ELSEVIER

*Carbohydrate Polymers* **25** (1994) 45–50
© 1994 Elsevier Science Limited.
Printed in Great Britain. All rights reserved
0144-8617/94/$07.00

# Chitosan from *Absidia coerulea*

**Riccardo A.A. Muzzarelli,[a] Pierluca Ilari,[a] Renato Tarsi,[b] Bruno Dubini,[c] & Wenshui Xia[a]**

[a]*Institute of Biochemistry,* [b]*Institute of Microbiology, and* [c]*Institute of Physical Sciences, Faculty of Medicine, University, IT-60100 Ancona, Italy*

(Received 21 April 1994; revised version received 31 May 1994; accepted 1 June 1994)

The chitosan–glucan complex system in *Absidia coerulea* grown in YM medium aerobically for 72 h at 25°C is more sensitive to alkali than the complex from other Mucoraceae previously studied. As a consequence, the chitosan can be isolated by boiling the biomass in 25% NaOH for 3 h, washing to neutrality and freeze-drying. Typically, the chitosan is highly filmogenic and has degree of acetylation 0.052, free amine 0.86 and average molecular weight $5 \times 10^5$; it is poorly susceptible to the hydrolytic action of lysozyme, papain and lipase. The reacetylated chitosan (degree of acetylation 0.23), besides being soluble in the usual pH range for chitosans, is exceptionally soluble at pH values over pH 9 and is promptly depolymerized by lysozyme, papain and lipase.

Early investigations on the isolation of chitosan from filamentous fungi indicated that it is relatively simple to recover the chitosan–glucan complex, a combination of chitosan and alkali-insoluble glucan. The chitosan–glucan complexes from *Streptomyces*, *Choanephora cucurbitarum*, *Phycomyces blakesleeanus* and *Mucor rouxii* were found to be effective in chelating transition metal ions, but poor filmogenic substances (Muzzarelli *et al.*, 1980*a*,*b*, 1981; Muzzarelli & Tanfani, 1982). For the isolation of chitosan from *Mucor rouxii*, an acetic acid extraction was proposed by White *et al.* (1979). Fungal microfibers made of chitosan–glucan complex from *Mucor* and *Phycomyces* were studied by Sagar *et al.* (1987). Rane and Hoover (1993*b*), examined the production of chitosan from mycelia of *Absidia coerulea, Absidia blakesleeana, Mucor rouxii, Gongronella butleri* and *Phycomyces blakesleeanus*. Nakamura *et al.* (1993) isolated chitin from *Epidermophyton* and Shimahara *et al.* (1989, 1990*b*) performed extended screenings of fungi for the same purpose.

*Absidia coerulea* was included in works by McGaren *et al.* (1984), Rane and Hoover (1993*a*,*b*), Shimomura *et al.* (1989) and Miyoshi *et al.* (1992), aimed at the isolation of chitosan by extraction with hot acetic acid solutions. By this approach it was demonstrated that pure fungal chitosan with low degree of acetylation (0.06–0.11) and high average molecular weight (450 000) could be obtained with yields of up to 0.99–1.34 g litre$^{-1}$ of culture broth and 10.4% per weight of dry fungal cells. Data for *Absidia butleri* obtained by Shimahara (1990*a*,*b*) were most similar to those for *Absidia coerulea*.

Authors agree on the convenience of the choice of *Absidia coerulea* for the production of chitosan, and the technological aspects for batch and continuous cultivation of *Absidia* spp. in submersed cultures were studied by Davoust and Hansson (1992). The morphological aspects have been studied by Pisarevskaya and Feofilova (1983) and by Pisarevskaya *et al.* (1983).

The proposed acetic acid extraction, however, seems hardly acceptable from the economic and environmental standpoints, because the chitosan has to be precipitated from the 2% acetic acid solution, washing and drying of the resulting amorphous chitosan being a serious problem. Actually, nobody has demonstrated that the acetic acid extraction is really necessary.

