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Polyuronans obtained by regiospecific oxidation of polysaccharides from *Aspergillus niger*, *Trichoderma reesei* and *Saprolegnia* sp.

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Abstract

The biomasses of *Aspergillus niger*, *Trichoderma reesei* and *Saprolegnia* sp. were submitted to sodium dodecylsulfate washing, mechanical disruption of the mycelia, stirring at 60°C for 3 h and protracted stirring overnight at room temperature. They were then oxidized regiospecifically at C6 with NaOCl and NaBr for 30 min at room temperature in the presence of Tempo® as a catalyst. The powders obtained were polyuronans in the sodium salt form, fully soluble in water over the pH range 3–12. Yields were much higher than for the chitosan extraction. The polyuronans were characterized by ^{1}H NMR spectrometry and were shown to contain 20 and >75% 6-oxychitin, for *A. niger* and *T. reesei*, respectively. The deconvolution of the FTIR spectra in the range 1500–1800 cm^{-1} confirmed the presence of a band at 1568 cm^{-1} that was assigned to chitin amide. This band was seen much more evidently in *T. reesei* than in the *A. niger* material. Since the fungi examined are representative of the three major types of cell walls, and are used industrially, it is concluded that the process is simple to follow and is of wide applicability. The process allows upgrading of the spent biomasses and the exploitation of their polysaccharides for industrial applications: examples are given where the polyuronans are reacted with glycerol or with poly(ethyleneglycol) to provide water soluble products with enhanced viscosity.

Keywords: Fungal polysaccharides; Polyuronans; Regiospecific oxidation; *Aspergillus niger*; *Trichoderma reesei*; *Saprolegnia*

1. Introduction

Huge quantities of mycelia from industrial fermentation processes are disposed off by incineration, addition to cattle feed and preparation of fertilizers.

There is much interest in upgrading fungal biomasses such as those of *Aspergillus niger* from citric acid production plants, *Streptomyces* from pharmaceutical industries and *Trichoderma* from cellulase production units, but so far the low value of the recovered products and the labor and chemical costs have prevented the development of commercially viable treatments.

The recovery of chitin, chitosan and chitin–glucan complex, from fungi containing a significant proportion of chitin, or from fungi that contain chitosan rather than chitin has been investigated (Jollès & Muzzarelli, 1999; Muzzarelli & Muzzarelli, 1998; Muzzarelli & Peter, 1997;). For example, chitosan was extracted from *Mucor rouxii* with the aid of acetic acid (Rane & Hoover, 1993; Sagar, Hamlyn & Wales, 1991; White, Farina & Fulton, 1979) and from *Absidia* via simplified alkali treatments with 30% yield (Muzzarelli, Ilari, Tarsi, Dubini & Xia, 1994) or 10% yield (Hu, Yeung, Ho & Hu, 1999); chitin was isolated from *Epidermophyton* (Nakamura, Yamada & Tomita, 1993; Shimahara, Takiguchi, Kobayashi, Uda & Sannan, 1989); the chitin–glucan complex was isolated from a variety of fungi including *Streptomyces* and *Aspergillus* and used in various applications (Muzzarelli, Tanfani & Scarpini, 1980a; Muzzarelli, Tanfani, Scarpini & Tucci, 1980b).

These methods do not lend themselves to scaling up because of the small yield, the large amount of chemical waste and the possible inability to compete with the well-established market for crustacean chitosans.

The use of the chitin–glucan complex for medical purposes has been investigated. This polysaccharide is not attractive due to its insolubility. Nevertheless, the biocompatibility of the fungal material was assessed, and limited information in terms of hydrogen peroxide generation (Schmidt, Chung, Andrews, Sagar, Hamlyn & Turner, 1993) and the effect on the fibroblast proliferation (Chung, Schmidt, Hamlyn, Sagar, Andrews & Turner, 1994) was provided for its use in wound management. Gram-positive bacteria were adsorbed onto the *A. niger* chitosan–glucan

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complex (Velichov, Nikolova & Veljanov, 1989) obtained after deacetylation (Benderliev, Alexieva & Ivanova, 1985), and *Chlorella vulgaris* was flocculated using the same product (Benderliev & Ratcheva-Kantcheva, 1988).

