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Short communication

Influence of nitrogen source on chitosan production carried out by *Absidia coerulea* CTCC AF 93105

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ABSTRACT

In the production of chitosan employing *Absidia coerulea* CTCC AF 93105, effect of different nitrogen sources in the medium containing soybean pomace, $(NH_4)_2SO_4$, $NaNO_3$, urea, or $(NH_4)_2CO_3$ on cell dry weight, chitosan molecular weight, chitosan production, and fermentation time for the maximum production of chitosan was investigated. Cell dry weight and the production of chitosan were greater with soybean pomace compared those with other nitrogen sources. The highest cell dry weight and production of chitosan by *A. coerulea* CTCC AF 93105 was 21.38 g L^{-1} and 5.88 g L^{-1}, respectively, with soybean pomace whereas those with $(NH_4)_2SO_4$ was 9.38 g L^{-1} and 3.14 g L^{-1}, respectively. Chitosan molecular weight was also affected by the nitrogen source in the medium and was higher with soybean pomace than that with any other nitrogen sources. The optimum fermentation time for chitosan production was also affected by the nitrogen source in the medium.

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1. Introduction

Chitosan, a linear copolymer of D-glucosamine and N-acetyl-D-glucosamine linked with β-1,4-glycosidic bond, is often derived by deacetylation of naturally occurring biopolymer chitin, which is present in the exoskeleton of crustacean such as crab, shrimp, lobster, crawfish and insects, and also can be found in the cell wall of most fungi, particularly zygomycetes (Chatterjee, Adhya, Guha, & Chatterjee, 2005; Muzzarelli, Ilari, Tarsi, Dubini, & Xia, 1994). It is polycationic, nontoxic as well as biodegradable, and has been reported to have numerous applications especially in food, pharmaceuticals and cosmetics (Kumar, 2000; Muzzarelli, 1996, 2009). The quantity and quality of chitosan extracted from the fungal mycelia depend on the fungal strain, fermentation type, agitator speed, fermentation temperature, pH of the medium and harvesting time (Nwe & Stevens, 2004).

It is thought that one of the most important factors in chitosan production is the nitrogen source. Nitrogen sources that have been used in chitosan production include $(NH_4)_2SO_4$, $NaNO_3$, urea, peptone, and yeast extract (Chatterjee et al., 2005; Kim, Lee, Theodore, & Chang, 2001; Nwe & Stevens, 2002, 2004; Wang et al., 2008). Soybean pomace, of which major components are carbohydrates and proteins, is an agro-industrial byproduct from the soybean plants of China and generated about 3000,000 tonnes annually. Most of the soybean pomace generated is discarded as waste due to high content of sodium chloride, resulting in serious environmental problems and a huge loss of natural resources. The utilization of agro-industrial byproducts as substrate for the production of chitosan has been investigated (Suntornsuk, Pochanavanich, & Suntornsuk, 2002).

Therefore, the objective of this study was to further investigate the effect of soybean pomace on chitosan production by *Absidia coerulea* CTCC AF 93105.

2. Materials and methods

2.1. Microorganism

A. coerulea was purchased from China Center for Type Culture (CTCC AF 93105). The strain was maintained on potato dextrose agar (PDA) slants at 4 °C and subcultured every 2 weeks.

2.2. Composition of the soybean pomace

The soybean pomace used was the agro-industrial byproduct from a factory (Soybean Plant, Lianyungang, China) which produces soybean curd and other soybean products. It is consisted of the following components (%, w/w): protein, 32.3 (N, 5.2); carbohydrate, 19.3; fat, 8.3; ash, 22.2; calcium, 0.3; phosphate, 0.6; moisture, 13.0.

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Table 1
Effect of different nitrogen sources (at 0.25 g N L^{-1}) on cell dry weight, chitosan production, and chitosan molecular weight. Fermentation conditions: fermentation temperature, 30 °C; initial pH, 4.5; fermentation time, 6 days.

