.24
Sucrose	3.24	$NaNO_3$	3.41
Glucose	2.17	$(NH_4)_2SO_4$	4.31
Fructose	2.21	Yeast extract	4.38
Molasses	2.76	Corn steep liquor	5.12

Table 3
Factor-level code of orthogonal experiment of culture medium $L_9(3^4)$

Level	Factor			
	A Urea (%)	B Sucrose (%)	C $MgSO_4{\cdot}7H_2O$ (%)	D K_2HPO_4 (%)
1	0.5	1	0.05	0.05
2	1	2	0.10	0.10
3	1.5	3	0.15	0.15

Table 4
Result of orthogonal experiment of culture medium

No.	A	B	C	D	LMW-chitosan (g/kg substrate)
1	1	1	1	1	4.78
2	1	2	2	2	5.04
3	1	3	3	3	3.36
4	2	1	2	3	6.12
5	2	2	3	1	5.55
6	2	3	1	2	4.37
7	3	1	3	2	4.04
8	3	2	1	3	4.23
9	3	3	2	1	3.07
$T_1(j)$[a]	13.18	14.94	13.38	13.40	Better factors and
$T_2(j)$[a]	16.04	14.82	14.23	13.45	levels shown as follows:
$T_3(j)$[a]	11.34	10.80	12.95	13.71	$A_2B_1C_2D_3$
$t_1(j)$[b]	4.39	4.98	4.46	4.47	
$t_2(j)$[b]	5.35	4.94	4.74	4.48	
$t_3(j)$[b]	3.78	3.60	4.32	4.57	
$R(j)$[c]	1.57	1.38	0.42	0.10	

[a] T_1, T_2, T_3 was summation of B/A (Chitosan/Biomass) of each factor-level.
[b] t_1, t_2, t_3 was the average of T_1, T_2, T_3, respectively.
[c] R was level difference ($R = t_{max} - t_{min}$).

an orthogonal array design table of $L_9(3^4)$ (Fan & Chen, 1996) shown in Tables 3 and 4.

Supplemented media (natural pH, 20 ml) in 500 ml conical flasks were thoroughly mixed with basal substrate (20 g) (see Section 2.2.1) to achieve uniformity and autoclaved at 121 °C for 30 min. They were then cooled, and were inoculated with spore suspension (1.8×10^9 spores/kg) and cultured at 30 °C for 7 days in a static mode.

2.3. Extraction method for LMW-chitosan

Dried mycelia were treated with 2% NaOH solution (1:30, w/v) at 121 °C for 20 min. The alkali insoluble materials (AIM) were centrifuged (4000*g*, 15 min), washed with distilled water, and treated (1:40; w/v) with 10% acetic acid at 60 °C for 6 h, closely following the method of Synowieki and Al-khateeb (1997). Through centrifugation, the acid-insoluble fraction was precipitated and the supernatant, containing the chitosan, was isolated. The supernatant was collected and adjusted to pH 10.0 with 2 M NaOH. The precipitate was centrifuged (4000*g*, 15 min) and washed with distilled water to pH 7, dried at 30 °C, and stored at room temperature in an air-tight vessel.

2.4. Analytical methods

2.4.1. Determination of purity of glucosamine content

Samples of LMW-chitosan (20 mg) were hydrolyzed in 4 M HCl (3.0 ml) in sealed ampoules (10 ml volume) for 18 h at 105 °C. The pH of the hydrolysate diluted with distilled water (40 ml) was adjusted to 7.0 with 4 M NaOH solution. The neutralized solutions were diluted to 100 ml in volumetric flasks and a further 1:5 dilution was made before measuring D-glucosamine content according to the Elson–Morgan procedure modified by Johnson (1971), with D-glucosamine hydrochloride as the standard reference.

2.4.2. Measurement of the degree of deacetylation (DD) and the molecular weight (M_w, M_n)

The DD was measured as follows (Tolaimate et al., 2000): the sample (0.1 g) was dissolved in a known excess of 0.1 M HCl (10 ml). From the titration of this solution with 0.1 M NaOH, a curve with two inflection points was obtained. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution.