The modification of native chitin in fungi, e.g. deacetylation and linkage to glucans, interferes with the crystallization process and the strength of the hydrogen bonding (Muzzarelli, 1993). Because of these variabilities among fungi, and documented differences in their enzymatic systems, it would be reasonable to expect that each fungus would produce a specific type of chitosan–glucan association. Recent data by Schmidt *et al.* (1993) substantiate this point: mycelia of *Agaricus bisporus*, *Fusarium graminearum*, *Phycomyces blakesleeanus*, *Rhizomucor miehei* and *Rhizopus oryzae*, upon identical alkali treatment, yielded materials with chitin content of 42, 19, 91, 70 and 72%, respectively. Differences in the mode and extent of (1-3)-β-D/(1-6)-β-D-glucan binding to chitin have been documented by Sietsma and Wessels (1981).

The acetic acid extraction (Arcidiacono & Kaplan, 1992; Matsubara & Kuroda, 1991) seems to be justified

for those fungi which are known to yield chitosan–glucan complexes upon NaOH treatment such as *Mucor* and *Phycomyces* (Knorr & Klein, 1986), but, for *Absidia coerulea*, in the absence of evidence that alkali-insoluble glucans prevail or are strongly linked to chitosan, it might prove to be avoidable. It should be underlined that the so-called 'alkali extraction' in the paper by Rane and Hoover (1993*a*) actually includes an extraction with 2% acetic acid for 12 h at 95°C.

We have therefore undertaken the present study aimed at the isolation of chitosan from *Absidia coerulea* using sodium hydroxide only, and also the chemical and enzymatic characterization of the product.

## MATERIALS AND METHODS

### Organism and culture conditions

*Absidia coerulea* 14076 was provided by American Type Culture Collection, Rockville, MD, USA and grown on two different media: medium YM (modified) containing glucose (1.0%), peptone (0.5%) and yeast extract (1.0%). A second medium was also used according to Rane and Hoover, containing glucose (2%), peptone (1%), yeast extract (0.1%), ammonium sulfate (0.5%), dipotassium hydrogen orthophosphate (0.1%), sodium chloride (0.1%), magnesium sulfate (0.5%), calcium chloride (0.01%) and cobalt sulfate heptahydrate (5 mg litre$^{-1}$). Prior to sterilization at 121°C, the medium pH was adjusted to 5.5 in both cases. The cobalt solution was sterilized by filtration. The *Absidia coerulea* was incubated in YM agar for 72 h at 30°C; by washing the mycelia with saline, spores were collected at the concentration of ca. $15 \times 10^6$ ml$^{-1}$. The media were inoculated with $1.0–1.5 \times 10^7$ spores per litre. The 2-litre flasks containing 400 ml of medium were incubated for 48 or 72 h at 25°C and 200 rpm, in a stream of air.

### Preparation of fungal chitosans

The fungal cells were inactivated with sodium hydroxide, extensively washed with demineralized water and used undried. A portion of the fungal mass (5 g dry weight) was disrupted with an homogenizer and then treated with boiling aqueous NaOH solution (100 ml, various concentrations) under reflux for various lengths of time to remove protein. The alkali-treated mass was collected by filtration, washed to neutral pH and lyophilized. For analytical purposes, the material was ground in a blade mill.

### Preparation of partially *N*-acetylated chitosan

The ground fungal mass (0.92 g) was dissolved into acetic acid (0.1%, 92 g) and water (98 g) and methanol (38 g) added to make up a solution to which acetic anhydride (0.54 ml molar ratio to the amine 1:1) was added. The solution was left standing overnight and was dialysed against three changes of demineralized water for 3 days. The product was recovered by lyophilization.

### Measurement of the degree of acetylation

The degree of acetylation of the fungal chitosans and the *N*-acetylated products was determined by first derivative UV spectrophotometry (Muzzarelli & Rocchetti, 1985), with a Kontron Uvikon 860 instrument. Two independent methods were used for the determination of the glucosamine units. The colloid titration method, based on the use of potassium polyvinyl sulfate (PVSK) with Toluidine Blue as an indicator, for the determination of cationic polymers (Korenaga, 1979). The spectrophotometric method based on the use of 3-methyl-2-benzotiazolinone hydrazone hydrochloride hydrate (MBTH) was used for the determination of the aldehyde groups generated by oxidative deamination (Vignon *et al.*, 1986). The ninhydrin method was found to be unreliable in the present case.