Large chemical differences among the alkali-treated mycelia of a variety of origins have been reported. A preparation from *Ganoderma tsugae*, was made from the residue of the fruiting body of *G. tsugae* after alkali treatment and used as a skin substitute as efficient as non-woven chitin dressings (Su, Sun, Juan, Ho, Hu & Sheu, 1997; Su, Sun, Juan, Hu, Ke & Sheu, 1999; Gorovoj, 2000).

While these studies indicate the potential of fungal polysaccharides in the medical field and encourage research intended for the exploitation of industrial biomasses, it should be noted that very few articles of relevance have been published during the last two years. The chitin–glucan complex from *A. niger* was carboxymethylated with monochloroacetic acid and sonicated to obtain a product still having anti-mutagenic properties but suitable for oral administration thanks to its low molecular weight (194 kDa). The degree of substitution was 0.43 and the nitrogen content 1.61% (Chorvatovicova, Machova & Sandula, 1998; Machova, Chorvatovicova, Kogan, Sandula & Stratilova, 1998; Machova, Kvapilova, Kogan & Sandula, 1999). Carboxylation reactions, widely applied to starch (Boruch, 1985), cellulose (Kennedy, Knill & Taylor, 1998) and chitin (Trujillo, 1968), appear to be an acceptable approach for transforming the *A. niger* chitin–glucan complex into a water-soluble product.

We have recently produced 6-oxychitin from crustacean chitin by regiospecific oxidation (Muzzarelli, 1997; Muzzarelli, Muzzarelli, Cosani & Terbojevich, 1999). Data have also been published on the possibility of derivatising other polysaccharides (Chang & Robyt, 1996; DeNooy, Besemer & van Bekkum, 1995). In that light, we have undertaken the present work with the intention of defining the conditions suitable for the production of C6-carboxylated polysaccharides from industrial *A. niger* spent biomasses, as well as from the model systems of *Trichoderma reesei* and *Saprolegnia* sp. These are well-studied fungi, *T. reesei* being a biocontrol agent against plant pathogens (Lambert, 1983) and a chitinase producer, and *Saprolegnia parasitica* being a pathogenic water mold of paramount importance in aquaculture activities (Bruno & Wood, 1999). The *Saprolegnia* mycelium looks like a white cotton-like plug, and is responsible for infection outbreaks involving fish and eggs.

While the chitin content of *A. niger* mycelia is very low and glucans predominate (Ruiz-Herrera, 1978), *T. reesei* contains a high ratio of chitin, typically greater than 75% of the cell dry weight (Berkeley, Gooday & Ellwood, 1979; Brown & Thornton, 1998). On the other hand, *Saprolegnia monoica* contains mostly cellulose accompanied by minor amounts of chitin (0.7%) (Gay, Chanzy, Bulone, Girard & Fevre, 1993). The present work therefore intends also to verify that the various associations of polysaccharides from these fungi (Rosenberger, 1976) are equally susceptible to regiospecific oxidation.

2. Experimental

2.1. Organisms and culture conditions

Aspergillus niger inactivated biomass was a gift of Cerestar, Casei Gerola, Italy. The mycelia were autoclaved, washed with dilute sodium hypochlorite and water before shipment. *T. reesei* was purchased from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *Saprolegnia* was from an aquaculture plant.