The number average molecular weight (M_n) and weight average molecular weight (M_w) of samples were measured by gel permeation chromatography combined with laser light scattering (GPC-LLS) on a multi-angle laser photometer ($\lambda = 633$ nm, DAWNDSP, Wyatt Technology Co., USA) combined with a P100 pump equipped with TSK-GEL G5000PWXL column and differential refractive index detector (RI-150) at 25 °C. The eluent was 0.1 M CH_3COOH–CH_3COONa at a flow rate of 0.5 ml/min. All the solutions used were first filtered with a sand filter and then with a 0.20 mm filter (Whatman, UK). Astra software was utilized for data acquisition and analysis. The refractive index increments (d*n*/d*c*) were determined by using an Optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 25 °C.

2.4.3. Other characterization

FT-IR spectra were taken as KBr discs on a Nicolet FT-IR 5700 spectrophotometer (Nicolet, Madison, USA).

X-ray diffraction patterns recorded by a Rigake X-ray diffractometer (D/max-rA, Tokyo, Japan) using Ni-filtered Cu Kα radiation at 30 kV and 30 mA. The sample was scanned from 5 to 40° of 2θ.

^{13}C NMR spectra were recorded on an INOVA-600 NMR 600 MHz spectrometer. Samples were dissolved in HCl/D_2O.

3. Results and discussion

3.1. Media optimization

3.1.1. Selection of basal substrate

Selection of a suitable substrate for the production of LMW-chitosan is a primary-key factor and an extremely significant step. Substrates provide the required energy

and substratum for the fungus to grow and produce the desired metabolite (Pandey, Soccol, Rodriguez-Leon, & Nigam, 2001). Meanwhile, LMW-chitosan is located in the cell wall of the fungal mycelia, so the basal substrate should be easily apart from the mycelia. The results of this study showed that the mycelia could be separated more easily from 1–1.5 × 4–7 cm potato pieces and cotton seed hulls than the other substrates studied. At the same time, LMW-chitosan by the strain on 1–1.5 × 4–7 cm potato pieces was the highest (Table 1), and it reached 2.34 g/kg substrate, followed by 2–4 × 2–5 mm potato pieces (2.19 g/kg substrate), corn residue (1.95 g/kg substrate), soybean residue (1.84 g/kg substrate) and cotton seed hulls (1.62 g/kg substrate). So 1–1.5 × 4–7 cm potato pieces were selected as the basal substrate for solid-state fermentation.

3.1.2. Effect of supplementation of carbon and nitrogen sources

The impact of supplementation of external carbon and nitrogen sources on LMW-chitosan production were studied and the results were shown in Table 2.

Addition of carbon sources with 2 wt% concentration to the medium showed different effects on LMW-chitosan production. The fungus, *A. coerulea* was grown on the medium with glucose or fructose more rapidly at the first 3 days, but it turn to autolysis soon, resulted in less mycelia and then less LMW-chitosan at last. While it was grown on the medium with sucrose more slowly at the first 3 days, then it was grown more rapidly in the following days, and it did not turn to autolysis until at the 7th day. So among all the compounds tested, sucrose yielded the highest LMW-chitosan production (3.24 g/kg substrate, see Table 2), followed by molasses (2.76 g/kg substrate), maltose (2.54 g/kg substrate), fructose (2.21 g/kg substrate) and glucose (2.17 g/kg substrate).

All the culture media with the additional nitrogen sources with 1 wt% concentration result in higher LMW-chitosan production compared to the basal substrate. The highest LMW-chitosan production was achieved (5.24 g/kg substrate, see Table 2) in the medium containing urea, which was the same as the effect of sucrose. It was much slower for the fungus to grow on the urea-supplemented media than on other nitrogen sources supplemented media within the first 3 days, but the growth became more rapid after 3 days and the fungus did not turn to autolysis until at the 7th day. The effect of the compounds on the yield of LMW-chitosan decreased in the following order: urea > corn steep liquor > yeast extract > $(NH_4)_2SO_4$ > $NaNO_3$. In comparison with organic nitrogen sources, inorganic nitrogen sources yielded low production.

So sucrose and urea can be added as supplementation of carbon and nitrogen sources in basal substrate to prolong cell growth and/or to improve LMW-chitosan formation.

