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Mycelia of *Mucor rouxii* as a source of chitin and chitosan

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Mycelia of *M. rouxii* may be used as a source of chitosan for medical, cosmetic and other purposes. The contents of chitin and chitosan in the mycelia from 2-day old cultures were 8.9 and 7.3% on a dry basis, respectively. Prolonged growth did not significantly influence the available amount of these polysaccharides. Chitin and chitosan isolation involved deproteinization of the mycelia with 2% NaOH solution at 90°C for 2 h, extraction of chitosan with 10% acetic acid at 60°C for 6 h and subsequent precipitation of chitosan at pH = 9.0. The recovery yield of aminosugars during the isolation process was about 94% of their total content in the mycelia. The chitosan contained 81.3% of glucosamine and its degree of acetylation was about 27.3%. © 1997 Elsevier Science Ltd

INTRODUCTION

Chitin and its deacetylated derivative, chitosan, have unique properties which make them useful for a variety of applications. Two main approaches for utilization of chitin/chitosan presently exist. One is the bioconversion of chitinous polymers to single-cell proteins by the use of *Pichia kudriavzevii* yeast, which grows on the hydrolysates obtained from chitin by chitinase digestion (Carroad & Tom, 1978; Cosio *et al.*, 1982). The other method is the utilization of chitin/chitosan for the removal and recovery of different waste materials such as heavy metals (Muzzarelli *et al.*, 1989; Ramachandran *et al.*, 1982), dyes (McKay *et al.*, 1982; Venkatrao *et al.*, 1986), pesticides (Thome & Van Daele, 1986), amino acids (No & Meyers, 1989), and proteins (Knorr, 1991). Chitosan can also be used for clarification of juices (Imeri & Knorr, 1988) and production of biodegradable packaging films (Mayer *et al.*, 1989). Chitosan may be employed in cosmetics, pharmaceuticals and agriculture. Its use as a component of toothpaste, hand and body creams, shampoo, cosmetics and toiletries, as well as pharmaceuticals for lowering of serum cholesterol, has been documented. Furthermore, its application in enzyme (Synowiecki, 1986) and cell immobilization, as a drug carrier, material for production of contact lenses, or eye bandages, as well as seed coats, etc., has been reviewed (Sandford, 1989; Muzzarelli, 1989).

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The traditional source of chitin is shellfish waste from shrimp, Antarctic krill, crab and lobster-processing (Shahidi & Synowiecki, 1991). However, the industrial isolation of this polymer is reduced by the problems of seasonal and limited supply in some countries and environmental pollution while collecting large amounts of shell waste. Moreover, the conversion of chitin to chitosan, using a strong base solution at high temperature, causes variability of the product properties, decreases the chitosan quality and increases the processing costs. This also produces waste liquid containing base, proteins and protein degradation products. The mycelia of various fungi including *Phycomyces blakesleeanus*, *Mucor rouxii*, *Colletotricum lindemuthianum* or *Absidia coerulea*, are alternative sources of chitin and chitosan (Muzzarelli, 1977; Knorr *et al.*, 1989). Among them, the fungus *Mucor rouxii* has been reported to contain significant quantities of chitosan and also chitin and acidic polysaccharides as cell wall components (Arcidiacono *et al.*, 1989; Knorr *et al.*, 1989; Shimahara *et al.*, 1989; Davis & Bartnicki-Garcia, 1984). This micro-organism can be readily cultured on simple nutrients and the cell wall chitosan can be easily extracted (Bartnicki-Garcia & Nickerson, 1962). Published data suggest that chitosans from fungi are suitable in agriculture because they are more effective in inducing plant metabolite production than chitosan originating from shellfish (Knorr *et al.*, 1989). In *M. rouxii*, being the most commonly researched fungi, however, the contents of chitosan reported by different

authors range from 8.9 to 35% of dry cell wall (Knorr *et al.*, 1989; White *et al.*, 1979; Bartnicki-Garcia & Nickerson, 1962; Arcidiacano *et al.*, 1989). The objectives of the present study were to examine the influence of growth time on the contents of chitosan and other main components of *M. rouxii* mycelia, as well as the yield of chitin and chitosan during the isolation process. Data reported in this paper could also be suitable for optimization of the isolation parameters of microbial chitosan as an alternative to the shellfish-derived product.