### Other analytical methods

The molecular weight determinations were made by gel permeation chromatography with a Spectra Physics Isocrom L.C. pump equipped with a Data Jet integrator, a Shodex RI SE-61 detector and a column TSK-GEL GMPW-XL, $7.8 \times 300$ mm operated at the flow rate of 1 ml/min. Chitosan samples (20 $\mu$l, 10 g litre$^{-1}$) were eluted with a solvent of acetic acid (0.5 M) and sodium acetate (0.5 M) at 27°C.

A rough estimation of the average molecular weight was also done by electrophoresis on a Biorad Protean II apparatus, at constant current (30 mA) for 7 h. Chitosan hydrolysates (100 $\mu$g) were placed on a 25% polyacrylamide, 7 M urea and 5.5% acetic acid slab gel. The buffer was 5.5% acetic acid. Gel staining was made by means of Coomassie Brilliant Blue as a 0.125% solution in a methanol–acetic acid–water (50:10:40) mixture, and destaining with the aid of methanol–acetic acid–water (10:10:80) mixture.

Alkalimetric titrations under nitrogen stream provided $pK_a$ values of $6.2 \pm 0.3$.

Infrared spectrometry was done on thin films cast from acetic acid solutions and treated with an alcoholic solution of KOH to remove the counterion.

X-ray diffraction spectra were obtained by using a vertical powder diffractometer; the source was a rotating anode generator Rigaku Denki RU-300 and Ni-filtered CuK$\alpha$ radiation (0.154 nm) was used.

Chemicals were supplied by Aldrich Chimica, Milan, Italy. Culture media were from Difco, Milan, Italy.

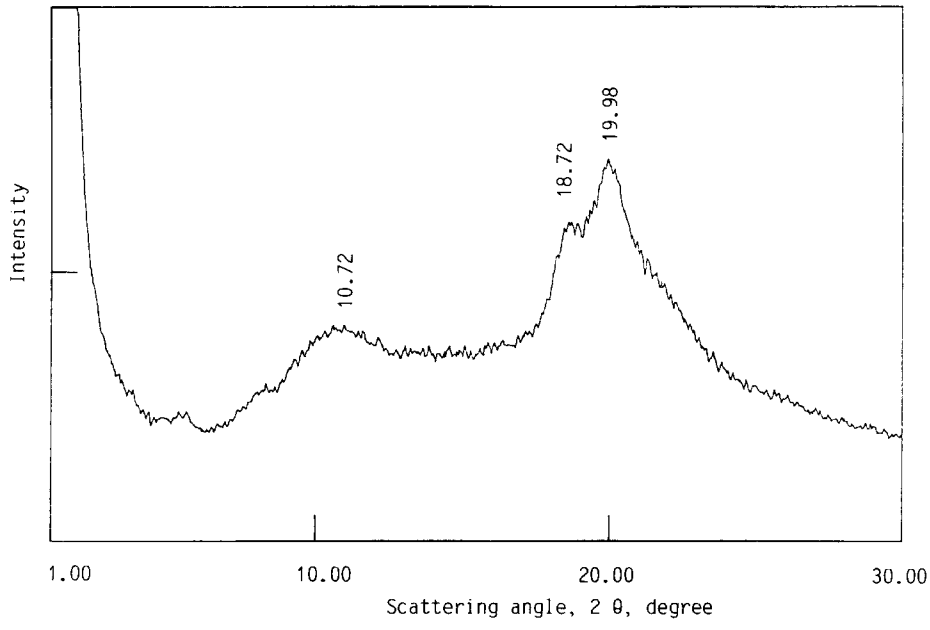


**Fig. 1.** X-ray diffraction spectrum for chitosan extracted under alkaline conditions (NaOH 250 g litre$^{-1}$).

### Enzymatic assays

The viscosity decrease for 2% fungal chitosan solutions (acetate buffer at pH 5.77) in the presence of lysozyme, papain or lipase was monitored by the rotational viscometry method (Muzzarelli *et al.*, 1994). Wheatgerm lipase was supplied by Sigma, Milan, Italy, hen egg white lysozyme and *Carica papaya* papain were supplied by Calbiochem, Milan, Italy, and endo-exo-$\beta$-1,3-glucanase (Quantazyme YLG) was supplied by Cellon Sarl, Luxembourg.