2.1.1. Growth conditions for Saprolegnia

Aliquots of the white slime enveloping *Saprolegnia*-infected rainbow trout fries (*Salmo iridea*) were collected with sterile scissors and transferred to Petri dishes made of Sabouraud agar containing the antibiotics gentamycine and cefuraxine (50 μl/ml each) necessary to prevent bacterial growth. After incubation overnight at 17°C, the culture was examined under the optical microscope to verify the absence of bacteria and to identify the fungus. Flasks (2 l each) were prepared with Sabouraud broth where *Saprolegnia* was inoculated at 25°C for 72 h.

Sabouraud broth in several 2 l flasks was inoculated with *Saprolegnia*; after incubation at 25°C for 72 h the culture was filtered on Millipore filters (0.45 μm). Similar conditions were adopted for the growth of *T. reesei* in Sabouraud broth, following the guidelines indicated by Brown and Thornton (1998). The harvested fungal mycelia were inactivated by washing with dilute NaOH and stored at −80°C.

2.1.2. Chemicals

To oxidize regiospecifically the polysaccharides, the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®) was used as a catalyst, together with NaBr, in 4% NaOCl solution. All chemicals, including poly(ethylene glycol) 400, were provided by Aldrich, Milano; culture media were supplied by Difco, Milano, Italy.

2.2. Instrumental analyses

The FTIR spectra were obtained with a Perkin–Elmer Spectrum 2000 FTIR spectrometer, or with a Nicolet 20-SX FTIR spectrometer equipped with a Spectra Tech "Collector" accessory for DRIFT measurements. Close spectral similarities could be seen between these spectra and those from Micro-ATR. ^{1}H NMR spectra were recorded on a Varian Gemini 200 spectrometer at 25°C. SEM observations were carried out with a Philips XL20 electron microscope equipped with an EDAX PV 9800 microanalysis system. A MeterLab CDM 210 conductivity meter made by Radiometer, Copenhagen was used in conjunction with the conductivity cell CDC641T. The Heto Drywinner 95

