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The chitosan yield of zygomycetes at their optimum harvesting time

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Fungi are a promising alternative source of chitosan. Fungi can be manipulated to give chitosan of more consistent and desired physico-chemical properties compared to chitosan obtained from crustacean sources. Chitosan was extracted from the mycelia of *Rhizopus oryzae* USDB 0602 at various phases of growth. The growth phase which produced the most extractable chitosan was determined to be the late exponential phase. In contrast to previous work on the screening of chitosan from fungal sources, mycelia of the fungi used in this study were harvested at their late exponential growth phase instead of at a fixed incubation time. The amount of extractable chitosan varied widely among the fungal strains. *Gongronella butleri* USDB 0201 was found to produce the highest amount of extractable chitosan per ml of substrate, followed by *Cunninghamella echinulata* and *Gongronella butleri* USDB 0428. However, in terms of yield of chitosan per unit mycelia mass, *C. echinulata* was the best strain among all fungi in the experiment. Therefore, besides *G. butleri* USDB 0201, *C. echinulata* can also be considered to be used in the commercial production of chitosan. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chitosan (β-1,4-D-glucosamine) is the deacetylated form of chitin (β-1,4-N-acetyl-D-glucosamine). It is less commonly found in living organisms than chitin and can be found in the cell wall of certain groups of fungi, particularly zygomycetes (Arcidiacono *et al.*, 1988). Chitosan has numerous applications especially in the agriculture, food and pharmaceutical industries (Knorr, 1991). Commercially, chitosan is obtained by chemically deacetylating crustacean chitin with strong alkali. The physico-chemical properties of the chitosan obtained by this method can be inconsistent due to the variability of raw materials and the harshness of the isolation and conversion processes (White *et al.*, 1979).

In order to obtain chitosan of a more consistent quality, the zygomycetes, has been considered as an alternative source of chitosan. There are several advantages of using these fungi to produce chitosan. The most important is that the cell wall of zygomycetous fungi contains a large quantity of chitosan and the physico-chemical properties of this chitosan can be manipulated and standardized by controlling the parameters of fermentation. For instance, chitosans of different molecular weights (MW) are produced by these fungi when they are grown on media comprising different pH and composition (Arcidiacono and Kaplan, 1992). Fungi are also easily cultured using relatively simple nutrients. Therefore, chitosan can be produced in a controlled environment all year round and be independent of the seasonal shellfish industry (White *et al.*, 1979). Some industrial by-products such as molasses from the sugar industry can be used as an inexpensive carbon source to grow fungi (McGahren *et al.*, 1984). This may also help to alleviate some environmental pollution concerns. Finally, the extraction process in obtaining chitosan from fungal cell wall is simpler and milder than the chemical process used to obtain chitosan from crustacean shells, and produces less waste materials (White *et al.*, 1979).

Most studies on the screening of the fungal chitosan content typically harvest the mycelia at a fixed incubation period. This is because chitosan is more difficult to extract from fungi after their active growth phase (McGahren *et al.*, 1984). However, this disregards the differences in the growth rate of different fungi (Rane and Hoover, 1993; Shimahara *et al.*, 1988). Furthermore, there are brief reports that allude to the late

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exponential phase as the most likely phase to give the highest yield of chitosan (Hang, 1990; Hansson *et al.*, 1992). In an effort to reconcile this disparity, this paper reports on the evaluation of the optimal harvesting time of the mycelia for chitosan production, the extractable chitosan content of certain zygomycetes at their late exponential phase and the selection of the most suitable strain for chitosan production.

MATERIALS AND METHODS

Organisms and cultivation

Thirteen strains of zygomycetes, namely *Rhizopus oryzae* USDB 0602, *R. oryzae* USDB 0263, *R. arrhizus*, *R. microsporus*, *R. stolonifer*, *R. oligosporus*, *Absidia glauca*, *Mucor* sp, *M. hiemellis*, *Zygorhynchus moelleri*, *Cunninghamella echinulata*, *Gongronella butleri* USDB 0489 and *G. butleri* USDB 0201 were obtained from the School of Biological Sciences, The National University of Singapore. They were maintained on 3.9% PDA (potato dextrose agar, Oxoid) slants at 5°C. Whenever required, all cultures were subcultured on PDA plates, incubated at room temperature (25°C) and exposed to blacklight to stimulate sporulation. The cultures were allowed to grow for 5–10 days for spores formation. Spores were harvested by flooding the culture plates with 5% Tween 80 in sterile distilled water, and collecting the spore suspensions in a sterile centrifuge tube. The spores were rinsed twice with sterile distilled water by alternate centrifuging at 1500 g for 5 minutes and gentle vortexing. A final spore suspension (1.8×10^6 spores/ml) was prepared for each fungal species and used to inoculate the nutrient broth.