3.1.3. Optimization of medium constituents

In order to search for the optimum combination, the following four factors, urea, sucrose, $MgSO_4{\cdot}7H_2O$ and K_2HPO_4 (these two bioelements have usually been recognized as favorable for mycelial growth), were selected as supplemental components of potato pieces for further optimization and their levels were modified through orthogonal experiment design $L_9(3^4)$ (Table 3). The final experiment results were shown in Table 4. According to the orthogonal method (Fan & Chen, 1996), the analysis of variance for the experimental designs was calculated by summation of LMW-chitosan yield of each factor-level and level difference (R). Based on the magnitude order of R (Table 4), the R value of urea (1.57) was higher than that of sucrose (1.38), $MgSO_4{\cdot}7H_2O$ (0.42) and K_2HPO_4 (0.10), hence urea and sucrose indicated significant influence. Compared to urea and sucrose, $MgSO_4{\cdot}7H_2O$ and K_2HPO_4 had little influence. The analytical results showed the best medium were $A_2B_1C_2D_3$. In the fifth experiment, the yield of LMW-chitosan reached 6.12 g/kg substrate which was better than other conditions, and the experimental conditions were $A_2B_1C_2D_3$ too. Therefore, the best supplementary media were urea 1%, sucrose 1%, $MgSO_4{\cdot}7H_2O$ 0.10%, K_2HPO_4 0.15%, and the mean yield of LMW-chitosan was 6.12 g/kg substrate. The LMW-chitosan production achieved at the optimized culture condition was about 2 times higher than that at the basal substrate.

3.2. Purity of the product, deacetylation and the molecular weights

The amount of glucosamine of LMW-chitosan was determined to be 83% (see Table 5), a little more than the report of Synowieki and Al-khateeb (1997). The relatively high content of glucosamine showed that the product contained mainly aminosugars.

The degree of deacetylation and calculated molecular weights of LMW-chitosan from *A. coerulea* were also shown in Table 5. The product with an average M_w of 6.4 kDa and a M_n of 5.9 kDa was prepared by *A. coerulea*, and the DD was 85%. The obtained M_w/M_n of 1.09 indicated that the product had a narrow molecular distribution.

Above-mentioned results showed that the molecular weight of extracted product from *A. coerulea* was as low as M_w of 6.4 kDa, and the quality of LMW-chitosan was high, its purity was up to 83% and the degree of deacetylation was as high as 85%.

3.3. Characterization of production

The structure of LMW-chitosan was confirmed by FT-IR, X-ray, and ^{13}C NMR.

Table 5
Purity, degree of deacetylation (DD) and molecular weights of LMW-chitosan

Sample	Purity (%)	DD (%)	M_w (kDa)	M_n (kDa)	M_w/M_n
LMW-chitosan	83	85	6.4	5.9	1.09

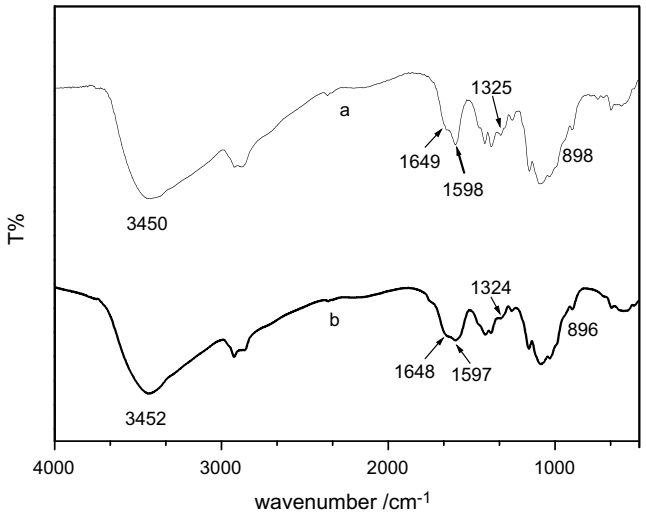

Fig. 1. FT-IR spectra of shrimp chitosan (a) and LMW-chitosan from *Absidia coerulea* (b).

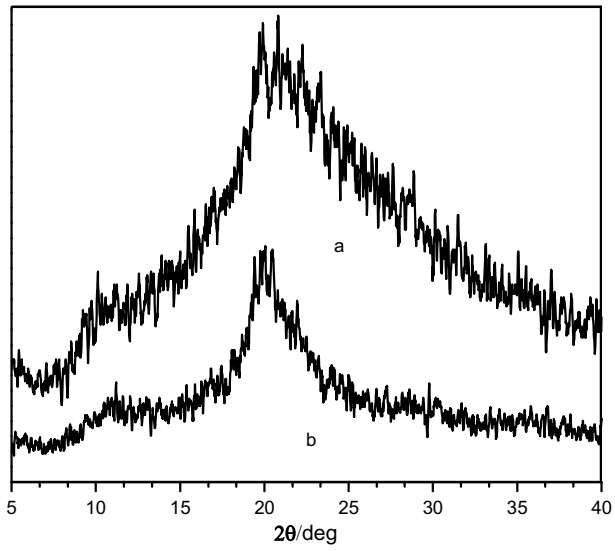

Fig. 2. X-ray diffraction patterns of shrimp chitosan (a) and LMW-chitosan from *Absidia coerulea* (b).