Nitrogen source	Cell dry weight (g L^{-1})	Chitosan (g L^{-1})	Chitosan molecular weight (kDa)
Soybean pomace	15.34 ± 0.57[a]	4.11 ± 0.21	650 ± 23
$(NH_4)_2SO_4$	11.37 ± 0.41	2.86 ± 0.17	221 ± 17
$NaNO_3$	7.69 ± 0.37	2.04 ± 0.09	216 ± 15
Urea	7.26 ± 0.28	1.97 ± 0.08	207 ± 16
$(NH_4)_2CO_3$	3.18 ± 0.19	1.13 ± 0.03	203 ± 11

[a] Mean ± S.D. from three independent experiments.

2.3. Preparation of medium

The medium contained 20 g glucose, 1 g NaCl, 5 g nitrogen source as indicated in Table 1, 1 g K_2HPO_4, 5 g $MgSO_4{\cdot}7H_2O$ and 0.1 g $CaCl_2$ in 1 L distilled water. The pH was adjusted to 4.5, and the medium was autoclaved at 121 °C for 15 min.

2.4. Fermentation

The seed culture was prepared by inoculating spores from a PDA slant into a 250-mL flask containing 50 mL medium, then incubating at 30 °C for 3 days with shaking at 200 rpm. Two milliliters of spore suspension (10^7 spores/mL) was transferred into a 250-mL flask containing 50 mL fermentation media. The culture was incubated at 30 °C and shaken at 200 rpm for up to 6 days.

2.5. Isolation and purification of chitosan

At the end of the desired incubation period, biomass was harvested through filtration and dried by lyophilization. Dried mycelia were treated with 1 M NaOH at 45 °C for 13 h. The alkali insoluble mass was treated with 0.35 M acetic acid at 95 °C for 5 h (Nwe, Stevens, Montet, Tokura, & Tamura, 2008). Chitosan was precipitated out from the supernatant by adjusting the pH to 8.5 with 1 N NaOH, and washed several times with chilled distilled water and triturated with acetone (Chatterjee et al., 2005).

2.6. Analytical methods

Under the optimized fermentation conditions, the resulting chitosan samples produced in $(NH_4)_2SO_4$ and soybean pomace containing media were characterized. Ash, moisture, and protein contents of the samples were determined by standard methods (Anon, 1984). The degree of deacetylation (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. Chitosan average molecular weight (CMW) was determined according to the method described by Wu, Jin, and Tong (2009) with slight modification, using HPGFC (LC-10A, Shimadzu, Japan) on an Ultrahydrogel Size Exclusion Column (LKB-Prodokter, AB, Bromma, Switzerland), which is capable to detect MWs in the range of 10^3–10^6. In the size exclusion chromatography studies, 0.1 M CH_3COOH–CH_3COONa (0.1 M CH_3COOH and 0.1 M CH_3COOH) was used as an eluent at a flow rate of 0.9 mL/min, employing High Sensitive Refractive Index Detector (ERC-7515 A, ERC Inc., Japan). The calibration of the detector was performed with known concentrations of commercially available dextran (Sigma–Aldrich, USA). An aliquot of 20 μL was injected into the column after filtration through 0.45 μm millipore filter, at ambient temperature. The procedure was repeated three times. The software used was the Multi-channel Chromatography Data Station (version 144A, 1993–1997 Ampersand Ltd.). Chitosan were dissolved in 0.1 mol/L CH_3COONa–0.2 mol/L CH_3COOH solutions. The intrinsic viscosities were determined using an Ubbelodhe viscosimeter by capillary viscometry at 30 °C. The intrinsic viscosities $[\eta]$ were calculated using the following equation:

$$[\eta] = \frac{\eta_{sp} + 3\ln\eta_r}{4c}$$

where η_r, η_{sp} were the relative viscosity and the incremental viscosity, respectively, and c is the concentration of chitosan (g/mL) (Wang, Huang, & Wang, 2005).