The composition of the nutrient broth used in the experiment consisted of the following per liter of distilled water: glucose (20g), peptone (10g), yeast extract (1g), $(NH_4)_2SO_4$ (5g), K_2HPO_4 (1g), NaCl (1g), $MgSO_4 \cdot 7H_2O$ (0.5g), $CaCl_2 \cdot 2H_2O$ (0.1g), and pH of the broth was adjusted to 4.5 (Shimahara *et al.*, 1988). Sterilization was done by autoclaving at 121°C, for 15 minutes. All broth cultures were incubated in a shaking incubator (New Brunswick Scientific, Innova 4340) at 25°C and 200 rpm.

Chitosan extraction

The chitosan extraction method was modified from Shimahara *et al.*, 1988. Mycelia were harvested by vacuum filtration and washed with distilled water until the filtrate was clear. The mycelia were freeze dried (Virtis, Unitop 200) and ground into powder form. Sodium borohydride (0.05 g) and 40 ml of 1 N sodium hydroxide were added to each g of dried powdered mycelia, and the mixture autoclaved for 15 minutes. The mixture was subsequently centrifuged at 16000 g for 5 minutes to sediment the alkali insoluble materials (AIM). The AIM was washed twice with distilled water, once with 95% ethanol and freeze dried. Chitosan was extracted from AIM by adding 200 ml of 2% acetic acid per g dry powdered AIM, stirring continuously at 25°C for 1 hour. The mixture was centrifuged at 16000 g for 5 minutes, the supernatant was retained and the pellets were subjected to one more round of extraction. The two supernatants were pooled and the pH was brought up to 8.5–9.0 by adding 2 N NaOH to precipitate the chitosan. The suspension was centrifuged at 16000 g for 5 minutes and the chitosan pellet retained. The chitosan pellet was washed twice with distilled water, once with 95% ethanol and freeze dried.

Determination of the cell wall chitosan content of *R. oryzae* Usdb 0602 at different growth phases and the cell wall chitosan content of *Mucor*, *Absidia*, *Gongronella*, *Cunninghamella* and *Zygorhynchus* species at their late exponential phase.

The cell wall chitosan content of *R. oryzae* USDB 0602 grown for 12, 24, 30, 36, 42, 48, 74, 96 and 120 hours was determined as described in (2) above. The culturing was done by inoculating 290 ml of nutrient broth with 10 ml of spore inoculum prepared as in (1) in a 500 ml conical flask. The experiment was carried out with 6 replicates for each incubation period.

Extractable chitosan of all the fungi evaluated were determined by inoculating 193 ml of nutrient broth in a 250 ml conical flask with 7 ml each of spore inoculum. Mycelia were harvested at their late exponential phase, based on the growth curve obtained from (4) and chitosan was extracted as in (2).

Determination of the growth curve of *Mucor* sp, *A. glauca*, *C. echinulata*, *G. butleri* USDB 0201 and *Z. moelleri*

The growth curves of *Mucor* sp, *A. glauca*, *C. echinulata*, *G. butleri* USDB 0201 and *Z. moelleri* were determined by culturing each fungus in the nutrient broth. This was done by inoculating 7 ml of spore inoculum prepared as in (1) above in 193 ml of nutrient broth. The mycelial dry weights after 16, 24, 30, 36, 42, 48, 74, 96 and 120 hours of growth were determined. Six replicate cultures were prepared for each incubation period.

RESULTS AND DISCUSSION

The dry weight of mycelia (biomass), AIM and extractable chitosan of *R. oryzae* USDB 0602 increased over a period of time. (Fig. 1). The fungal biomass increased rapidly during the first 74 hours of incubation and continues to increase until 100 hours. Beyond this point,

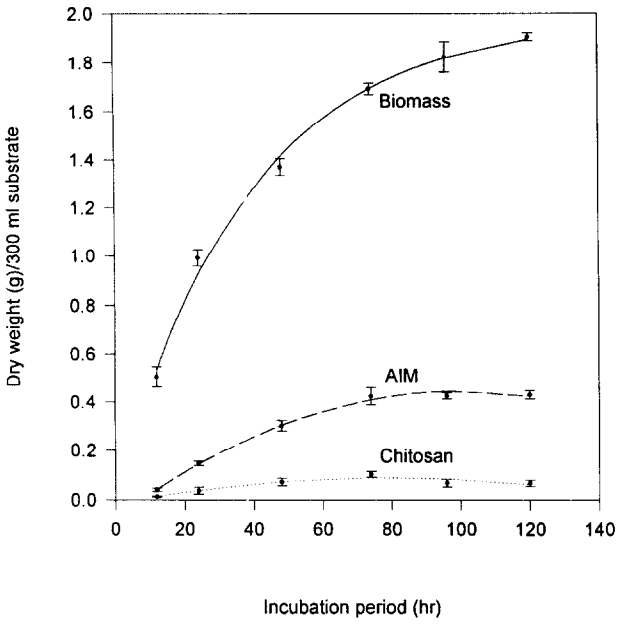

Fig. 1. Dry weight of mycelia, alkaline insoluble materials and extractable chitosan of *Rhizopus oryzae* USDB 0602 with incubation time.

the growth slowed down and the fungus appeared to enter the stationary phase. The amount of AIM also increased steadily with incubation time to 74 hours, although its maximum was reached only at about 100 hours. In the case of chitosan, the absolute amount of extractable chitosan increased slowly with incubation time, peaking at the 74th hour, followed by a slow decline when the cultures were incubated beyond this point.