MATERIALS AND METHODS

Cell cultivation

Mucor rouxii (ATCC 24905) was stored at 4°C on solid medium containing 2% of agar, 0.3% yeast extract, 1.0% peptone, 2.0% glucose and 94.7% distilled water adjusted to pH 4.5 with H_2SO_4 and autoclaved for 20 min at 117°C. Glucose was added to the medium separately after sterilization. Cultures were grown in 500 ml Erlenmeyer flasks containing 200 ml of liquid medium (0.2% yeast extract, 1.0% peptone, 2.0% glucose and 96.7% distilled water, pH 4.5) and autoclaved for 20 min at 117°C. The flasks were inoculated with a spore suspension (5.0×10^7 cells per flask) and stirred in a water bath (agitation rate 170 rpm) at 28°C for 12 to 96 h without additional aeration.

After the desired growth time, the mycelium of *M. rouxii* was separated by vacuum filtration on Whatman No2 filter paper, washed with distilled water until a clear filtrate could be obtained and lyophilized.

Isolation of chitin and chitosan

The chitin and chitosan from 3–10 g lyophilized mycelia were isolated according to the flowsheet given in Fig. 1. The overall process involved: deproteinization with 2% w/v sodium hydroxide solution (30:1 v/w, 90°C, 2 h), separation of alkali-insoluble fraction (AIF) by centrifugation (4000×*g*, 15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid, 40:1 v/w, 60°, 6 h), separation of crude chitin by centrifugation (4000×*g*, 15 min), and precipitation of chitosan from the extract at pH 9.0, adjusted with 4 M NaOH solution. Crude chitin and chitosan were washed on a coarse sintered-glass funnel (G-4) with water, ethanol and acetone and air-dried at 20°C.

ANALYSES

Moisture

Water was determined by oven-drying of approximately 1 g of the sample at 105°C until a constant weight was obtained (AOAC, 1990).

Proteins

Crude protein in the mycelia was determined (in three replicates) by extracting (2–3 g) samples with 10% w/v NaOH solution for 2 h at 90°C, separating the insoluble matter in NaOH by filtration on a coarse sintered-glass funnel, and dilution to 100 ml with distilled water. The extract (10 ml) was used for protein determination ($N\times6.25$) according to the Kjeldahl procedure (AOAC, 1990).

Glucosamine

Samples (20 mg) of lyophilized mycelia, deproteinized mycelia and chitin or chitosan were hydrolysed in 3.0 ml of 4M HCl in sealed 10 ml ampoules for 18 h at 105°C. The pH of hydrolysates diluted with 40 ml of distilled water was adjusted to 7.0 with 4M NaOH solution. The neutralized solutions were diluted to 100 ml in volumetric flasks. A further 1:5 dilution was made before analysis. The concentration of D-glucosamine in the investigated samples was determined using the Elson-Morgan procedure modified by Johnson (1971). The calibration curve was prepared from 5 to 30 mg of D-glucosamine (Sigma Chemical Co., St. Louis, USA) hydrolysed with 4 M HCl, neutralized and diluted as described for test samples. Final concentrations of glucosamine after dilution ranged from 10 to 60 μg ml^{-1}.

Degree of acetylation

The degree of acetylation (DA) of chitin and chitosan was determined according to Roberts (1992) from their infra-red spectra recorded on a Bruker IFS66 apparatus using the absorbances ratio A_{1655}/A_{3450}. DA was calculated using the equation:

$$DA(\%) = (A_{1655}/A_{3450}) \times 100/1.33$$

STATISTICAL ANALYSIS

Analysis of variance and Tukey's studentized range test (Snedecor & Cochran, 1980) were used to determine differences in mean values of the data from 3–4 replicates. Significance was determined at 95% probability.

