FUNGAL BIOMASS AS AN ALTERNATIVE SOURCE OF CHITIN AND CHITOSAN

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Abstract

Basidiomycetes contain large quantities of chitin that constitutes the structure of both mycelia and carpophores. Obtaining chitin from mushrooms could be an advantageous perspective for chitosan production. To evaluate this possibility, chitinous material was extracted from mycelia of *Agaricus bisporus* (SMR 13), *Auricularia auricula-judae* (SMR 54), *Lentinula edodes* (SMR 90), *Trametes versicolor* (SMR 117), *Armillaria mellea* (SMR 439), *Pleurotus ostreatus* (SMR 684), *Pleurotus eryngii* (SMR 755). The extracted materials were characterised for purity, degree of acetylation and cristallinity, and compared with standard products. The yield of fungal chitin ranged between 8.5 and 19.6 %, chitosan yield was about 1%. The degree of acetylation of fungal chitins was similar to the standard one; the glucosamine content ranged between 64.5 and 26.4 %. Fungal chitins showed a cristallinity about 40% lower than the standard. Chitosans with a degree of acetylation comparable with that of the standard chitosan (7.5%) were obtained by chemical deacetylation of fungal chitins.

Introduction

Nowadays the most convenient available source for the commercial production of chitin are the shells of crustaceans [1]. Chitin and chitosan, its deacetylate product, are used by many Companies on a commercial scale for a variety of applications, i.e. cosmetics, pharmaceuticals, agriculture, water treatment and recovery of heavy metals. The industrial extraction of chitin from this source presents some drawbacks due to the seasonal availability and environmental pollution. Chitin in crustaceans is present as a mucopolysaccharide, intimately associated with calcium carbonate, proteins, lipids and pigments. Its purification involves hard treatments that cause variability of the final product often decreasing its quality because of the possible chemical changes such as deacetylation and depolymerisation of chitin. Moreover, environmental pollution can also be produced because of the storage of large amounts of the raw material and the release of waste containing alkali and degradation products. Due to its high cristallinity, chitin is insoluble in aqueous solutions and organic solvents [2] so, to be best utilised, it must be transformed into chitosan. Chitosan can be obtained from chitin either by homogeneous or heterogeneous alkaline N-deacetylation [3] or by enzymatic hydrolysis [4]. Recently, many organisms have been taken into consideration as alternative sources of chitin. Chitin, a biopolymer composed of β-1,4-linked 2-acetamido-2-deoxy-D-glucose residues, is widely distributed in fungi, occurring in Basidiomycetes, Ascomycetes, and Phycomycetes, where it is a component of the cell wall and structural membrane of mycelia, stalks, and spores. The amounts vary between traces and up to 45% of the organic fraction, the rest being mostly proteins, glucans and mannans [5]. Several Basidiomycetes, i.e. *Pleurotus spp, Lentinus edodes* and *Agaricus spp* have been proposed as possible alternative

sources of chitin and chitosan due to the high content of chitin in the cell walls [6-12] that can be easily extracted for its subsequent deacetylation [13].

Recently, Wu et al. [14] proposed the by-products of *Agaricus bisporus* farm as an alternative source for chitin extraction. This study is aimed at determining the content and properties of chitinous materials in different species of Basidiomycetes in view of their use for chitosan production.

Material and Methods

Organisms

Mycelia from *Agaricus bisporus* (SMR 13), *Auricularia auricula-judae* (SMR 54), *Lentinula edodes* (SMR 90), *Trametes versicolor* (SMR 117), *Armillaria mellea* (SMR 439), *Pleurotus ostreatus* (SMR 684), *Pleurotus eryngii* (SMR 755), stored in the International Bank of Edible Saprophytic Mushrooms (IBESM) of the Italian National Council of Research, were used.

Fungal growth

Inocula were prepared by growing each organism in three Erlenmeyer flasks containing 50 ml of liquid medium (MEP: 3% malt extract, 0,5% peptone) at 25°C, stirred at 150 rpm for 10 days. Afterwards mycelia were homogenised aseptically in an omni-mixer homogeniser for 3 seconds. 50 ml of mycelial suspension (equivalent to 400 mg of dry weight) were inoculated into flasks containing 1000 ml of MEP.

