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Production and Characterization of Chitosan from Ginseng-Steaming Effluents by *Mucor miehei*

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Abstract *Mucor miehei* KCTC 6011, which grew successfully in ginseng-steaming effluents and produced a large amount of chitosan efficiently, was selected from various fungi. Approximately 120 mg of chitosan per g-dry mycelium was maximally produced in 84 h at 25°C when grown in the ginseng-steaming effluent (pH 8.0) supplemented with 0.5% yeast extract and 0.002% $CuSO_4$. Chitosan produced by *Mucor miehei* KCTC 6011 was identified by the IR-spectra to have deacetylated approximately 56%. Viscosity and molecular weight of the chitosan were 80 cps and 1.07×10^3 kDa, respectively. The chitosan at 1.5 mg/ml inhibited 73.9% of the mycelium growth of *Rhizoctonia solani* in 60 h.

Key words: Chitosan, ginseng-steaming effluents, *Mucor miehei*, characterization

Chitin is identified as an insoluble mucopolysaccharide which comprises materials of crab shells, crustaceans, and cuticle of insects along with cell wall of some fungi [2, 16, 22, 23, 24]. It contains a polymer of glucose derivative in which -OH on the second carbon atom of D-glucose is replaced by amine groups (N-acetyl-D-glucosamine linked by β-1,4 bonds; Poly-β-1, 4-N-acetyl-D-glucosamine) [3]. Chitin and chitosan, the partially deacetylated chitin, have advantages of being biodegradable, biocompatible, nontoxic, and immunopotentiating. In fact, they have numerous food, cosmetic, and medical applications and are used as sources of fiber or oligosaccharides (bifidobacteria growth factor), functional ingredients (texture modifying agents), immobilizers of enzymes or plant cells, microencapsulators of flavors, intermediate matrix of some drug, and flocculants for proteinaceous liquid wastes, etc. [3, 6, 7, 10].

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Even though crabs and crustaceans have a large amount of chitin, some problems exist to obtain chitin from these sources, because of environmental pollution due mostly to chemical treatments. Microbial chitin has been found in some fungi such as *Absidia*, *Gongronella*, *Mucor*, and *Rhizopus* species. However, the amount of chitin produced from fungi has been quite low [5, 11, 13, 17, 19, 20]. Therefore, it will be very useful to find microorganisms which can produce high amounts of chitin.

Various ginseng products including ginseng tea and drinks are produced from ginseng extracts by ginseng processing companies. A large amount of ginseng-steaming effluents containing high concentration of useful substances are discharged during the manufacturing process of ginseng extracts. However, only a small portion of ginseng-steaming effluents is recently utilized to extract useful ginsenosides or to produce maltooligosaccharides [9] and mononucleotides [8].

Therefore, it is imperative to improve efficiency and develop high-value added products from ginseng-steaming effluents. In this paper, optimal conditions for production of chitosan by *M. miehei* KCTC 6011 grown on ginseng-steaming effluents (GSE) and characteristics of chitosan are described.

Materials and Methods

Materials and Chemicals

Ginseng-steaming effluents (GSE), produced as a waste from the manufacturing process of ginseng extract [8], were obtained from a ginseng-processing company in Geumsan, Korea. The GSE were 1,025.9 mg/l of COD_{Mn} and pH 6.67, and its solid contained 63.8% sugar, 33.9% crude protein, and 2.3% ash.

All chemicals used in this study were of the analytical grade. Chitosan as a standard was purchased from Pronova

Inc. (Oslo, Norway) and potato dextrose agar was from Difco (Detroit, U.S.A.).

Microorganisms and Culture Conditions

In order to screen chitosan-producing fungus in the GSE, *Mucor miehei* KCTC 6011, *Mucor ambiguus* KCTC 6142, *Mucor racemosus* KCTC 6119, *Rhizopus japonicus* KCTC 6945, *Rhizopus formosaensis* KCTC 6947, and *Rhizopus nigricans* KCTC 6062 were all obtained from the Korea Collection for Type Cultures. They were cultivated in the GSE at 30°C for 2–4 days.