RESULTS AND DISCUSSION

Figure 2 shows the cultivation profile of *M. rouxii*. Similar to the results of Arcidiacono *et al.* (1989), the biomass yield increased rapidly up to 48 h of growth. However, the final density of the culture reached during this time was about 4 g of dry mycelia per litre of medium. This is significantly lower than the values (8 to 14 g litre^{-1}) obtained by White *et al.* (1979), perhaps due to the existing differences in aeration of the samples. According to White *et al.* (1979), increasing aeration

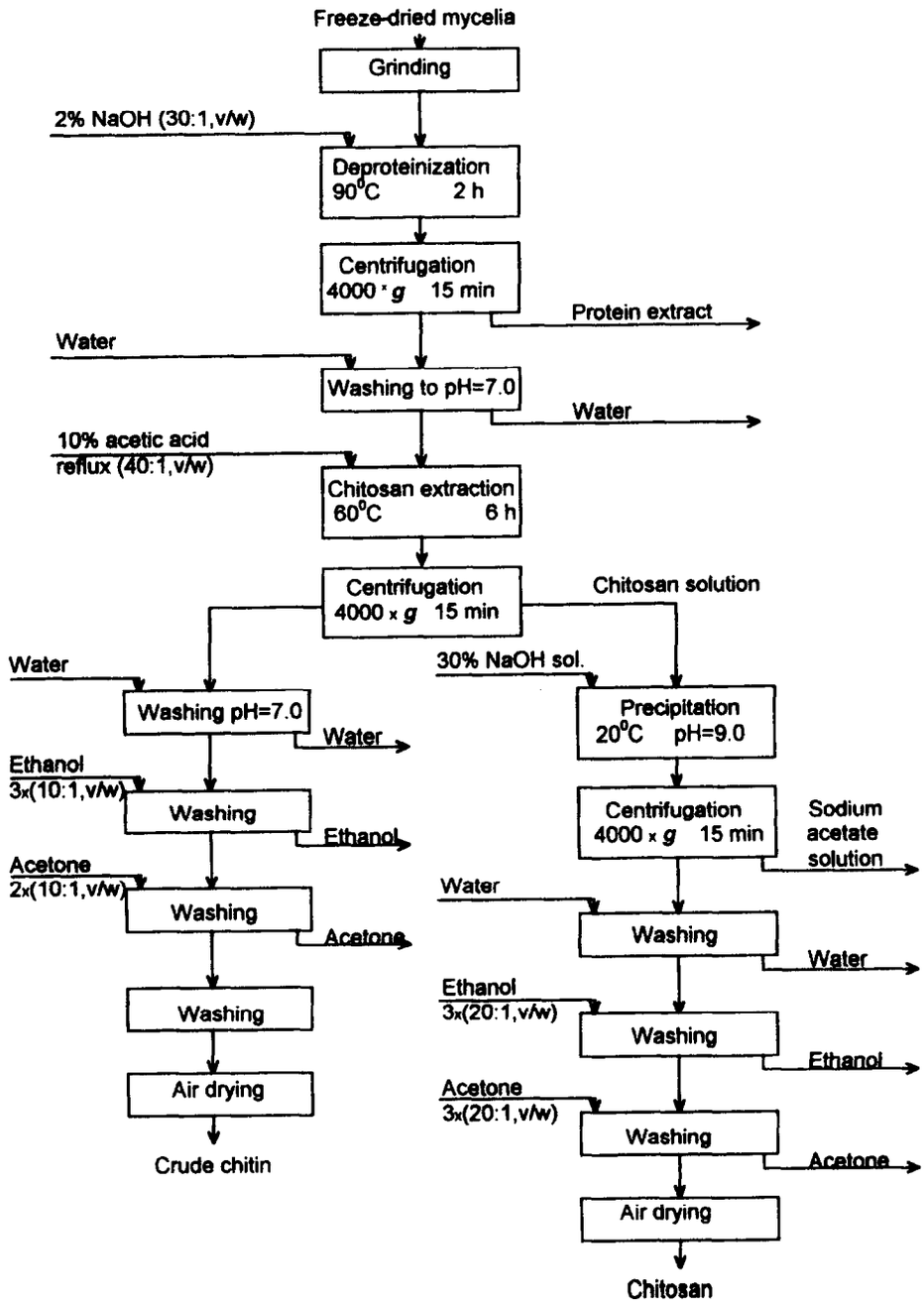

Fig. 1. Flowsheet for the isolation of chitin and chitosan from *M. rouxii*.

