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Production and Characterization of Mushroom Chitosan under Solid-State Fermentation Conditions

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Abstract: Chitosan was extracted from 15 days old biomass of *Agaricus* Sp, *Pleurotus* Sp and *Ganoderma* Sp. It was subjected to different alkaline and acid treatment in the extraction protocol. In both alkaline and acid treatment, the temperature, incubation period and concentration of acid or alkaline significantly affects the production and quality of the chitosan produced ($P<0.05$). The type of acid used was also important in the production of chitosan. Usage of hydrochloric acid in the extraction of chitosan gave a significantly higher degree of deacetylation (DD) compared to acetic acid. Employment of strong acid, high acid concentration and high temperature produced darker coloured chitosan whereas milder treatments gave lighter coloured chitosan.

Key words: Chitosan · *Agaricus* Sp · *Pleurotus* Sp · *Ganoderma* Sp and Degree of deacetylation

INTRODUCTION

Chitin (poly-N-acetyl glucosamine) is a ubiquitous biopolymer which occurs naturally as a major component in the skeletal or exoskeletal structures of lower animals. Chitin is also present in the vast majority of fungi as the principal fibrillar polymer of the cell wall [1]. The deacetylated form of chitin, chitosan (polyglucosamine), has unique properties which make it useful for a variety of industrial applications such as a viscosity control agent, adhesive, paper-strengthening agent and flocculating aid, among others Chitosan has recently been used in many areas, for example, in cosmetics, pharmaceuticals, food additives and agriculture. Its use as, a component of toothpaste, hand and body creams, shampoo, lowering of serum cholesterol, cell and enzyme immobilizer, as a drug carrier, material for production of contact lenses, or eye bandages, permeability control agent, as chromatographic support, antimicrobial compounds, seed coats and flocculating and chelating agents in wastewater treatments [2]. The traditional industrial source of chitin is shellfish waste from shrimp, crab and lobster processing [1]. However, problems with seasonal and limited supply, confined production locations, product variability and high processing costs associated with the chemical conversion of chitin to chitosan appear to have limited the potential industrial acceptance of this polymer [3]. Several alternative industrial raw material sources of chitin have been suggested, including insects, Antarctic krill and diatoms for reasons inherently similar to those described previously, or because of untested culturing or engineering techniques, no significant progress has been made in establishing new technologies leading to the large-scale controlled production of chitosan. Recent advances in fermentation technology suggest that large-scale culturing of an organism that contains chitosan might be an attractive route to the production of this polymer. Reports showed that this organism can be readily cultured on simple nutrients [4] and that the cell wall chitosan can be readily extracted, have led us to study the production and isolation of microbial chitosan as an alternative to the shellfish-derived product.

On a commercial scale, chitosan is extracted from the exoskeleton of crustaceans employing harsh chemical treatments. However, this extraction process, together with the variability in source material leads to inconsistent physicochemical characteristics of the chitosan produced. These characteristics make fungi a promising chitosan

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source as the physical properties of the extractable chitosan from fungi can be manipulated through the regulation of factors such as growth media composition and processing parameters in the extraction protocol [5].

The present study was investigating the increase fungal chitosan production through the modified protocol [6]. In addition, this study was conducted to ascertain the effects of the alkaline and acid treatment used in the extraction protocol towards the quality of chitosan in terms of degree of deacetylation and colour of the chitosan produced.

MATERIAL AND METHODS

Source of the Used Basidiomycetes: Three mushroom species *Agaricus* Sp, *Pleurotus* Sp and *Ganoderma* Sp. were used in this study. The mushrooms were collected from different areas within the Western Ghats of Tirunelveli range, Tamil Nadu, India. These basidiomycetes were identified by their spore prints and, by comparing their morphological, anatomical and physiological characteristics with the standard descriptions [7]. The basidiomycetes were used for producing the grain spawn by the convenient method. The prepared spawn were stored at 5°C until using them for cultivation.

Growing Media and Cultivation: Hard wood sawdust and rice straw was used (1:1) for preparation of growing media as Follows

Sawdust + 1% CaCo + 1% sugar
Rice straw + 1% CaCo + 1% sugar
Sawdust + rice straw + 1% CaCo + 1% sugar

The moisture content of the aforementioned media formulae were adjusted to approximately 63 - 64%. Then each formula was filled in polypropylene bags (1kg each) and autoclaved at 121°C for 1 hour. After the sterilized media was cooled down, the bags were inoculated by the previously prepared grain spawn 2% (w/w), then being incubated at 22 - 27°C for spawn run (mycelium growth). At the end of incubation time (spawn run) the bags were opened and subjected to the fruiting conditions i.e. exposure to scattered light, watering by daily water spraying, good ventilation, adjusting relative humidity to 85-90% and temperature to 20-25°C. The crop was picked after 14-20 days from the end of incubation time in consecutive flushes at intervals of 15-20 days.