Fusarium oxysporum, *Fusarium solani*, *Rhizoctonia solani*, and *Alternaria gaisen*, obtained from the Laboratory of Plant Pathology of Chungnam National University, Daejeon, Korea, were used in the test for antifungal activity of the chitosan, and they were cultivated in a potato dextrose agar (PDA) medium at 28°C for 3 days.

Extraction of Chitosan

Chitosan from *M. miehei* KCTC 6011 was extracted by the method described by Park and Kim [20]. *M. miehei* KCTC 6011 was cultivated in GSE at 30°C for 3 days. After mycelium was harvested by filtration (Whatman No. 41), it was dried and milled. The powder was suspended in 1 N NaOH (1:40) and hydrolyzed at 121°C for 15 min, followed by filtration. Acetic acid (0.5 N) was added to the filtrates to a 1% final concentration, and chitosan was extracted at 35°C for 5 h. After filtration, chitosan in the filtrate was precipitated by adding 1 M of NaOH, (pH adjusted to 7.0), centrifuged (3,500 ×*g* for 20 min), and then dried to obtain the purified chitosan. The content of chitosan was described as mg/g-dry mycelium (Fig. 1).

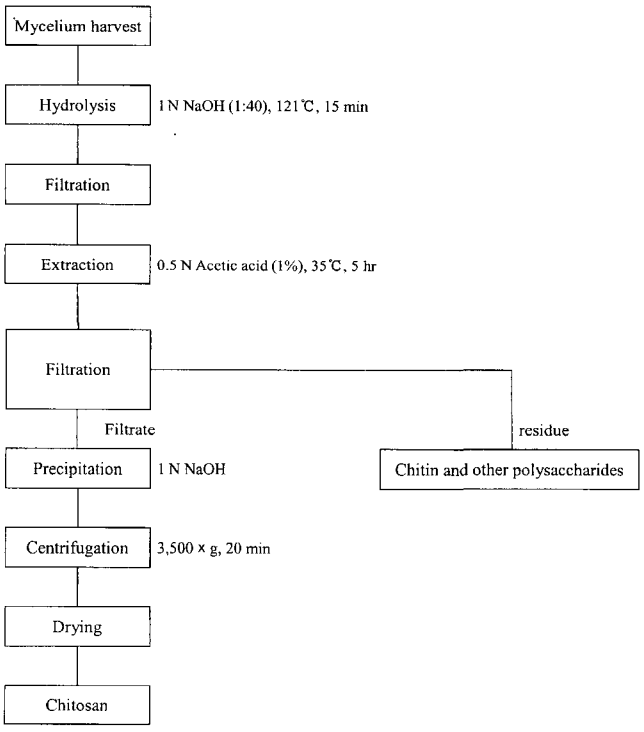

Fig. 1. Schematic diagram of procedure for chitosan extraction from *M. miehei* KCTC 6011.

Characterization of the Chitosan

The IR spectra of the chitosan were measured by a FT-IR spectrophotometer (Shimadzu FTIR-8200, Japan) with KBr tablets [5]. Water-holding capacity of the chitosan was determined and described as ml-H_2O per g-dry chitosan, according to the methods of Knorr [3]. Also, 5 g of the chitosan powder was dissolved in 100 ml of 1% acetic acid, and its viscosity was then determined by using a Brookfield viscometer (Model DV-II, U.S.A.) and described as cps.

Molecular weight of the chitosan was determined by the modified Standinger equation as follows [21]:

$$[\eta]=K\times M_w^a$$

where, η is viscosity, M_w is Molecular weight, and K and a are 8.93×10^{-4} and 0.71, respectively [15].

The degree of deacetylation of the chitosan was determined from the IR spectra of the chitosan and its calibration curve [23] by applying the ratio of absorbance of the band at 1,550 cm^{-1} to that of the band at 2,878 cm^{-1} (A_{1550}/A_{2878}).