Chitinous material extraction

After 21 days, when the maximum of chitin is reached [15], cultures were filtered through gauze, washed with distilled water and lyophilised. 3g of every lyophilised mycelium was added to 100 ml N NaOH and stirred overnight at 40°C. The suspension was centrifuged at 5,000 g for 30 min. This treatment was repeated 3 times. The precipitate was suspended in distilled water and stirred at 95°C overnight. These treatments removed proteins and resulted in alkali insoluble material. The precipitate obtained after centrifugation at 5,000 g for 20 min at room temperature (chitin) was then washed 3 times with distilled water and lyophilised (Fig.1).
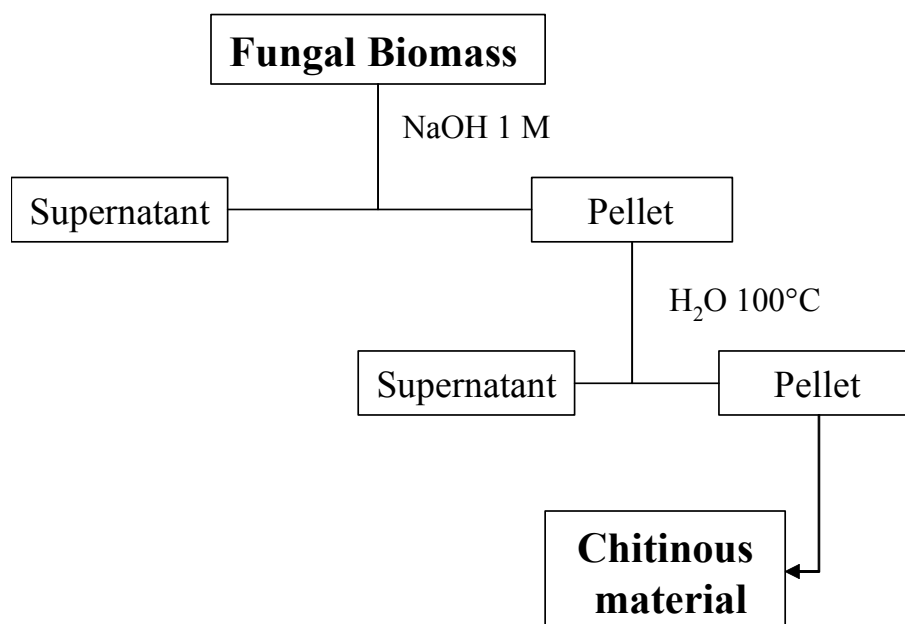

Figure 1: Chitin extraction from fungal biomass

The yield of chitin was determined by weight (Tab.1).

Characterisation of extracted chitin and chitosan
Chitin (Sigma) and chitosan (Pharmitalia) were used as standard.
Fourier transform IR spectra were performed from 4000 to 700 cm^{-1} on a FT-IR Perkin Elmer mod.16F PC. The samples were prepared for the analyses by mixing 10 mg of KBr with 1 mg of lyophilised chitin or chitosan.
The degree of acetylation (DA) was performed according to Neugebauer et al. [16]. Chitin and chitosan cristallinity was determined according to Focher et al. [17]. Glucosamine content was determined according to Plassard [18]. Proteins were determined according to Bradford [19].

Chitin deacetylation
Chitin deacetylation was performed on both Sigma and fungal chitins according to Mima et al. [20].

Results and Discussion
Recent advances in fermentation technology indicate the cultivation of selected fungal strains as a suitable alternative source of chitin that can be easily extracted from both carpophore and mycelial biomasses. Unlike the conventional industrial isolation of chitin from crustacean shells, the fungal materials do not require harsh processes of extraction. The main fungal components are water, proteins and a NaOH-insoluble fraction mainly containing chitin and acidic polysaccharides in addition to a low quantity of chitosan. Unlike crustacean chitinous extracts, the alkali insoluble fraction contained no residual proteins. The total amounts of chitin extracted from fungal mycelia ranged between 8.5 and 19.6 % of dry weight, the best yields being obtained from *Auricularia auricula-judae* and *Pleurotus ostreatus* (Tab.1).