Chitosan Extraction: The fungal biomass obtained were dried in a freeze dryer (Thermo scientific) and weighed. Lyophilized fungal biomass was then subjected to chitosan extraction protocol employing the standard method [3]. This extraction protocol includes an alkaline treatment which is followed by an acid treatment. In the optimization of the extraction protocol, freeze-dried fungal biomass were subjected to modified alkaline treatment followed by the acid treatment method of extraction. Mean while in the acid treatment, fungal biomass was subjected to the standard alkaline treatment prior to the modified acid treatment [4].

Alkaline Treatment: For the study on the effects of alkaline concentration on chitosan extraction, IM, 2M, 3M and 4M sodium hydroxide (NaOH) solutions were used to treat samples at 121°C for 15 minutes. For the study on the effect of temperature and incubation period, three temperatures, 95°C, 110°C and 121°C and five incubation periods, 10, 15, 20, 25 and 30 minutes were used with NaOH 1M as the treatment solution.

Acid Treatment: Three different acids, acetic acid (AA), and hydrochloric acid (HCl) were used as the extracting solution. Acid treatment were performed at 2%, 6% and 10% acid concentrations; incubation period at 3, 6 and 12 hours; temperature at 60°C and 95°C. The acid treatment was performed using a general linear experimental design.

Determination of Degree of Deacetylation: The degree of deacetylation was determined by the first derivative UV spectrophotometry method (Simenzd) [5]. The degree of deacetylation for the chitosan samples was determined based on percentage calculations of the glucosamine content in the samples.

RESULTS AND DISCUSSION

During Mushroom cultivation the substrate saw dust had long incubation time and differ significantly than the other substrate composition. On the other hand sawdust + paddy straw had the shortest spawn runtime and differ significantly than the other substrate formulae. As for the second and third seasons, sawdust has the same trend and recorded the longest spawn other formulae. Also, sawdust + ricestraw had the same trend as first season and recorded significantly short spawn runtime compared to other formulae.

Table 1: Effect of concentration of NaoH solution on the extraction yield of chitosan at temperature 121°C

Alkaline treatment molarity of NaoH	*Agaricus* sp	*Pleurotus* sp	*Ganoderma* sp
1M	0.608	0.597	0.628
2M	0.745	0.715	0.742
3M	1.428	1.488	1.448
4M	1.116	1.006	1.106

In the alkaline treatment, it was observed that significantly higher amounts ($P<0.05$) of chitosan was extracted with the increase in incubation period and temperature. The highest yield of chitosan was obtained at incubation temperature 121°C and incubation period of 30 minutes (19.7% chitosan / biomass). The utilization of different NaOH concentrations (1M, 2M, 3M and 4M) did not give significant differences to the chitosan yield (Table 1).

It was observed that the yield of chitosan increased significantly with the increase of temperature and incubation period especially for 25 minutes and 30 minutes incubation period. Chitosan production showed significant differences even with small increments of temperature and incubation period ($P<0.05$). At 121°C, a raise of the incubation period from 15 minutes to 30 minutes yielded a significant increase ($P<0.05$) of chitosan yield (41.29%). A decrease in the temperature to 95°C at standard incubation period (15 minutes) showed a reduction of 35.37% in the chitosan produced. Meanwhile, alkaline treatment at 115°C and incubation period of 15 minutes did not render significant differences when compared to the standard alkaline treatment (121°C, 15 minutes). Higher temperatures and longer incubation periods were necessary to enable effective interactions between NaOH and the constituents of the fungal cell wall thus making it possible to extract higher levels of chitosan [8]. Longer incubation period also gave longer reaction time for NaOH to act on the chitin and chitosan structure in order to separate chitosan from other cell wall polysaccharides [7]. Thus it can be concluded that increasing the incubation period to 30 minutes and maintaining the temperature at 121°C yielded high amounts of chitosan when alkaline treatment was used (Table 2).

In acid treatment, it was observed that the utilization of acetic acid as the extracting solution yielded higher amounts of chitosan as compared to hydrochloric acid. The highest chitosan yield was obtained with 6% *formic* acid at incubation period of 12 hours at 95°C. This study also observed that the same incubation period, temperature and acid concentration rendered different effects and interactions when different acids were used as the extracting solution.

Table 2: Effect of temperature and incubation period on the extraction yield of chitosan with 1M NaoH

	Chitosan (mg)		
Incubation Period (Min)	95°C	115°C	121°C
10	0.701	0.635	0.654
15	0.725	0.781	0.817
20	0.832	0.849	0.928
25	0.944	0.966	0.976

Table 3: Extraction of chitosan using acetic acid at different concentration and temperature

Acetic acid	Incubation time	Chitosan
3%	12 hrs	0.641%
5%	12 hrs	0.782 %
7%	12hrs	0.891 %

Table 4: Extraction of chitosan using Hcl at different concentration and temperature

Hcl	Incubation time	Chitosan
3%	12 hrs	0.621 %
5%	12 hrs	0.660 %
7%	12hrs	0.825 %

Table 5: Effect of concentration of NaoH solution of the degree of deacetylation of chitosan

Alkaline treatment Molarity of NaoH	Degree of deacetylation
1M(standard treatment)	0.82%
2M	0.83%
3M	0.87%
4M	0.89%

All treatments done at 121°C for 15 minutes. Treatment with 1M NaoH is considered the standard method

For example, in the utilization of acetic acid, it was found that only the incubation period played a significant role in affecting the amount of extractable chitosan ($P<0.05$) (Table 3). Whereas for hydrochloric acid, acid concentration was found to significantly affect chitosan extraction ($P<0.01$) (Table 4).