Inhibitory effect of the chitosan on mycelium growth of some plant pathogenic fungi was investigated by the modified method of Lee *et al.* [14] as follows: Chitosan in 0.5 M acetic acid was added to potato dextrose agar (PDA) medium in the range of 0.1–1.5 mg/ml. Agar inoculum containing *F. oxysporum*, *F. solani*, *R. solani*, or *A. gaisen* was incubated at 28°C for 3 days. Colony diameter of each fungus was determined, and then the inhibitory rate of mycelium growth of each fungus was obtained from the following equation.

$$\text{Inhibitory rate of mycelium growth (\%)} = \{(A-B)/A\}\times100$$

where, A is colony diameter in chitosan-free medium and B is colony diameter in chitosan-containing medium.

RESULTS AND DISCUSSION

Screening of a Fungus for the Chitosan Production

As shown in Table 1, *M. miehei* KCTC 6011 showed the most satisfactory growth in the GSE without any added nutrients and it produced maximally 0.4 mg of chitosan per g-dry mycelium after 3 days of culture at 25°C. While *Rhizopus* species showed poor growth in the GSE, *Aspergillus* species grew very well in the GSE without producing chitosan. Therefore, in this study, *Mucor miehei* KCTC 6011 was selected for the production of chitosan in the GSE.

Table 1. Growth and chitosan productivity of various fungi in GSE medium.

Strains[a]	Dry mass of mycelium (g/l)	Chitosan productivity (mg/g-dry mycelium)
Rhizopus japonicus KCTC 6945	0.1	–[b]
Rhizopus formosaensis KCTC 6947	0.1	–
Rhizopus nigricans KCTC 6062	0.1	–
Mucor miehei KCTC 6011	0.6	0.4
Mucor ambiguus KCTC 6142	0.3	0.2
Mucor racemosus KCTC 6119	0.3	0.1
Aspergillus niger	0.4	–
Aspergillus nidulance	0.4	–

[a]The strains were cultivated in GSE medium at 25°C for 3 d.
[b]–; <0.1 mg/g-dry mycelium.

Bartnicki-Garcia and Nickerson [1] first reported the presence of significant quantities of chitosan in *M. rouxii*. Since it was known that Mucorales contained a high content of chitin in the cell walls, the production of microbial chitosan might depend on the cell wall composition [11, 17]. It has been reported that some fungi such as *Absidia atrospora* IFO 09471, *A. coerulea* IFO 4011, *A. glauca* IFO 4002, *A. glauca* IFO 4003, *A. glauca* var. *paradoxa* IFO 4431, *Gongronella butleri* IFO 8080, and *Rhizopus japonicus* produced chitosan [4, 12, 22]. Indeed, *G. butleri* IFO 8081 produced 730 mg of chitosan/l after a 5-day culture in the sweet potato-shoshu medium [5].

Optimal Conditions for the Chitosan Production

The effect of nitrogen sources on the chitosan production from the GSE by *M. miehei* KCTC 6011 is shown in Table 2. A considerable amount of chitosan (57.2 mg of chitosan/g-dry mycelium) was produced by adding yeast extract into the GSE, and tryptone with peptone were also good sources of nitrogen for the production of chitosan. However, urea was not utilized, and the addition of inorganic nitrogen sources did not affect the production of chitosan at all.

Figure 2 shows the effect of concentration of yeast extracts on the production of chitosan. A maximal amount of up to 60.0 mg of chitosan/g-dry mycelium was produced by adding 0.5% yeast extract into the GSE, but the growth was maximal with 2.0% yeast extract. Similarly, 10% yeast extract was also effective in both growth and chitosan yield.

The effect of various minerals in the production of chitosan by *M. miehei* was investigated, and 79 mg of chitosan/g-dry mycelium was produced by adding 0.002% $CuSO_4$ into the GSE (data not shown).