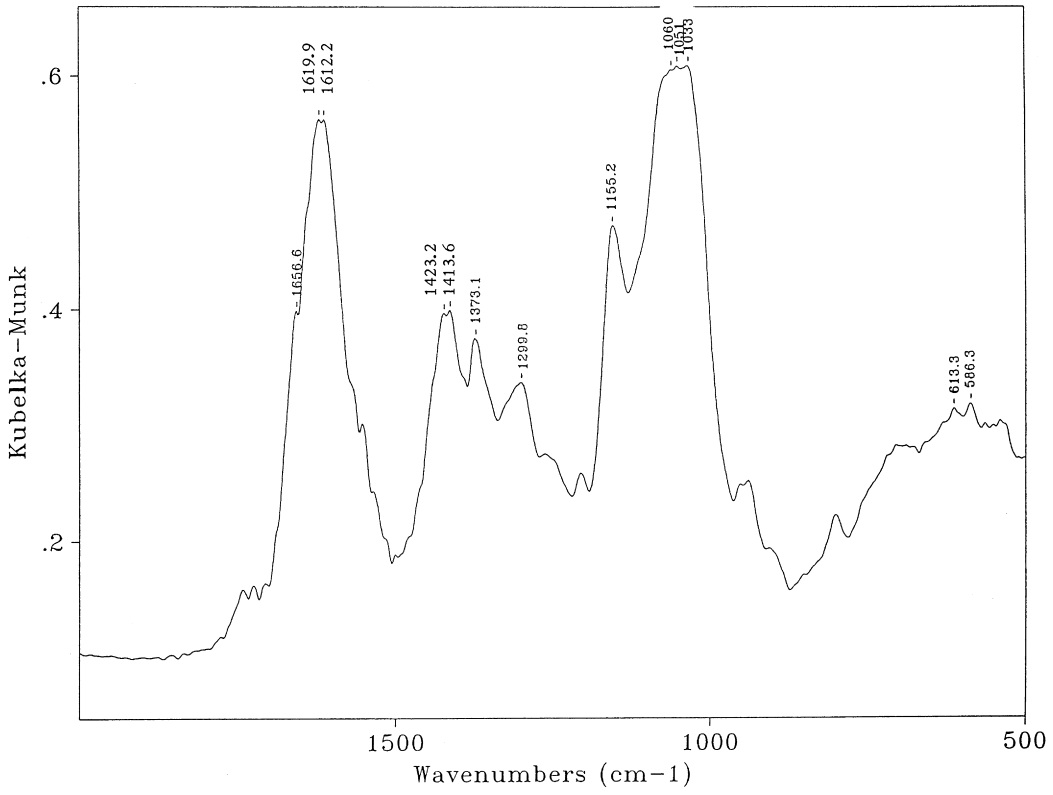

Fig. 1. FTIR spectrum of *A. niger* polyuronans, obtained after regiospecific oxidation. The carboxyl group contributes the band peaking at 1612.2 cm^{-1}.

freeze-drier was operated at −95°C and 1.50 mbar; lower pressures caused loss of the product.

3. Results and discussion

The following protocols were executed in order to prepare regiospecifically oxidized polysaccharides from *A. niger*. As a preliminary step, the dry content of the frozen material was determined: in the case of *A. niger*, 10 g of the frozen mycelia corresponded to ca. 1 g of dry matter. This was done separately, by drying at 80°C. The bulk of the mycelia necessary for the preparation of the polyuronans was never exposed to thermal treatments, in order to avoid aggregations that would limit the extent of the subsequent reactions. The crucial condition that the biomass should never be dried was in fact verified.

Proteins were removed with sodium dodecylsulfate (1 g in 50 ml water) by stirring for 5 min at 20°C. Lipids were removed with chloroform + methanol (2:1) by stirring for 15 min at 20°C. The material, extensively washed with demineralized water, was treated with an emulsifier in order to disrupt the hyphal structure; observations in the electron microscope confirmed that this technique effectively triturates the hyphae. The material was swollen in water at 50°C for 4 h and kept in water at 20°C for 24 h.

The suspension (50 ml, pH 6.9) was oxidized after addition of Tempo® (12 mg), NaBr (0.4 mg) and NaOCl (20 ml, 4%), in this order, according to the standard procedure (Muzzarelli et al., 1999), and the reaction conducted at pH 10.8 with 0.5 M NaOH (11 ml). At the end (30 min) the turbid suspension had turned into a pale yellow clear solution, pH 9.6, the yellow color being due to Tempo®.

The solution was dialyzed against various changes of demineralized water in a dialysis tube with a cut off value 2500 Da for 36 h (final weight 150 g), frozen and then freeze-dried, to yield polyuronans as a white powder.

The *T. reesei* frozen material (12 g, corresponding to 1 g dry weight) was submitted to the same procedure and the final pH of the oxidized product solution was 9.9. The same procedure was adopted for *Saprolegnia* sp. In subsequent preparations, the chloroform + methanol extraction was omitted and replaced with ethyl acetate extraction, but no appreciable advantage was noticed. On the contrary, the SDS treatment seemed to be indispensable. In fact, omission of the SDS washing in the preparation from *T. reesei* material, yielded a dark product with poor yield.

3.1. Instrumental evaluation of the products

The FTIR spectra for the various polyuronan preparations from all fungi were recorded. For *A. niger*, the FTIR spectrum exhibited bands at 1618 (assigned to carboxylate, with a weak shoulder at 1656), 1413, 11375, 1301, 1153 and 1066–1049 cm^{-1} (Fig. 1). The FTIR spectrum for *T. reesei* exhibited bands at 1612 (assigned to carboxylate with