## RESULTS AND DISCUSSION

The yield of the mycelium produced in the YM medium was 6.2 g litre$^{-1}$; from the YMS medium it was 7.0 g litre$^{-1}$. The overall average yield of chitosan was 1.87 and 1.80 g litre$^{-1}$, respectively (dry weight), corresponding to 30 and 26% of the mycelial dry weight (comparable to those obtained by Park *et al.* (1991) and definitely higher than in other studies).

During X-ray diffraction spectrometry, the *Absidia coerulea* material was poorly crystalline, showing a broad peak at 20° 2$\theta$ value, but the spectrum for the alkali-treated material showed a broad peak at 10.72° and two peaks at 18.72° and 19.98° 2$\theta$, with close similarity to the spectrum of authentic chitosan (Fig. 1). At the optical microscope, the alkali-treated products stained with Saphranine or with other stains showed the preserved morphology of the fungus, with flattened and empty structures (Fig. 2). Based on literature data, the chitosan was considered deprived of protein.

As shown in Table 1, two concentrations of NaOH (25

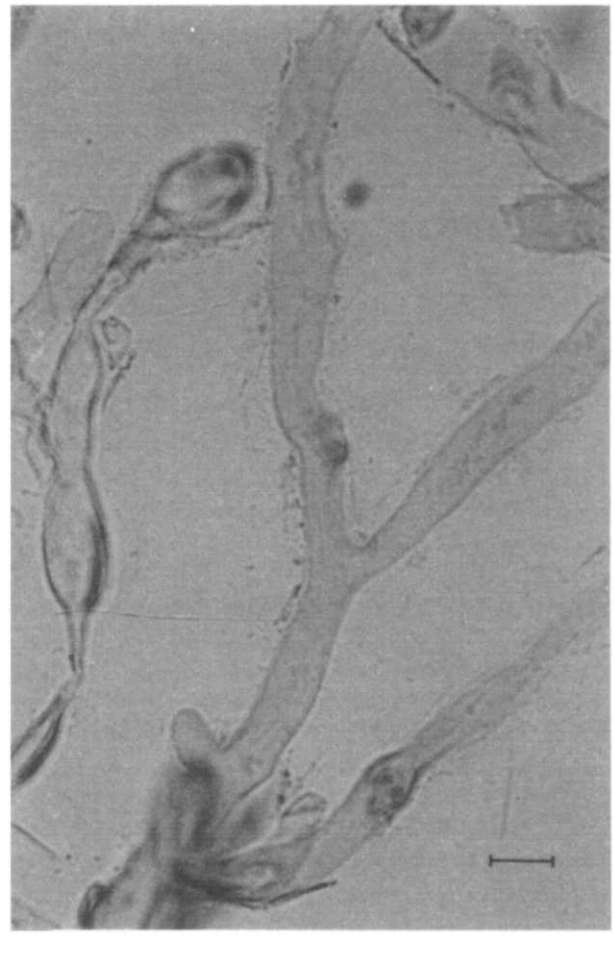

**Fig. 2.** *Absidia* chitosan after isolation in NaOH (250 g kg$^{-1}$), as observed at the optical microscope after staining with saphranine (bar = 10 $\mu$m).

**Table 1. Characteristic properties of the *Absidia coerulea* chitosans obtained by alkali treatment at boiling temperature, washed to neutrality and freeze-dried before analysis**

| Treatment time, h | 25% NaOH aqueous solution | | | 40% NaOH aqueous solution | | |
|---|---|---|---|---|---|---|
| | d.a. | Free amine % | Viscosity mPa·s | d.a. | Free amine % | Viscosity mPa·s |
| 1 | 0.065 | 0.73 | 194 turbid | 0.046 | 0.84 | 53 |
| 2 | 0.062 | 0.77 | 112 turbid | 0.040 | 0.90 | 30 |
| 3 | 0.052 | 0.86 | 78 | 0.030 | 0.94 | 20 |
| 5 | 0.046 | 0.93 | 34 | 0.020 | 0.93 | 13 |
| 7 | 0.046 | 0.94 | 30 | 0.009 | 0.95 | 10 |

d.a. = degree of acetylation determined by first derivative spectrophotometry. Free amine determined by colloid titration with PVSK. Viscosity determined by rotatory viscometry at shear rate 200 $s^{-1}$ on 10 g $litre^{-1}$ solutions.