The effect of initial pH on the production of chitosan was examined in the range of pH 4.0 to 9.0. Approximately 90 mg of chitosan/g-dry mycelium was produced when the initial pH of the GSE was adjusted to 8.0 and cultured for 3 days at 25°C (Table 3). These results were quite different from those of *R. japonicus* (pH 5.5) and *R. acetoinus* HUT 1219 (pH 4.5–5.5) [20].

The effect of temperature on chitosan productivity by *M. miehei* ACTC 6011 showed that cell growth and chitosan productivity were at their maximum level at 25°C (101.2 mg/g-dry mycelium), and 40.5 mg/g-dry mycelium

Table 2. Effect of nitrogen sources on the production of chitosan from *M. miehei* KCTC 6011.

Nitrogen source[a]	Dry mass of mycelium (g/l)[b]	Chitosan productivity (mg/g-dry mycelium)
Urea	–	–
Tryptone	2.6	44.7
Yeast extract	2.9	57.2
Peptone	2.3	43.1
NH_4Cl	0.9	0.5
NH_4NO_3	0.8	0.4
$(NH_4)_2SO_4$	0.7	0.4
KNO_3	0.9	0.6
$NaNO_3$	0.9	0.5
Ammonium citrate	1.2	0.8
Ammonium oxalate	0.8	0.7

[a]Organic nitrogen sources were added to 0.5% and inorganic nitrogen sources were added to 0.2%, except ammonium citrate and ammonium oxalate (0.1%).
[b]Cultivation was carried out for 3 days at 25°C in ginseng-steaming effluent (GSE) medium.

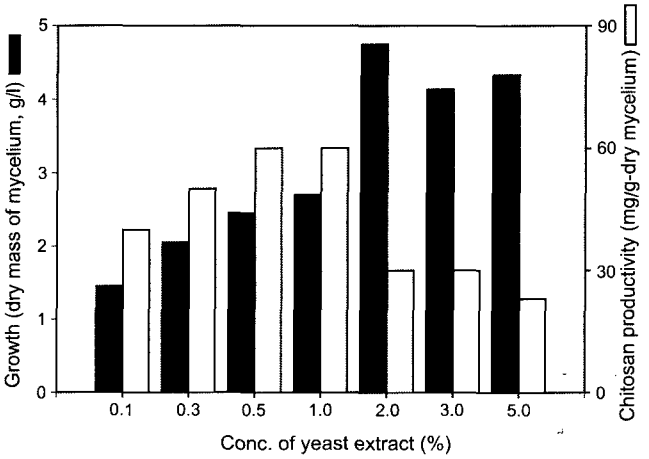

Fig. 2. Effect of yeast extract on the production of chitosan from *M. miehei* KCTC 6011.

Table 3. Effect of pH on the production of chitosan from *M. miehei* KCTC 6011.

pH	Dry mass of mycelium (g/l)	Chitosan productivity (mg/g-dry mycelium)
4.0	0.2	4.3
5.0	1.9	53.6
6.0	2.1	75.0
7.0	2.4	82.9
8.0	3.3	90.0
9.0	2.0	50.3

Cultivation was carried out for 3 days at each pH in the GSE medium containing 0.5% yeast extract and 0.002% $CuSO_4$.

and 50.1 mg/g-dry mycelium of chitosan were also produced by cultivation at 20°C and 30°C, respectively. However, chitosan was not produced when it was cultured at 37°C (data not shown).

This result was different from that of Park and Lee [20] who showed that optimal temperature for producing chitosan by *R. japonicus* was 30°C and a high level of chitosan was also produced at 37°C.

Figure 3 shows the time course for the production of chitosan in batch culture. The chitosan production was gradually increased with the growth rate, and the maximum chitosan production (120 mg/g-dry mycelium) level was observed after 84 h of cultivation. The amount of chitosan produced by *M. meihei* in the GSE was higher than those of *R. japonicus*, *R. acetoinus* [20], *Aspergillus awamori* [18], and *Gongronella butleri* [5].