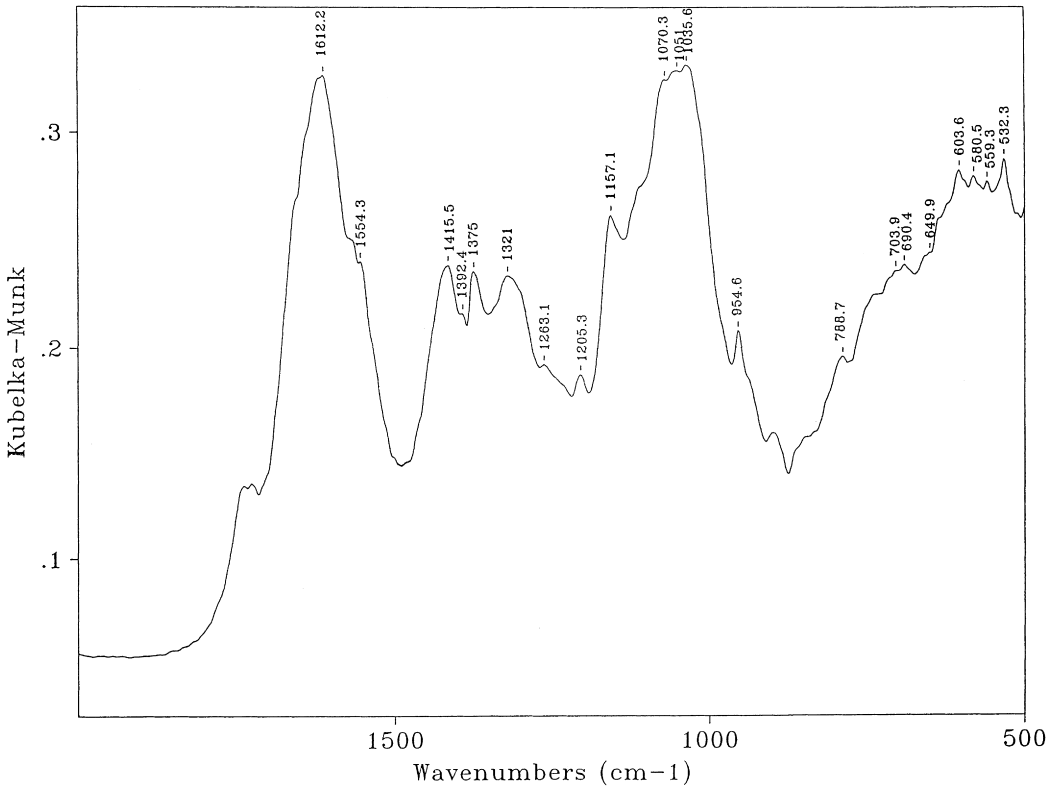

Fig. 2. FTIR spectrum of *T. reesei* polyuronans, obtained after regiospecific oxidation. The carboxyl group contributes the band peaking at 1612.2 cm^{-1}.

evident shoulders at 1554), 1656 (assigned to chitin amide), 1415, 1375, 1312, 1157 and 1070–1035 cm^{-1} (Fig. 2). Differences were observed in the region 1554 and 1321 cm^{-1}, possibly reflecting the different ratios of chitin and glucan in the two products of different origin. Deconvolution of the FTIR spectrum in the region 1800–1500 cm^{-1} showed that the major difference between the *A. niger* and *T. reesei* polyuronans was a band at 1568 cm^{-1} assigned to chitin amide (Fig. 3).

The ^{1}H NMR spectra for the *A. niger* polyuronan (Fig. 4) showed the presence of the acetyl–CH_3 group remarkably split into two signals at 1.96 and 1.98 ppm, and the well-known group of signals from H2, H3, H4 and H5 in the region 3.4–3.9 ppm. The integrals were in the ratio $0.90/13.35 + 0.90 = 0.06$ indicating that oxychitin probably, accounted for 20% of the polyuronan. The splitting of the $-CH_3$ signal presumably arises because of the relatively short chains and hence the relatively high number of terminal units (Heinze, 1998).