and 40%) were used to remove the glucans. The *Absidia coerulea* chitosans thus obtained were weighed as freeze-dried materials and were most easily dissolved in dilute acetic acid for analytical purposes. Turbid solutions were only those relevant to short treatments in 25% NaOH (1 and 2 h). Viscosity was found to depend on the concentration of NaOH and the duration of the treatment. The flow curve indicated non-Newtonian behaviour.

The degree of acetylation was measured by the first derivative spectrophotometry method: using the hypothesis that some glucan would accompany the chitosan this method is still reliable, the determination being directed to the *N*-acetylglucosamine content. The degree of acetylation was found to be small, ranging from 0.009 to 0.065, and depending of course on the duration of the alkali treatment: it dropped from 0.065 to 0.046 in the case of 25% NaOH during the 7-h treatment, and to 0.009 in the case of 40% NaOH. The free amine contents were coincident at 0.94 in both systems after 5–7 h. If one assumes that no protein is present in the latter samples and that the water content is 4.5%, then the glucan content after 1, 2 and 3 h of treatment with 25% NaOH would be approximately 0.017, 0.014 and 0.005. The sample combining in itself ease of preparation and the best set of characteristics (degree of acetylation 0.052, free amine 0.86, viscosity 78 mPa·s, glucan < 0.05) was the one obtained after 3 h in 25% NaOH. This product is referred to as *Absidia* chitosan.

Clear and mechanically strong films could be cast from the *Absidia* chitosan acetic acid solutions, according to standard procedure. They could be made thin enough to permit direct chitosan examination by IR spectrometry, as acetate salt as well as after treatment with alcoholic KOH. The IR spectra were most similar to the spectra for the authentic polyaminosaccharides: in the case of chitosan (lower spectrum in Fig. 3), the shape of the 1594 $cm^{-1}$ band was typical for the highly deacetylated (d.a. 0.04) chitosans (compare Mima *et al.*, 1982, Fig. 1, p. 23), as well as the shoulder at 1650 $cm^{-1}$. Other spectral regions essentially matched the authentic chitosan spectra, especially the 1036, 1093 and 1154 $cm^{-1}$ bands. Perhaps the band at 1417 $cm^{-1}$ was somewhat enhanced in the *Absidia* chitosan. As for the reacetylated chitosan, the IR spectrum was identical to that for crustacean chitin (compare Shimahara *et al.*, 1982, Fig. 2, p. 11).

As expected on the grounds of their low degree of acetylation, the *Absidia* chitosans were found to be poorly susceptible to enzymatic attack. When submitted to partial *N*-acetylation a final overall degree of acetylation of 0.23 was obtained, which falls in the range of maximum susceptibility to lysozyme. Thus, the reacetylated chitosans proved to be highly degradable under the action of lipase, papain and lysozyme, as indicated by the viscosity vs time curves in the initial 10-min interval. Here, the viscosity decrements observed after 10 min were 67, 66 and 53%, respectively. The HPLC data showed that the reacetylated chitosan was polydisperse with fractions in the range $2 \times 10^5$–$1 \times 10^6$, average $5 \times 10^5$; after 10 min contact with papain, the average value was 43 000, and after 100 h was 600. The HPLC and electrophoretic results showed that the *Absidia* chitosans were polydisperse, due to the alkali treatment, and, compared to the LMW Fluka chitosan, contained an higher proportion of high m.w. fractions. Therefore, the alkaline extraction led to the same average mw ($5 \times 10^5$) already reported by other authors for the acetic acid extraction.

The reacetylated chitosans were fully soluble in water and yielded nearly clear solutions at all usual pH values as well as at alkaline values (pH 9). Attempts at removing the slight cloudiness present in both chitosan and reacetylated chitosan solutions with the aid of a glucanase (Quantazyme YLG) did not lead to any improvement. Of course, the *Absidia* chitosan could be reacetylated to greater extents by using 5% v/v acetic anhydride in methanol, as described by East & Qin (1994) for chitosan fibers.