enhanced the achievable dry cell mass of *M. rouxii* cultures from 2 to 5 g per litre. The pH of the media drops gradually during growth of *M. rouxii* from the starting value of 4.5 to about 4.1 after 96 h of the experiment. This low pH prevents undesired microbial contamination of the *M. rouxii* cultures.

The main components of the mycelia are water, proteins and an AIF containing chitin, chitosan and acidic polysaccharides (Table 1). The glucosamine content in the dry mycelia after 48 h of cultivation was 11.2%. The first step of chitin and chitosan isolation is deproteinization in alkaline solution. Our preliminary study shows that the maximal yield of protein extraction (60.1% on a dry basis of mycelia) was achieved at 90°C for 2 h at a mycelium to 2% NaOH solution ratio of 1:30 (w/v). These conditions are quite similar to the best parameters for deproteinization of the shrimp shells (Shahidi & Synowiecki, 1991). However, the amount of protein extracted at 60°C decreased to 55.2% of the dry mycelia. During the growth period of *M. rouxii* (12 to 96 h) the protein contents gradually decreased from 63.7 to 55.5% on a dry weight basis of the mycelia (Table 1). Moreover, up to 48 h of cultivation, the amount of AIF increased rapidly from 11.8 to 16.4% of the dry mycelia, as compared with an AIF value 17% reported by White *et al.* (1979). Prolonged growth up to 96 h only slightly influenced the yield of AIF (Fig. 2). Although the amount of AIF continued to increase (Fig. 2) the

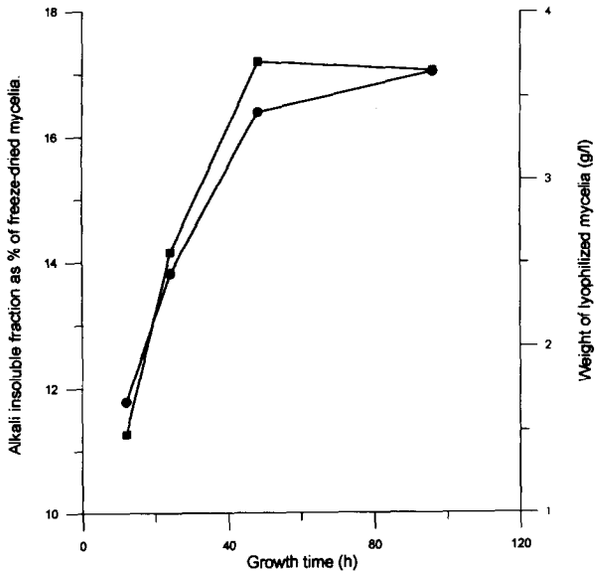

Fig. 2. Changes in total amounts of *M. rouxii* mycelia (●) and alkali-insoluble fraction contents (■) during growth at 28°C. Results are mean values of three replicates. Standard deviations did not exceed 8% of the recorded values.

content of extractable chitosan reached a maximum (7.3% of the dry mycelia) in 2 day-old cultures (Table 1). These data show that the optimal growth time of *M. rouxii* should not exceed 48 h. The amount of glucosamine recovered in the residue after deproteinization was about 94% of this component present in the starting material (Table 2). The yield of chitosan corresponds to the amount of extractable polysaccharide (4–8% of dry cells) reported by Shimahara *et al.* (1989) in the *M. rouxii* cultures. For chitosan isolation, the procedure of White *et al.* was used, but modified by elimination of the homogenization of deproteinized cell with Waring blender, replacement of 1M HCl solution with 10% acetic acid to avoid changes in acetyl content and degree of polymerization, and increase of pH of precipitation to 9.0. The glucosamine content in chitosan isolated after 48 h of *M.rouxii* cultivation was 82.3%. This corresponds to the results (79% of glucosamine) of White *et al.*