Furthermore, 92% of COD_{Mn}, 89% of sugar, and 94% of crude protein in the GSE were reduced by 84 h of cultivation of *M. miehei* KCTC 6011, together with chitosan production (data not shown). Therefore, it can be said that this process could be used to prevent pollution. In fact, the

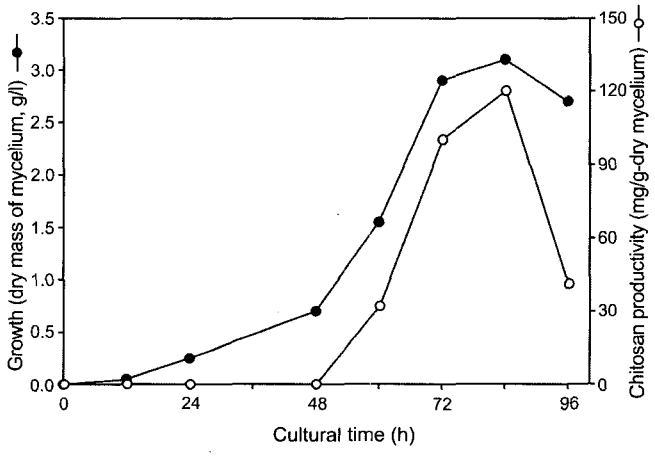

Fig. 3. Time course of cell growth and chitosan production of *M. miehei* KCTC 6011.
Cultivation was carried out in the GSE medium (pH 8.0) containing 0.5% yeast extracts and 0.002% $CuSO_4$.

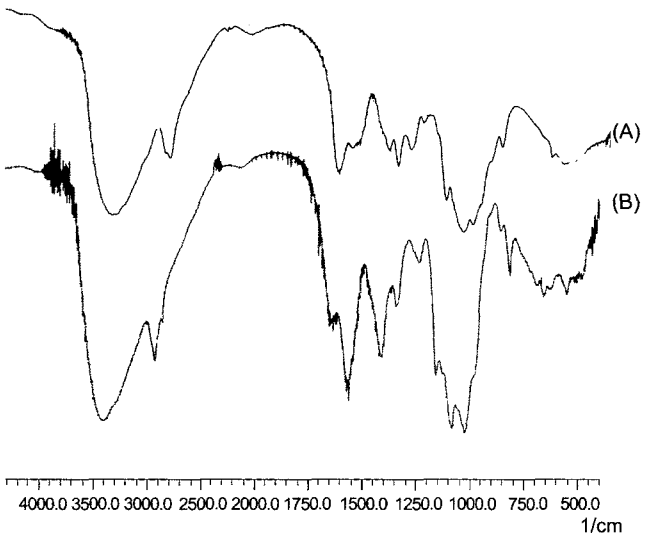

Fig. 4. Infrared spectra of authentic chitosan (A) and chitosan of *M. miehei* KCTC 6011(B).

results are similar to those obtained with *Aspergillus awamori* [18] and *Gongronella butleri* [5] in the shochu distillery wastewater.

Characteristics of the Chitosan

The infrared spectrum of the chitosan, harvested from *M. miehei* KCTC 6011 and grown in the GSE, was compared with the authentic chitosan (Chitosan 1000) using a FT-IR spectrophotometer with KBr tablets [5, 12].

As shown in Fig. 4, characteristic bands of chitosan such as the hydroxyl band at 3,450 cm^{-1}, amine band at 1,630 cm^{-1} to 1,550 cm^{-1}, and C-H band at 3,250 cm^{-1} were observed in the IR spectrum of the chitosan from *Mucor miehei* KCTC 6011. The pattern of the spectrum was found to be identical with those of the authentic chitosan (commercial) and chitosan from *Gongronella butleri* IFO 8081 [5].

The deacetylation degree of the chitosan was approximately 56%, according to the method described by Takanori *et al.* [23] by using the ratio of absorbance at 1,550 cm^{-1} and 2,878 cm^{-1}. This value was lower than those of chitosan from *G. butleri* IFO 8081 [5] and commercial chitosan.

Table 4. Comparison of main characteristics with authentic chitosan and the chitosan from *M. miehei* KCTC 6011.