3. Results and discussion

3.1. Effect of different nitrogen sources on chitosan production

Effect of different nitrogen sources on chitosan production by *A. coerulea* is shown in Table 1. The highest cell dry weight (15.34 g L^{-1}), chitosan production (4.11 g L^{-1}), and CMW (650 kDa) were observed in the media containing soybean pomace as a nitrogen source: the lowest cell dry weight (3.18 g L^{-1}), chitosan production (1.13 g L^{-1}), and the CMW (203 kDa) were observed in the medium containing $(NH_4)_2CO_3$. Although being used as a universal nitrogen source for chitosan production, $(NH_4)_2SO_4$ was proved not the best one, and cell dry weight (11.37 g L^{-1}), chitosan production (2.86 g L^{-1}), and CMW (221 kDa) were lower than those resulted in the medium containing soybean pomace, but higher than those observed in the medium containing other nitrogen sources. The molecular polydispersity of chitosan (MPC) was varied from 1.97 to 2.53 and showed no casual relationship among nitrogen sources. These results clearly showed that the type of nitrogen source in the culture medium affected cell dry weight, chitosan production, and CMW. As to CMW, Chatterjee et al. (2005) reported that the MW of chitosan from molasses salt medium was the smallest with respect to those obtained from potato dextrose broth and yeast peptone glucose medium. However, Wang et al. (2008) observed that low-MW of chitosan (5–10 kDa) was produced when urea was used as nitrogen source. Therefore, it is possible to produce different MW chitosan using different nitrogen sources, as well as by different fungal strains.

3.2. Effect of the concentration of nitrogen source on fermentation

Because of being used as universal nitrogen source and the second optimum nitrogen source for chitosan production as shown in this study (Table 1), $(NH_4)_2SO_4$ was used as control nitrogen source for further study. In Fig. 1, when $(NH_4)_2SO_4$ was used as the nitrogen source, cell dry weight and chitosan production increased as the level of $(NH_4)_2SO_4$ increased, but decreased when excess $(NH_4)_2SO_4$ was present. The maximum cell dry weight and chitosan production was observed in medium containing 0.3 g N L^{-1} of $(NH_4)_2SO_4$ (Fig. 1a). However, CMW and MPC (about 2.52) did not change as the level of $(NH_4)_2SO_4$ increased. Similar results were observed when soybean pomace was used as a nitrogen source except that the highest cell dry weight (21.38 g L^{-1}) chitosan production (5.48 g L^{-1}) was obtained in medium containing 0.4 g N L^{-1} of soybean pomace (Fig. 1b).

3.3. Kinetics of chitosan production

As shown in Fig. 2, time-course studies on the production of chitosan by *A. coerulea* CTCC AF 93105 were carried out for a period of 6 days in the fermentation media. With $(NH_4)_2SO_4$, cell dry weight and chitosan yield increased to reach a maximum within

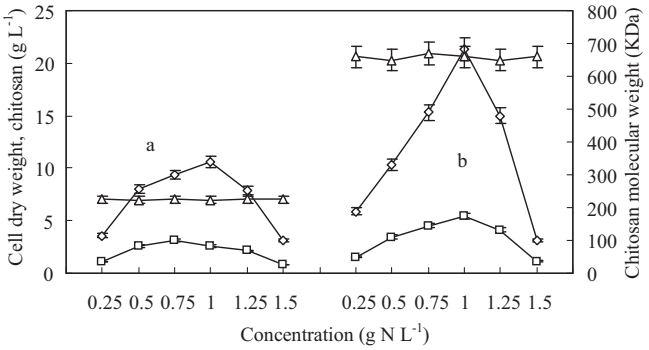

Fig. 1. Effect of level of nitrogen source on cell dry weight (◊), chitosan production (□), and molecular weight (△). (a) Nitrogen source, $(NH_4)_2SO_4$; (b) nitrogen source, soybean pomace. Fermentation conditions: fermentation temperature, 30 °C; initial pH, 4.5; fermentation time, 6 days.