The FT-IR spectrum of LMW-chitosan from *A. coerulea* was similar to that of the chitosan from shrimp (Fig. 1) and similar to fungal chitosan such as chitosan from *Mucor rouxii* (Synowieki & Al-khateeb, 1997). The absorption band at 1597 cm^{-1} was characteristic of the amino deformation mode (Brugnerotto et al., 2001), the peak at 3452 cm^{-1} was NH bond stretching, and the absorption bands at 1648, 1324 cm^{-1} were referenced as amide I and III bands, respectively. The amide II band had disappeared in the FT-IR spectrum mainly because it overlapped with the band of amino deformation vibratin. The band at 898 cm^{-1} was referenced as the β-anomer. These absorption bands preliminary ascertained the product to be chitosan.

Both the X-ray diffraction patterns (Fig. 2) of shrimp chitosan (a) and LMW-chitosan by SSF (b) showed diffraction angle ranging from 5 to 40°. They had similar characteristic peaks at 10.5 and 20.0, indicating the extracted product from the mycelia was chitosan. But the intensity of this peak was less than the peak of shrimp chitosan around 20°, showing that the LMW-chitosan from *A. coerulea* had lower crystallinity.

The structure of LMW-chitosan was further investigated by means of ^{13}C NMR (Fig. 3). In the ^{13}C NMR spectra, it could be seen that LMW-chitosan had the same signals as shrimp chitosan at 55.8(C2), 60.0(C6), 70.0(C3), 74.7(C5), 76.2(C4) and 97.4(C1) (ppm), further ascertaining the extracted product from the mycelia was LMW-chitosan.

4. Conclusions

In summary, the preparation of LMW-chitosan with M_w of 5–10 kDa from *A. coerulea* mycelia would appear to be commercially feasible. For solid-state fermentation, the best media for *A. coerulea* to produce LMW-chitosan were as follows: in 500 ml conical flasks, potato pieces of 1–1.5 × 4–7 cm 20 g, urea 1%, sucrose 1%, $MgSO_4 \cdot 7H_2O$ 0.10%, K_2HPO_4 0.15%, water 20 ml, the pH was natural.

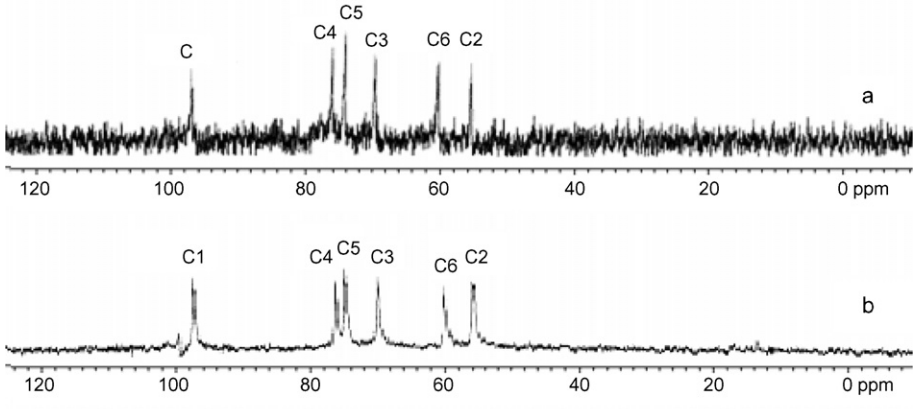

Fig. 3. ^{13}C NMR spectra of shrimp chitosan (a) and LMW-chitosan from *Absidia coerulea* (b).

Determination of the optimal concentrations of nutrient components leads to decrease expenses of raw materials and achievement of maximal LMW-chitosan production. Maximum LMW-chitosan production in optimized media was 6.12 g/kg substrate, nearly 2 times higher than that in the basal substrate. The glucosamine content and degree of deacetylation of LMW-chitosan were 83% and 85%, respectively, and the molecular weight of which was as low as M_w of 6.4 kDa. These would lay a foundation for lessening the pollution of strong alkaline from traditional chitosan production, turning two-step methods of LMW-chitosan preparation to one-step way, and providing a new, simple and green technology to highly produce LMW-chitosan directly.

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