items	Chitin yield (% dry weight)	Glucosamine (% of chitin)	D.A. %
Standard chitin (Sigma)	--	47.1 ± 0.9	97.0 ± 1.5
Auricularia auricula-judae (SMR 54)	19.6 ± 1.1	64.5 ± 1.3	95.1 ± 1.3
Lentinula edodes (SMR 90)	10.1 ± 1.0	46.3 ± 0.5	92.0 ± 1.2
Trametes versicolor (SMR 117)	13.1 ± 0.7	26.4 ± 0.4	97.0 ± 1.6
Armillaria mellea (SMR 439)	11.1 ± 1.3	38.1 ± 0.6	92.7 ± 1.7
Pleurotus ostreatus (SMR 684)	15.3 ± 2.2	30.7 ± 0.8	98.7 ± 2.3
Agaricus bisporus (SMR 13)	8.5 ± 1.4	51.1 ± 0.7	91.0 ± 1.4
Pleurotus eryngii (SMR 755)	8.7 ± 1.1	44.76 ± 0.6	94.2 ± 1.3

Tab.1: Yield, glucosamine content and degree of acetylation of chitins extracted from the different mycelia in comparison with the standard product.

Chitin is a linear polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine in a different ratio. The glucosamine content, index of chitin purity, has been determined after a complete hydrolysis of chitin. The glucosamine percentage in some fungal strains (*L. edodes*, *A. bisporus, P. eryngii*) is comparable with that determined in the standard chitin. A consistently greater percentage was determined in *A. auricula-judae* chitin whereas lower amounts were recorded in the other strains (*P. ostreatus, A. mellea, T. versicolor*). However, based on the yield of chitin and its glucosamine content, fungal strains can be positively considered as an alternative source for chitin production. That is confirmed by the FT-IR spectra characterised by the amide bands at approximately 1655, 1555 cm^{-1}. The high degree of similarity recorded among IR fungal spectra and when they are compared with that of the standard chitin lead us to suppose the congruity of the considered chitins (Fig. 2).
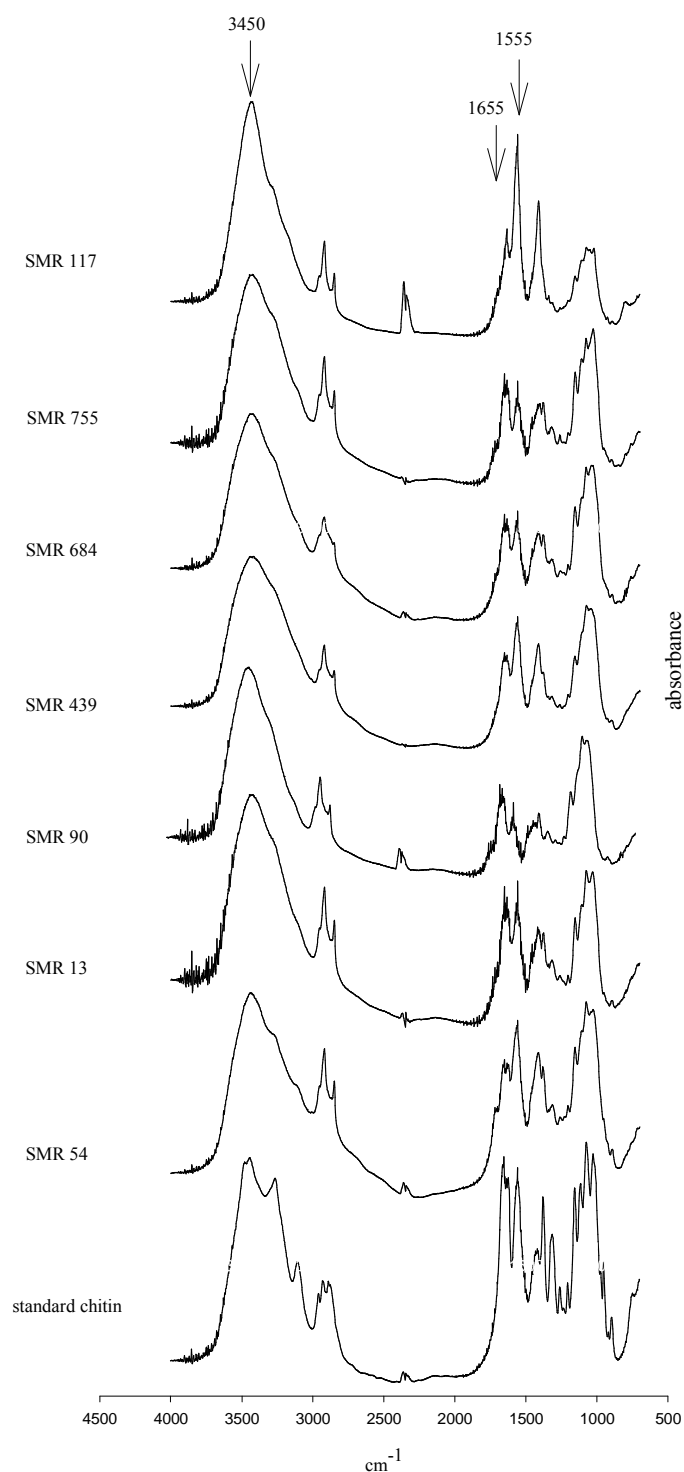
Figure 2: FT-IR spectra of fungal and standard chitins.