Table 6: Degree of de- acetylation for mushroom chitosan extracted using different acid treatments

Acid concentration	Incubation period	Temperature	Acetic acid	Hydrochloric acid
6%	6hrs	60°C	0.76%	0.86%
8%	6hrs	60°C	0.82%	0.88%
6%	12hrs	95°C	0.82%	0.87%
8%	12hrs	95°C	0.88%	0.89%

Degree of Deacetylation: The increase of the alkaline concentration in the alkaline treatment increased the degree of deacetylation (DD) of the chitosan extracted (Table 5). This is in accordance with the previous study [7] which stated that the level or extent of deacetylation is influenced and controlled by alkaline concentration, temperature, incubation period, particle size and density. Higher alkaline concentrations may cause alkaline hydrolysis to occur at a higher rate in the sample resulting in a higher degree of deacetylation [9]. Repetition of the standard alkaline treatments to the same samples for up to 3 times also produced chitosan with a higher DD as compared to chitosan extracted using the standard alkaline treatment (Table 5) [9].

The degree of deacetylation (DD) for chitosan extracted by the various acid treatment were also determined (Table 6). The general trend observed was that DD increased with the increase in incubation temperature, incubation period and acid concentration. DD was also found to be significantly higher ($P<0.01$) when hydrochloric acid was used as the extracting solution compared to acetic acid. The highest DD (89%) was obtained with 8% hydrochloric acid and 12 hours incubation period at 95°C. Meanwhile, the lowest DD (76.16%) was obtained with 6 % *formic* acid and 6 hours incubation period at 60°C. Hydrochloric acid, being a strong acid in comparison to acetic acid, caused a greater extent of hydrolysis towards the acetyl moieties, in addition to the hydrolysis within the network of monomers in the chitosan polymer [6]. DD was found to be highest for all acids at 8% acid concentration. In the acid treatment, it was observed that varying individual factors (acid concentration, temperature and incubation period) did not seem to give consistent results whereas the varying/manipulation of particular parameter factor gave mixed results. However, the 3 way ANOVA performed showed that the trend of the DD values was dependent on the interaction between factors and also the type of acid utilized (Table 6). Multiple paired comparison shows that the DD of chitosans extracted using hydrochloric acid were significantly different compared to chitosan extracted using acetic acid ($P<0.05$). There was no significant difference between the DD values of chitosan extracted using acetic acid ($P>0.05$) (Table 6).

REFERENCES

1. Jeuniaux, C., 1978. Distribution and quantitative importance of chitin in animals, pp: 5-10. In R.A.A. Muzzarelli and E.R. Pariser (ed.), Proceedings of the First International Conference on Chitin/Chitosan. MIT Sea Grant Report MITSG 78-7, Index no. 78-307-Dmb. Massachusetts Institute of Technology, Cambridge.
2. Muzzarelli, R.A.A., 1977. Chitin. Pergamon Press, New York.
3. Ashford, N.A., D. Hattis and A.E. Murray, 1977. Industrial prospects for chitin and protein from shellfish wastes. MIT Sea Grant Program. Report no. MITSG 77-3, Index no. 77-703-Zle. Massachusetts Institute of Technology, Cambridge.
4. Miyoshi, H., K. Shimura, K. Watanabe and O. Kasuki, 1992. Characterization of some fungal chitosans. Biosci. Biotechnol. Biochem., 56: 1901-5.
5. Di, Lena, G., A.D. Annibate and G. Giovannozzi-Sermanni, 1994. Influence of the age growth conditions on the mycelial chitin content of *Lentinus edodes*. J. Basic Microbiol., 34: 11-6.
6. White, S.A., P.R. Farina and I. Fulton, 1979. Production and Isolation of Chitosan from Mucor rouxii. Applied and Environmental Microbiol., 38: 323-328.
7. Roberts, G.A.F., 1992. *Chitin Chemistry*. London: The Macmillan Press Ltd.
8. Win, N.N. and W.F. Stevens, 2001. Shrimp Chitin as Substrates for Fungal Chitin Deacetylase. Applied Microbiology and Biotechnol., 57: 334-341.
9. Rinaudo, M. and A. Domard, 1989. Solution Properties of Chitosan. In *Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications*. Edited by G. Skjak-Braek, T. Anthonsen and P. Sandford. London: Elsevier Science Publisher, pp: 71-86.