Characteristics	Chitosan preparation	Authentic chitosan[a]
Appearance	Yellow and brown powder	White and yellow flake
Degree of deacetylation	56%	71.5%
Viscosity	80 cps	2,020 cps
pH	6.6	7.0
Odor	No taste and smell	No taste and smell

[a]Pronova chitosan from shrimp-shell.

Table 5. Effect of chitosan on the mycelial growth of some phytopathogenic fungi.

Concentration (mg/ml)	Inhibition rate (%)			
	Fusarium oxysporum FCU 428	*Fusarium solani* FSG 503	*Rhizoctonia solani* 920102	*Alternaria gaisen* CNU 5080
0.1	3.6	0	21.8	7.1
0.5	7.1	4.6	34.8	14.3
1.0	14.3	13.6	54.4	17.9
1.5	21.4	27.3	73.9	21.4

Strains were incubated for 60 h at 25°C on PDA media containing various concentrations of chitosan.

Water-holding capacity and viscosity of the chitosan from *M. miehei* KCTC 6011 were 0.8 ml-H_2O/g-dry chitosan and 80 cps, respectively. Although this water-holding capacity was lower than that of the commercial chitosan [3], the difference in the protein content of the chitosan might affect water-binding capacity [3]. Molecular weight of the chitosan was 1.07×10^3 kDa. When comparison was made between the commercial chitosan from crab shells and the chitosan extracted from *M. miehei* KCTC 6011 (Table 4), the main properties of the chitosan except viscosity were similar to those of commercial chitosan.

Table 5 shows the inhibitory effects of the chitosan on mycelium growth of *F. oxysporum*, *F. solani*, *R. solani*, and *A. gaisen*. The chitosan inhibited mycelium growth of *R. solani* as the concentration increased, and its inhibitory rate was approximately 74% at 1.5 mg/ml of the chitosan. Several studies on antifungal activity of chitosan have been published [4, 14]; Lee *et al.* [14] reported that more than 90% of the growth of *Botryosphaeria dothidea* were inhibited at 1.0 mg/ml of commercial chitosan, and Yun *et al.* [25] also reported that growth of *Fusarium culmorom*, a plant pathogenic fungus was significantly inhibited by 0.025% of chitosan after 8 days of culture.

Overall, these results suggest that utilization of *M. miehei* grown in the GSE may be useful in the production of chitosan, thereby eliminating pollution problem as well as in inhibiting plant pathogens. However, further studies are necessary on biomedical or physiological functionalities of the chitosan from *M. miehei*.