Many methods, including elemental analysis, enzymatic degradation, titration of free amino groups, IR, UV and NMR spectroscopy [9, 21-23] have been proposed to determine the degree of acetylation (DA) of chitin and chitosan. To avoid possible drawbacks due to the interpretation of FT-IR spectra, the method of Neugebauer et al. [16] was used. This method measures the DA by the bounds of picric acid with amino groups of chitin and chitosan. The results obtained in this way were highly reproducible. The DA of the fungal chitins are reported in table 1. Although DA of fungal chitins are very similar to the value obtained for standard chitin they differ for cristallinity that can be assumed as an index of the resistence of chitin to hydrolysis. A high cristallinity would

reduce accessibility of chitin molecules to HCl and result in incomplete hydrolysis [24]. The cristallinity of chitins increased as follows: *Pleurotus eryngii, Lentinula edodes, Trametes versicolor, Agaricus bisporus* (0.80) < *Pleurotus ostreatus, Auricularia auricula-judae* (0.91) < *standard chitin* (1.54).

Chitins were then converted into chitosan by alkaline N-deacetylation with concentrated sodium hydroxide (40 – 50 %) at 80 – 150°C [3]. A strong decrease of DA was generally obtained. Values comparable with the standard of chitosan (DA=7.5 %) were obtained for the standard chitin and some fungal chitin (*A. auricula-judae* (SMR 54), *P. ostreatus* (SMR 684), *A. bisporus* (SMR 13), *T. versicolor* (SMR 117) (Fig.3).
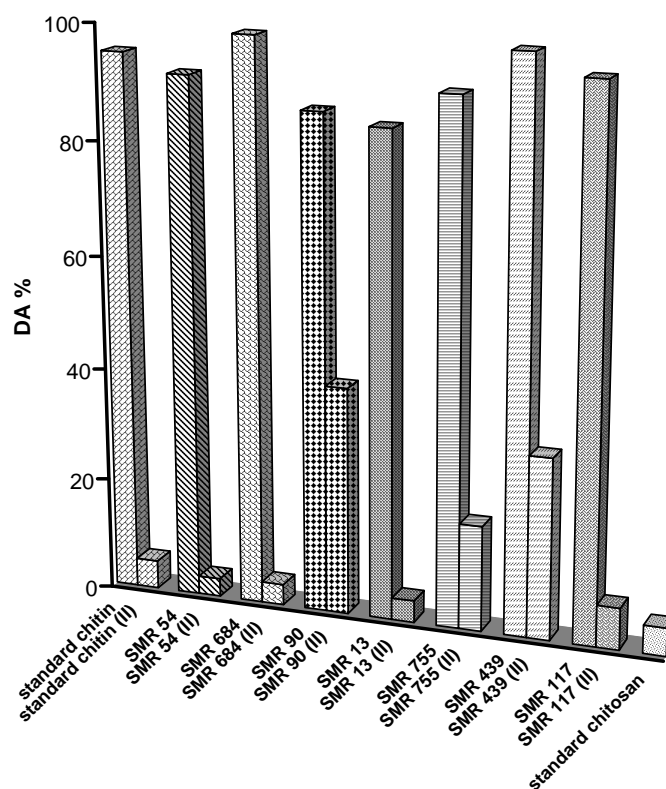

Figure 3: Effect of alkaline deacetylation (DA) of standard and fungal chitins. For each chitin, initial DA and DA after second treatment (II) are reported. As reference, the DA of standard chitosan is also reported.