In the case of *T. reesei*, the split $-CH_3$ signal was the most prominent, but the signals in the region 3.4–3.9 were more altered than in the *A. niger* polyuronan samples (Fig. 4). The integrals were in the ratio $19.9/39.6 + 19.9 = 0.33$ indicating that most of the polyuronan was 6-oxychitin, in agreement with the presence of the amide bands in the FTIR spectra.

The preparation from *Saprolegnia* sp. proceeded smoothly in agreement with published data on cellulose oxidation at C6 (Heinze, 1998); the final product retained a yellow tinge.

3.2. Recommended simplified preparations

For scaling up some simplifications were introduced. The following recommendations would save chemicals and labor while providing the polyuronans with good yield and purity.

Table 1
Yields and characteristic properties of the polyuronans obtained according to the simplified procedure

	A. niger	*T. reesei*	*Saprolegnia parasitica*
Biomass wet weight (g)	100	120	100
Estimated dry matter (g)	~ 10	~ 10	~ 10
pH values			
After SDS treatment	6.2	6.7	5.8
After oxidation	10.2	9.8	9.8
After dialysis	6.2	6.8	7.1
Overall yield as Na salt (g)	~ 10	~ 9	~ 10
Color of freeze-dried powder	White	White	Yellowish
Solubility in water	Complete	Complete	Complete
Chitosan lactate flocculation	Immediate	Immediate	Immediate
Degree of carboxylation[a]			
Conductimetric	0.30	0.35	0.30
Alkalimetric	0.38	0.43	0.40

[a] After conditioning in ethanol + HCl, washing with ethanol and drying.