Infra-red spectra of chitin and chitosan from *M. rouxii* are similar to those reported in the literature (Muzzarelli, 1977) and also to the IR spectra of these polysaccharides produced from krill shells in the Sea Fisheries Institute in Gdynia (Fig. 3). The most significant parts of these spectra are those showing the amide bonds at approximately 1655, 1555 and 1313 cm^{-1}. As shown in Fig. 3 the chitosan spectrum differs from that of chitin in that the band at 1555 cm^{-1} is absent. The DA has been found to influence the physical and chemical properties and biological activity of chitosan. The DA value for chitosan isolated from *M. rouxii* was 27.3%, which corresponds to the acetyl groups content of 9–22% determined in different microbial chitosans (Shimahara *et al.*, 1989). However, DA values for chitosans produced from shellfish chitin by deacetylation with concentrated NaOH solutions ranged from near zero to 50% with variable degrees of acetylation (Roberts, 1992). The extracted chitosan contained 48.4% of the total glucosamine present in the starting material (Table 2).

The main components of the insoluble residue in acetic acid are chitin, un-extracted chitosan and other glucans, which caused relatively small DA value

Table 1. Changes in the main components of the mycelia during growth of *M. rouxii*

Component	Growth time (h)			
	12	24	48	96
Water(%)	81.1 ± 2.2[a]	82.9 ± 2.3[a]	84.0 ± 0.9[a]	81.0 ± 2.1[a]
Proteins(%db)	63.7 ± 1.2[a]	61.7 ± 0.5[b]	60.1 ± 1.0[c]	55.5 ± 0.0
Deproteinized mycelia (%db)	11.8 ± 0.3[a]	13.8 ± 0.4[b]	16.4 ± 1.0[c]	17.1 ± 1.2
Residue insoluble in CH_3COOH (%db)	7.0 ± 0.9[a]	7.7 ± 0.7[a]	8.9 ± 0.3[b]	9.6 ± 0.0[c]
Chitosan (%db)	4.4 ± 0.5[a]	6.1 ± 1.0[b]	7.3 ± 0.1[c]	7.0 ± 0.1[d]

Results are mean values of three determinations ± standard deviation. Values in each row with the same superscript are not significantly different ($p > 0.05$) from one another. db, dry weight basis of mycelia.

Table 2. Glucosamine recovery during isolation of chitin and chitosan from *M. rouxii*[a]

Component	Glucosamine as % db of mycelia	Weight of fraction (g)	Glucosamine amount (g)	Recovery yield[b] (%)
Freeze-dried mycelia	11.2 ± 0.80	2.90 ± 0.25	0.33	100.0
Deproteinized mycelia	62.4 ± 1.07	0.49 ± 0.08	0.31	93.9
Residue insoluble in acetic acid	68.7 ± 0.37	0.25 ± 0.08	0.17	51.5
Chitosan	81.3 ± 0.29	0.20 ± 0.05	0.16	48.4

[a]Results are mean values of data from three separate samples (cultivated 48h) ± standard deviation.
[b]Recovery yield = $(B/A) \times 100\%$ where A is amount of glucosamine in the investigated component and B is amount of glucosamine in freeze-dried mycelia.