A comparison among the FT-IR spectra of some different chitosans is reported in Fig.4. In this figure, the spectra of standard chitin and chitosan as a reference terms are also reported. The infrared spectra of chitosans from mushrooms are very similar among them and to the standard chitosan spectrum although some subtle differences in the absorption intensity were recorded. As expected N-deacetylation is associated with a progressive weakening of the band occurring at 1665 cm^{-1} (Amide I vibrational mode) and at 3265 and 3100 cm^{-1}(NH amide bond stretching).
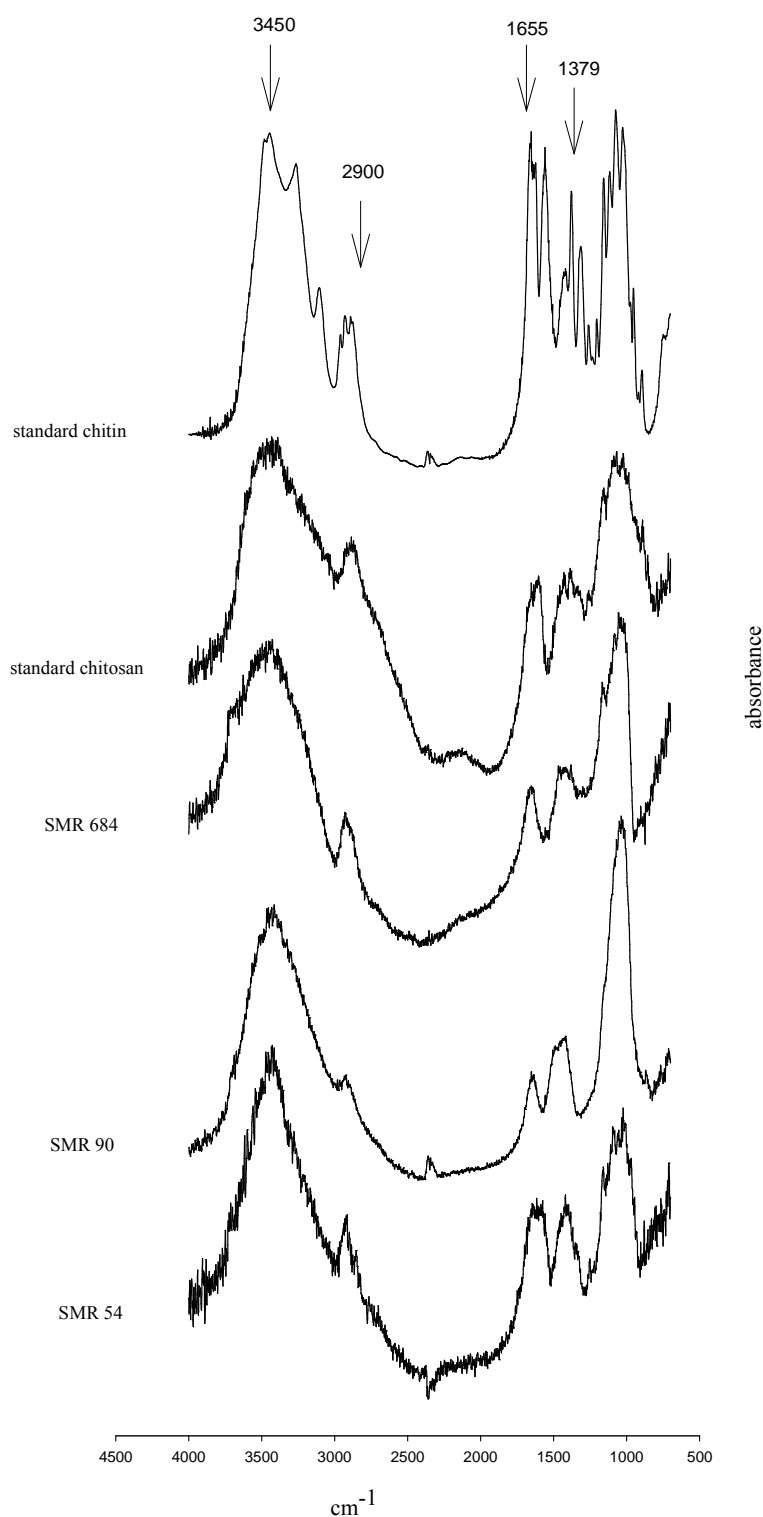
Figure 4: FT-IR spectra of some fungal chitosans compared with standard chitin and chitosan.

Conclusion

New prospects are open by fungal biotechnologies which offer advantages in the production of chitin and chitosan over classical procedures since fungal biomasses can be easily obtained and can be processed avoiding harsh treatments.

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