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References

1. Bartnicki-Garcia, S. and C. A. Nickerson. 1962. Isolation, composition and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochim. Biophy. Acta* **58:** 102–119.
2. Chung, G. H., H. S. Kim, J. W. Hur, and H. K. No. 1996. Physicochemical properties of chitin and chitosan prepared from lobster shrimp shell. *Kor. J. Food Sci.* **28:** 870–876.
3. Dietrichi, K. 1982. Functional properties of chitin and chitosan. *J. Food Sci.* **47:** 593–595.
4. Ghaouth, A. E., J. Arul, J. Grenier, and A. Asselin. 1992. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology* **82:** 398–402.
5. Haruhiko, Y., A. Tomoteru, N. Shuichi, H. Jun, H. Sachio, and T. Yoshiyuki. 1998. Chitosan production from *shochu* distillery wastewater by funguses. *J. Ferment. Bioeng.* **85:** 246–249.
6. Hiromichi, O., K. Hideo, and T. Takahiro. 1997. Antihypertensive and antihyperlipemic action of chitosan. *J. Chitin Chitosan* **2:** 49–59.
7. Jeon, Y. J. and S. K. Kim. 2001. Effect of antimicrobial activity by chitosan oligosaccharide N-conjugated with asparagine. *J. Microbiol. Biotechnol.* **11:** 281–286.
8. Kim, J. H., B. H. Lee, and J. S. Lee. 2002. Production of ribonucleotides from autolysates of *Hansenula anomala* grown on Korean-ginseng-steaming effluents. *J. Biosci. Bioeng.* **93:** 318–321.
9. Kim, N. M., J. S. Lee, and B. H. Lee. 2000. Enzymatic hydrolysis of Korean ginseng starch and characteristics of produced maltooligosaccharides. *J. Ginseng Res.* **24:** 41–45.
10. Kim, S. K. and Y. J. Jeon. 1997. Chitin and chitosan as materials of functional cosmetics. *J. Chitin Chitosan* **2:** 5–13.
11. Kim, S. K. and E. H. Lee. 1997. Food industrial application of chitin and chitosan. *J. Chitin Chitosan* **2:** 43–59.
12. Kobayashi, T., Y. Takiguchi, K. Shimahara, and T. Sannan. 1998. Distribution of chitosan in *Absidia* strains and some properties of the chitosan isolated. *Nippon Nogeikagaku Kaishi* **62:** 1463–1469.
13. Lee, J. C., K. Na, J. M. Yun, and J. K. Hwang. 2001. *In vitro* bifidogenic effect of nondigestible oligosaccharides isolated from red ginseng marc. *J. Microbiol. Biotechnol.* **11:** 858–862.
14. Lee, S. J., J. Y. Uhm, and Y. H. Lee. 1996. Effect of chitosan on the growth of *Botryosphaeria dothidea*, the causal fungus of apple white rot. *Kor. J. Appl. Microbiol. Biotechnol.* **24:** 261–267.
15. Lee, V. F. P. 1974. *Solution and Shear Properties of Chitin and Chitosan*. Ph. D. Dissertation, University of Washington, Xerox Univ. Microfilms, Ann Arbor, MI, U.S.A.

16. Minoru, T., I. Shinya, and Y. Sumito. 1996. Alternation in cell wall chitin of *Zygosaccharomyces rouxii. J. Ferment. Bioeng.* **81:** 171–173.
17. Miyoshi, H., K. Shimura, K. Watanabe, and K. Onodera. 1992. Characterization of some fungal chitosan. *Biosci. Biotech. Biochem.* **56:** 1901–1905.
18. Morimura, S., K. Kida, M. Nakagawa, and Y. Sonoda. 1994. Production of fungal protein by *A. awamori* wastewater. *J. Ferment. Bioeng.* **78:** 160–163.
19. Oh, C. H., H. C. Yang, and H. Y. Cho. 1995. Effect of chitosan on cell flocculation in soybean curd wastewater treated by photosynthetic bacteria. *Kor. J. Appl. Microbiol. Biotech.* **23:** 763–769.
20. Park, H. K. and K. H. Lee. 1996. Production of microbial chitosan from *Rhizopus japonicus. J. Food & Nutr.* **9:** 336–340.
21. Rutherford, F. A. and P. R. Austi. 1978. Marine chitin properties and solvents, p. 182. *In* Pariser, E. R. (ed.), Proceedings of the First International Conference on Chitin/Chitosan, Muzzarelli. MIT Sea Grant Program, Cambridge, MA, U.S.A.
22. Stephen, A. W., R. F. Peter, and F. Inge. 1979. Production and isolation of chitosan from *Mucor rouxii. Applied Env. Microbiol.* **38:** 323–328.
23. Takanori, S., K. Keisuke, O. Katsuyuki, and I. Yoshio. 1978. Studies on chitin: 7. I.R. spectroscopic determination of degree of deacetylation. *Polymer* **19:** 458–459.
24. Yoon, H.-G., S.-C. Ha, Y.-H. Lim, and H.-Y. Cho. 1998. New thermostable chitosanase from *Bacillus* sp.: Purification and characterization. *J. Microbiol. Biotechnol.* **8:** 449–454.
25. Yun, Y. S., K. S. Kim, and Y. N. Lee. 1999. Antibacterial and antifungal effect of chitosan. *Kor. J. Chitin Chitosan* **4:** 8–14.