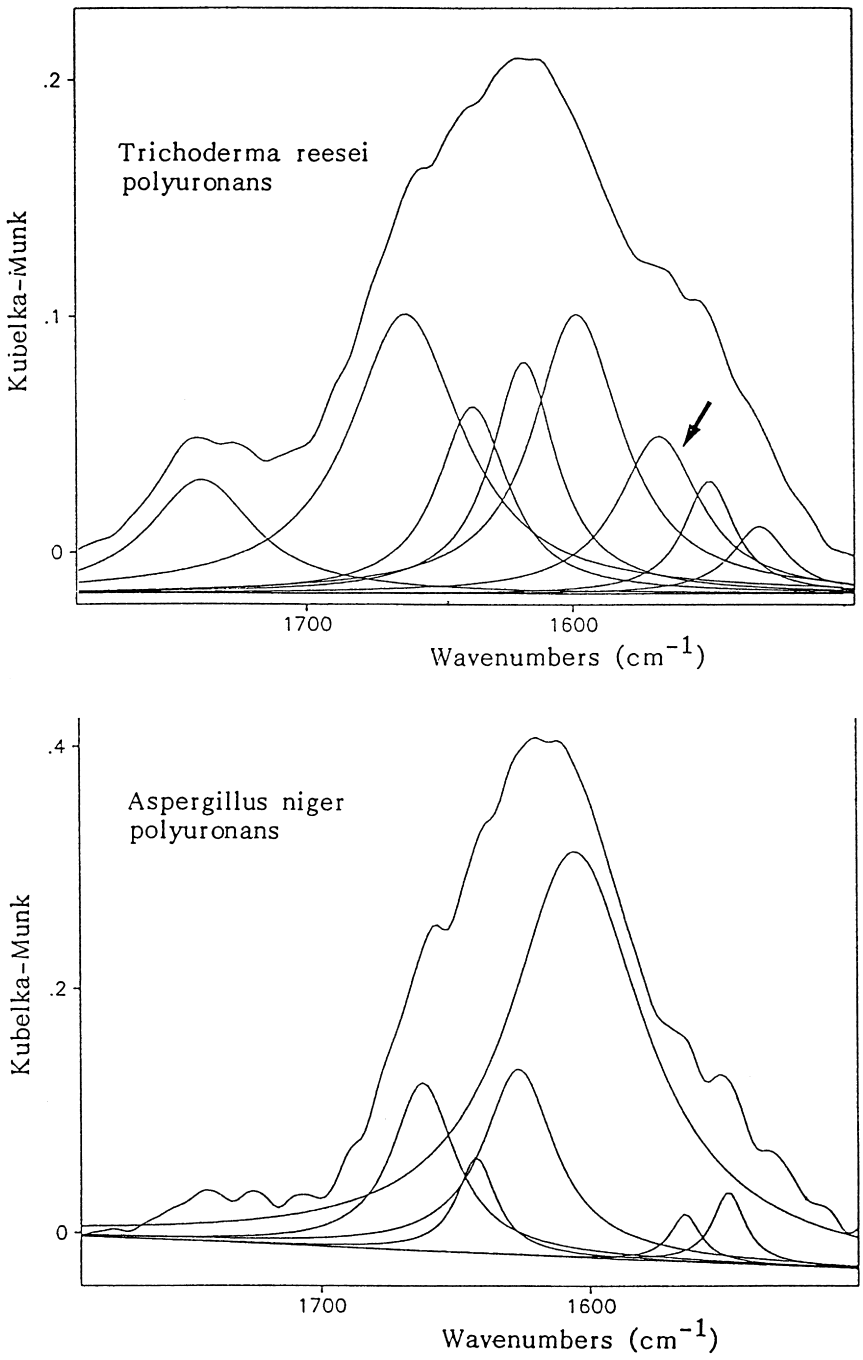

Fig. 3. Deconvolution of the FTIR spectra of *T. reesei* polyuronans and *A. niger* polyuronans, exhibiting a substantial difference between the chitin amide bands at 1568 cm^{-1}. The polyuronan from *A. niger* contains ca. 20% of 6-oxychitin; the polyuronan from *T. reesei* contains over 75% of 6-oxychitin.

The moist mycelia, preferably stored at −80°C (100 g) are washed with sodium dodecylsulfate (20 g) in water (0.6 l) with vigorous stirring for 10–15 min. They are then disrupted mechanically, filtered and washed. The material resuspended in 0.5 l of water is stirred at 60°C for 3 h and kept under stirring at least until the temperature again reaches the room temperature. The oxidation is then carried out as described above. The characteristic properties of the products are those listed in Table 1.

3.3. Esterification with glycerol and poly(ethyleneglycol)

Oxychitin, as well as the polyuronans described above, is soluble in glycerol; they also give cloudy solutions in poly-(ethylene glycol) PEG-400. The polyuronan (1.0 g) was dissolved in glycerol (100 g); then concentrated HCl was added (0.3 ml). The sealed vessel was kept on a roll bar stirrer for 7 days. A noticeable viscosity increase took place over this period. The glyceryl ester was isolated with the aid of a five-fold excess of isopropanol, and centrifuged