## CONCLUSIONS

*Absidia coerulea* has been found to produce chitosans of satisfactory yield and quality by simply boiling the harvested biomass with 25% NaOH. There is no need to extract the chitosan with the aid of boiling acetic acid, because the chitosan is not in combination with

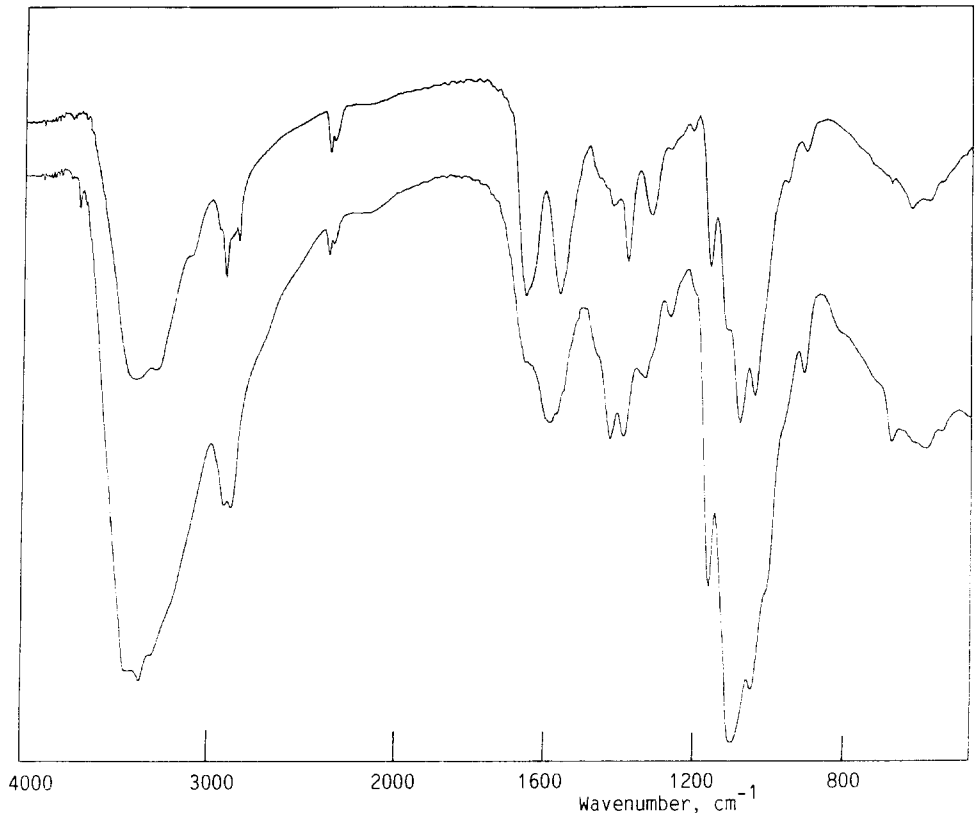


**Fig. 3.** FTIR spectra of *Absidia* chitosan. Lower curve: obtained after treatment with boiling 25% NaOH for 3 h. *Upper curve*: same after reacetylation with acetic anhydride (degree of acetylation: 0.23). Spectra taken on films conditioned with alcoholic KOH.

quantities of glucan that would impart detrimental characteristics; rather, the fungal chitosan in its final presentation is partly crystalline and preserves the original structure of the fungus thus offering enhanced ease of handling and versatilty (for instance for non-vowen fabrics). No anomalous behaviour of this fungal chitosan would prevent its use in alternative to animal chitosan: the chitosan provides clear solutions and transparent and resistant films. As for the biological/immunological aspects involved with the presence of minor amounts of glucan, it should be noted that fungal chitosan–glucan complexes have been tested *in vivo* for several years. The glucan itself has been proposed as a wound dressing (Cassone *et al.*, 1991) and an immunomodulator (DeFelippe *et al.*, 1993). The *Absidia* chitosans tested according to ASTM F813–83 and F619–79 with murine fibroblasts were found to be biocompatible in terms of cytotoxicity and lactate dehydrogenase determinations; these data were in agreement with those by Chung *et al.* (1994).