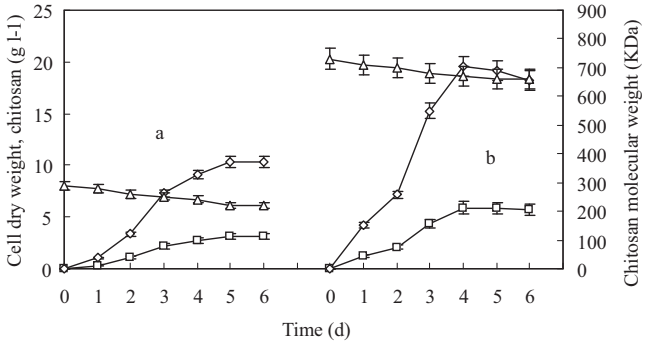

Fig. 2. Effect of time course on cell dry weight (◊), chitosan production (□), and molecular weight (△). (a) Nitrogen source, 0.3 g N L^{-1} $(NH_4)_2SO_4$; pH, 4.5; fermentation temperature, 30 °C. (b) Nitrogen source, 0.4 g N L^{-1} soybean pomace; pH, 4.5; fermentation temperature, 30 °C.

5 days (9.37 g L^{-1} and 3.14 g L^{-1}, respectively), and thereafter chitosan production leveled off (Fig. 2a). When soybean pomace was the nitrogen source in the medium, the maximum cell dry weight (21.38 g L^{-1}) and chitosan production (5.88 g L^{-1}) occurred at day 4 (Fig. 2b), and were higher than those of $(NH_4)_2SO_4$. In other reports, chitosan production were 0.61 g L^{-1} (Chatterjee et al., 2005), 2.3 g L^{-1} (Kim et al., 2001), and 4.3 g L^{-1} (Suntornsuk et al., 2002), respectively, and lower than that observed in this study. These different results were probably due to different fungal strains and fermentation conditions. CMW slightly decreased probably due to some hydrolytic enzyme that was secreted from the fungal during fermentation time (Fig. 2), but MPC slightly increased (data not shown).

3.4. Characterization of the product

The chitosan obtained from the mycelia of *A. coerulea* cultured in the medium containing $(NH_4)_2SO_4$ and soybean pomace was characterized. As shown in Table 2, although there were not much differences in chitosan content, ash content, moisture content, and protein content between the samples from the two media, the M_w of chitosan and viscosity obtained from soybean pomace containing medium was much higher than those obtained from $(NH_4)_2SO_4$ containing medium. The products were white and water insoluble powder.

Table 2
Comparison of quality of chitosan produced from $(NH_4)_2SO_4$ and soybean pomace media.

Parameter	Source of chitosan	
	$(NH_4)_2SO_4$	Soybean pomace
Chitosan content (%, w/w)	95.07 ± 0.67[a]	95.01 ± 0.57
Protein (%, w/w)	0.21 ± 0.03	0.23 ± 0.04
Ash content (%, w/w)	1.2 ± 0.20	1.3 ± 0.21
Moisture content (%, w/w)	5.3 ± 0.30	5.1 ± 0.31
DD (%)	87 ± 0.82	88 ± 0.79
M_w ($\times 10^5$)	2.2 ± 0.24	6.7 ± 0.13
Viscosity (g/mL)	183 ± 1.24	561 ± 1.31

[a] Mean ± S.D. from three independent experiments.

4. Conclusions

The influence of nitrogen source on the chitosan production and CMW in the culture of *A. coerulea* CTCC AF 93105 was explored. The optimal nitrogen sources for both high CMW and chitosan production were soybean pomace and $(NH_4)_2SO_4$. With $(NH_4)_2SO_4$, the optimal concentration for chitosan production was 0.3 g N L^{-1}, and with soybean pomace, the optimal concentration was 0.4 g N L^{-1}. The chitosan production was obtained on day 5 in medium containing $(NH_4)_2SO_4$. In comparison, in medium containing soybean pomace, the optimal chitosan production occurred at day 4. The type of nitrogen source affected CMW. However, the concentration and fermentation time had little effect on CMW.

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