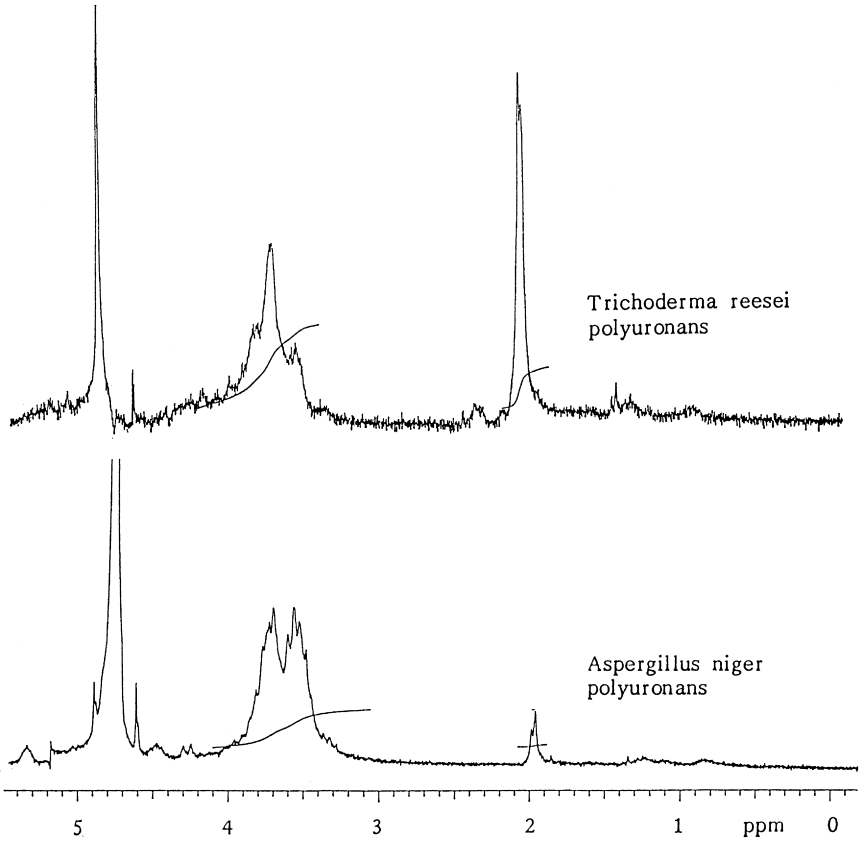

Fig. 4. ^{1}H NMR spectra for the *T. reesei* polyuronans and *A. niger* polyuronans.

(5 min, 3000 rpm). After washing with methanol, the sample was treated at low pressure (0.1 mbar) to eliminate volatile compounds. The isolated dry product was soluble in water. Analogous preparations were carried out with poly(ethylene glycol). The FTIR spectra showed a very prominent band at 1747.2 cm^{-1} assigned to the ester function, accompanied by enhanced bands at 2881–2892 cm^{-1} assigned to the methylene group; the band at 1379 cm^{-1}, because of the ester contribution, became more prominent compared to the one at 1415 cm^{-1}.

4. Conclusions

The *A. niger* spent biomass, of major industrial interest, is easily converted to a refined polyuronan that includes ca. 20% of 6-oxychitin. This product, in the form of sodium salt, is an off white powder immediately soluble in aqueous solutions over the pH range 3–12. A typical reaction of 6-oxychitin in admixture with other polyuronans is the coagulation of chitosan from its lactate solutions; in all cases chitosan was precipitated.

The other two fungi studied here as models for chitin–glucan complex and cellulose–glucan complex bearing fungi, lend themselves to the same treatment and provide polyuronans with satisfactory yields and analytical characteristics, in particular *T. reesei*.

It should be underlined that an advantage of the present work is the high yield close to 100%, mainly due to the formation of carboxylate groups from a large portion of primary alcohol groups, and to the total derivatization of the polyuronan to a sodium salt. In other works (for instance, Hu et al., 1999) chitosan was isolated with yields as low as 10% because most of the biomass (glucan) was discarded.

In conclusion, this study offers the opportunity for exploitation of industrial spent fungal biomasses making use of common chemicals, i.e. sodium dodecylsulfate, NaOCl, NaBr and a catalyst. The fungi treated here are amply representative: in fact, three types of cell walls have been defined for zoosporic fungi: chitin–β-glucan of Chytridiomycetes, chitin–cellulose of Hyphochytridiomycetes and β-glucan–cellulose of Oomycetes (Rosenberger, 1976). The present method, therefore, seems widely applicable.

Of course, the final products were mixtures of polyuronans, and for certain purposes, might require refinement. Nevertheless, these polyuronan mixtures may be suitable for major applications, since once standardized biomasses and protocols are used, the final polyuronan composition should be reproducible. The crosslinking esterification with glycerol and with poly(ethyleneglycol) provided products with increased viscosity properties and these might find applications in various fields.

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