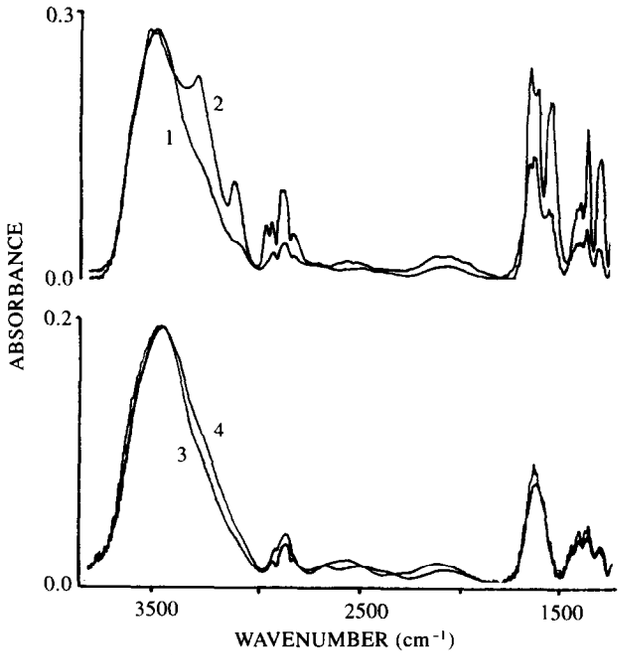

Fig. 3. IR spectra of chitin from *M. rouxii* (1), krill chitin (2), chitosan from *M. rouxii* (3) and krill chitosan (4).

(45.9%) of this fraction. The difficulties in extracting high molecular weight chitosan from arthrospores of *M. rouxii* were also reported by Arcidiacono *et al.* (1989). The amount of the insoluble residue in acetic acid ranged, during growth of *M. rouxii*, from 7.0 to 9.6% of dry mycelia (Table 1). This corresponds to a chitin content of 9% reported by Knorr (1991). However, the relatively small content of glucosamine (68.7% of dry sample) shows that this fraction contained not only aminosugars, but also other polysaccharides.

In conclusion, the mycelia of *M. rouxii* from 2 day-old cultures may serve as a source of chitin and chitosan obtained with yields 8.9 and 7.3%, on a dry weight basis of mycelia, respectively. Prolonged growth did not significantly influence the available amounts of these polysaccharides. The recovery yield of aminosugars during the proposed isolation process was about 94% of their total content in the mycelia. The chitin and chitosan contained 68.7 and 81.3% of glucosamine, respectively.

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REFERENCES

AOAC (1990). *Official Methods of Analysis*, 13th edn. Association of Official Analytical Chemists, Washington, DC, USA.

Arcidiacono, S., Iombardii, S. J. & Kaplan, D. L. (1989). Fermentation processing and enzyme characterization for chitosan biosynthesis by *Mucor rouxii*. In *Chitin and Chitosan*, ed. G. Skjak-Braek, T. Anthonsen & P. Sandford. Elsevier Appl. Sci., London–New York, pp. 319–332.

Bartnicki-Garcia, S. & Nickerson, W. J. (1962). Nutrition, growth, and morphogenesis of *Mucor rouxii*. *J. Bacteriol.*, **84**, 841–858.

Carroad, P. A. & Tom, R. A. (1978). Bioconversion of shellfish chitin wastes. Process conception and selection of micro-organisms. *J. Food Sci.*, **43**, 1158–1162.

Cosio, I. G., Fisher, R. A. & Carroad, P. (1982). Bioconversion of shellfish chitin waste. Waste pretreatment, enzyme production, process design and economic analysis. *J. Food Sci.*, **47**, 901–903.

Davis, L. L. & Bartnicki-Garcia, S. (1984). Chitosan synthesis by the tandem action of chitin synthetase and chitin deacetylase from *M. rouxii*. *Biochem.*, **23**, 1065–1073.

Imeri, A. G. & Knorr, D. (1988). Effects of chitosan on yield and compositional data of carrot and apple juice. *J. Food Sci.*, **53**, 1707–1709.

Johnson, A. R. (1971). Improved method of hexosamine determination. *Analyt. Biochem.*, **44**, 628–635.

Knorr, D. (1991). Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.*, **45**, 114–122.

Knorr, D., Beaumont, M. D. & Pandya, Y. (1989). Potential of acid soluble and water soluble chitosan in biotechnology. In *Chitin and Chitosan*, ed. G. Skjak-Braek, T. Anthonsen, & P. Sandford. Elsevier Apl. Sci., London–New York, pp. 101–118.