## ACKNOWLEDGEMENTS

This work was carried out with the financial contribution of Consiglio Nazionale delle Ricerche, Progetto Finalizzato Chimica Fine II, Roma (Contract no. 93.02886PF72, assigned to Prof. R. Muzzarelli).

## REFERENCES

Arcidiacono, S. & Kaplan, D.L. (1992). Molecular weight distribution of chitosan from *Mucor rouxii* under different culture and processing conditions. *Biotechnol. Bioengng*, **39**, 281–6.

Cassone, A., Bistoni, F. & Marconi, P.F. (1991). Process for preparing a glucan-containing product starting from *Candida albicans* BMM-12. *Eur. Pat. Appl.*, 416,343 A2.

Chung, L.Y., Schmidt, R.J., Hamlyn, P.F. & Sagar, B.F. (1994). Biocompatibility of potential wound management products: fungal mycelia as a source of chitin/chitosan and their effect on the proliferation of human F1000 fibroblasts in culture. *J. Biomed. Mater Res.*, **28**, 463–9.

Davoust, N. & Hansson, G. (1992). Identifying the conditions for development of beneficial mycelium morphology for chitosan producing *Absidia* spp. in submersed cultures. *Appl. Microbiol. Biotechnol.*, **36**, 618–20.

DeFelippe J., Silva, M.D.E., Maciel F.M.B., Soares, A.D. & Mendes, N.F. (1993). Infection prevention in patients with severe multiple trauma with the immunomodulator β-1-3-polyglucose (glucan). *Surg. Gynecol. Obstet.*, **177**, 383.

East, G.C. & Qin, Y. (1993). Wet spinning of chitosan and the acetylation of chitosan fibers. *J. Appl. Pol. Sci.*, **50**, 1773–9.

Knorr, D. & Klein, J. (1986). Production and conversion of

chitosan with the cultures of *Mucor rouxii* or Phycomyces blakesleeanus. Biotechnol. Lett., **8**, 691–4.

Korenaga, T. (1979). Studies on the formation of polyion complexes by means of colloid titration method. *Mikroch. Acta*, **II**, 421–31.

Matsubara, M. & Kuroda, H. (1991). Isolation and physiological activity of the chitosan from conidia and mycelia of *Mycosphaerella pinodes*. *Chem. Pharm. Bull.*, **39**, 1484–8.

McGaren, W.J., Perkinsons, G.A., Growich, J.A., Leese, R.A. & Ellestad, G.A. (1984). Chitosan by fermentation. *Process Biochem.*, **19**, 88–90.

Mima, S., Miya, M., Iwamoto, R. & Yoshikawa, S. (1982). Highly deacetylated chitin and its properties. In *Chitin and Chitosan*, eds S. Hirano & S. Tokura. Jpn. Soc. Chitin and Chitosan, Sapporo, pp. 21–5.

Miyoshi, H., Shimura, K., Watanabe, K. & Onodera, K. (1992). Characterization of some fungal chitosans. *Bios. Biotech. Biochem.*, **56**, 1901–5.

Muzzarelli, R.A.A. (ed.) (1993). *Chitin Enzymology*. Eur. Chitin Soc., Ancona.

Muzzarelli, R.A.A., Ilari, P. & Tomasetti, M. (1994). Depolymerization of chitosans with the aid of papain. *Enzym. Microb. Technol.*, **16**, 110–4.

Muzzarelli, R.A.A. & Rocchetti, R. (1985). The determination of the degree of acetylation of chitosan by first derivative ultraviolet spectrophotometry. *Carbohydr. Polym.*, **5**, 461–72.

Muzzarelli, R.A.A. & Tanfani, F. (1982). The chelating ability of chitinous materials. *Proc. 2nd Int. Conf. Chitin/Chitosan*, eds S. Hirano & S. Tokura. Jpn. Chitin Society, Sapporo, pp. 183–6.

Muzzarelli, R.A.A., Tanfani, F. & Emanuelli, M. (1981). The chelating ability of chitinous materials from *Streptomyces, Mucor rouxii, Phycomyces blakesleeanus* and *Choanephora cucurbitarum*. *J. Appl. Biochem.*, **3**, 322–7.

Muzzarelli, R.A.A., Tanfani, F. & Scarpini, G. (1980*a*). Chelating, film-forming and coagulating ability of the chitosan-glucan complex from *Aspergillus niger* industrial wastes. *Biotechnol. Bioengng*, **22**, 885–96.

Muzzarelli, R.A.A., Tanfani, F., Scarpini, G.F. & Tucci, E. (1980*b*). Removal and recovery of cupric and mercuric ions from solutions using chitosan-glucan from *Aspergillus niger*. *J. Appl. Biochem.*, **2**, 54–9.

Nakamura, K., Yamada, C. & Tomita, S. (1993). Process for producing chitin and chitosan from dermatophyte. *PCT Int. Appl.*, WO 9314216.

Park, O., Miyoshi, H., Watanabe, J., Chiba, T. & Endo, I. (1991). Manufacture of a chitosan with Mucoraceae. *Eur. Pat. Appl.*, EP 531,991.

Pisarevskaya, I.V. & Feofilova, E.P. (1983). Changes in the content and fatty acid composition of lipids from whole cell walls and the process of cytodifferentiation of *Absidia coerulea*. *Microbiology (Moscow)*, **52**, 444–8.

Pisarevskaya, I.V., Feofilova, E.P. & Marchenko, I.V. (1983). Changes in the composition of cell walls in the process of *Absidia coerulea* cytodifferentiation. *Microbiology (Moscow)*, **52**, 124–30.

Rane, K.D. & Hoover, D.G. (1993*a*). An evaluation of alkali and acid treatments for chitosan extraction from fungi. *Process Biochem.*, **28**, 115–8.

Rane, K.D. & Hoover, D.G. (1993*b*). Production of chitosan by fungi. *Food Biotechnol.*, **7**, 11–33.

Sagar, B., Hamlyn, P. & Wales, D. (1987). Wound dressing. *Eur. Pat. Appl.*, 460,774 A2.

Schmidt, J., Chung, L.Y., Andrews, A.M., Sagar, B.F., Hamlyn, P.F. & Turner, T.D. (1993). Hydrogen peroxide generation by chitin/chitosan of fungal origin and its relationship to their pro- and anti-proliferative effects on fibroblasts in culture. *3rd Eur. Conf. Adv. Wound Management*, Oct. 19–22, Harrogate, UK.

Shimahara, K. (1990*a*). Novel methods for production of chitin and chitosan. *Sen'i Gakkaishi*, **46**, P547–52.

Shimahara, K. (1990). Production of chitosan by cultivation of molds. *Kenkyu Hokoku - Asahi Garasu Zaidan*, **57**, 423–9.

Shimahara, K., Okhouchi, K. & Ikeda, M. (1982). A new isolation method of crustacean chitin using a proteolytic bacterium. In *Chitin and Chitosan*, eds S. Hirano & S. Tokura. Jpn. Soc. Chitin and Chitosan, Sapporo, pp. 10–4.

Shimahara, K. Takiguchi, Y., Kobayashi, T., Uda, K. & Sannan, T. (1989). Screening of mucoraceae strains suitable for chitosan production. In *Chitin and Chitosan*, eds G. Skjak-Braek, T. Anthonsen & P. Sandford. Elsevier, Amsterdam, pp. 171–83.

Shimomura, T., Takahashi, Y., Yamada, M., Tsukamoto, Y. & Kawamura, K. (1989). Manufacture of chitosan hydrolysates from filamentous fungi and food preservatives containing them. *Jpn. Kokai Tokkyo Koho, JP*, 02,215,393.

Sietsma J.H. & Wessels, J.G.H. (1981). Solubility of (1-3)-β-D/(1-6)-β-D-glucan in fungal walls: importance of presumed linkage between glucan and chitin. *J. Gen. Microbiol.*, **125**, 209–12.

Vignon, C., Plassard, C., Mousain, D. & Salsac, L. (1986). Assay of fungal chitin and estimation of mycorrhizal infection. *Physiol. Veg.*, **24**, 201–7.

White, S.A., Farina, P.R. & Fulton, I. (1979). Production and isolation of chitosan from *Mucor rouxii*. *Appl. Environ.*, **38**, 323–8.