McKay, G., Blair, H. S. & Gardner, J. R. (1982). Absorption of dyes on chitin. I. Equilibrium studies. *J. Appl. Polym. Sci.*, **27**, 3043–3045.

Mayer, J., Wiley, B., Henderson, K. & Kaplan, D. (1989). Physical properties of films produced from the biopolymers pollulan and chitosan produced by *Aureobasidicum pullulans* and *M. rouxii*. Abstracts, Annual Meeting of the Am. Soc. for Microbiology, ASM, Washington, DC, USA.

Muzzarelli, R. A. A. (1977). *Chitin*. Pergamon Press, Oxford, UK, pp. 11–14.

Muzzarelli, R. A. A. (1989). Amphoteric derivatives of chitosan and their biological significance. In *Chitin and Chitosan*, ed. G. Skjak-Braek, T. Anthonsen & P. Sandford. Elsevier Appl. Sci., London–New York, pp. 87–99.

Muzzarelli, R. A. A., Wecks, M. & Filipini, O. (1989). Removal of trace metal ions from industrial waters, unclear effluents and drinking waters, with the aid of cross-linked N-carboxymethyl chitosan. *Carbohydr. Polym.*, **11**, 293–297.

No, H. K. & Meyers, S. P. (1989). Recovery of amino acids from seafood processing waste water with a dual chitosan-based ligand exchange system. *J. Food Sci.*, **54**, 60–70.

Ramachandran, N. K. & Madhaven, P. (1982). Metal binding properties of chitosan from different sources. In *Proceedings of 2nd International Conference on Chitin/Chitosan*, ed. S. Hirano & S. Tokura. Japanese Soc. of Chitin and Chitosan, Sapporo, pp. 314–322.

Roberts, G. A. F. (1992). Chitin Chemistry, ed. G. A. E. Roberts, Macmillan Press, Ltd, London, pp. 85–91.

Sandford, P. A. (1989). Chitosan: commercial uses and potential applications. In *Chitin and Chitosan*, ed. G. Skjak-Braek, T. Anthonsen & P. Sandford. Elsevier Appl. Sci. London–New York, pp. 51–69.

Shahidi, F. & Synowiecki, J. (1991). Isolation and characterisation of nutrients and value-added products from Snow Crab (*Chinoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *J. Agric. Food Chem.*, **39**, 1527–1532.

Shimahara, K., Takiguchi, Y., Kobayashi, T., Uda, K. & Sannan, T. (1989). Screening of mucoraceae strains suitable for chitosan production. In *Chitin and Chitosan*, ed. G. Skjak-Braek, T. Anthonsen & P. Sandford. Elsevier Appl. Sci. London–New York, pp. 319–332.

Snedecor, G. W. & Cochran, W. G. (1980). *Statistical Methods* 7th edn. The Iowa State University Press, Ames, IA.

Synowiecki, J. (1986). Use of krill chitin as an enzyme support. In *Chitin in Nature and Technology*, ed. R. A. A. Muzzarelli, C. Jeuniaux & G. Gooday. Plenum Press, New York, pp. 417–420.

Thome, J. P. & Van Daele, Y. (1986). Adsorption of polychlorinated biphenyls (PCB) on chitosan and application to decontamination of polluted stream waters. In *Chitin in Nature and Technology*, ed. R. A. A. Muzzarelli, C. Jeuniaux & G. Gooday. Plenum Press, New York, pp. 551–554.

Venkatrao, B., Baradajaran, A. & Sastry, C. (1986). Adsorption of dyestuffs on chitosan. In *Chitin in Nature and Technology*, ed. R. A. A. Muzzarelli, C. Jeuniaux & G. Gooday. Plenum Press, New York, pp. 554–559.

White, S. A., Farina, P. R. & Fulton, I. (1979). Production and isolation of Chitosan from *Mucor rouxii*. *Appl. and Envir. Microbiol.*, **38**, 323–328.