The decline of the extractable chitosan seen in the time-culture curve might be due to physiological changes in the fungal cell wall (McGahren *et al.*, 1984). Chitosan is produced in the fungal cell wall by deacetylating its precursor, nascent chitin (Herrera, 1992). During the exponential phase, the ratio of free chitosan molecules is relatively high, due to the active growth. Once the culture enters the stationary growth phase, more of the chitosan is anchored to the cell wall of the zygomycetes by binding to chitin and other polysaccharides and extraction becomes more difficult (Davis and Bartnicki-Garcia, 1984; Bartnicki-Garcia, 1968). Therefore, although the fungal biomass was highest during the stationary growth phase, less chitosan is obtained.

The results in Fig. 1 indicate that extracting for chitosan at the late exponential growth phase of the fungus would give the best yield for chitosan. This correlates well with the chitosan production profile of *Absidia butleri* HUT 1001 reported by Shimahara *et al.* 1988. Their results showed that the amount of AIM from *A. butleri* HUT 1001 kept increasing until day 10, although fungal growth had ceased at day 7. However, the amount of chitosan extracted reached its maximum at day 4 (late exponential phase) despite the increase in AIM. Therefore, all fungi should be harvested at their late exponential growth phase and the content of extractable chitosan determined. This is because different fungi have different growth rates, and the time needed for them to reach their late exponential growth phase will also different. If mycelia were harvested at a fixed incubation time, the amount of extractable chitosan obtained might not be the maximum.

In light of the above results, the late exponential growth phase was determined for all genera of fungi used in this study. The results of this determination are presented in Fig. 2. For *Mucor* sp, *C. echinulata* and *Z. moelleri*, the exponential and stationary growth phases could be easily identified from their growth curves. The late exponential phase of *Mucor* sp was at 74 hours, and that of *C. echinulata*, *G. butleri* USDB 0201 and *Z. moelleri* were at 96 hours. However, the growth curve of *A. glauca* was very gradual without a defined late exponential growth phase. In the case of *A. butleri* HUT 1001, the maximum amount of extractable chitosan was obtained during the late exponential growth phase at 96 hours of incubation (Shimahara *et al.*, 1988). We adopted 96 hours as the time for *Absidia* to reach the late exponential growth phase. As stated above, the maximum amount of chitosan was obtained at the late exponential growth phase in *R. oryzae* USDB 0602 in this study and in *A. butleri* HUT 1001 (Shimahara *et al.*, 1988). Therefore, the extractable chitosan content was determined for the remaining 12 zygomycetous fungi harvested at their late exponential phase and the results presented in Table 1.

The extractable chitosan content varied widely among the fungi evaluated; even between species of the same genus. This variation is clearly seen in *Rhizopus*, the

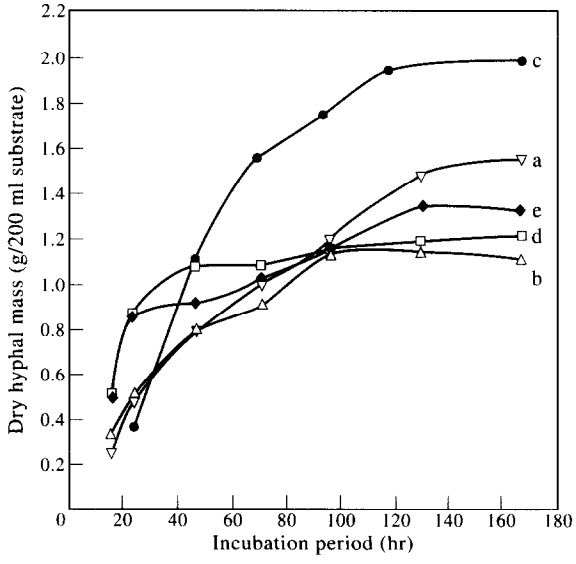

Fig. 2. Growth curves of zygomycetes. a: *Absidia glauca* ▽; b: *Cunninghamella echinulata* △; c: *Grongonella* ●; d: *Mucor* sp □; e. *Zygorhynchus moelleri* ◆.

Table 1. Weight of Mycelia, AIM and yield of extractable chitosan of zygomycetes

Fungi	A: Biomass (g)	B: AIM (g)	C: Chitosan (mg)	C/A (%)	C/B (%)
Rhizopus oryzae 0602	1.13 ± 0.19	0.28 ± 0.01	55.70 ± 8.53	4.91	20.14
Rhizopus oryzae 0263	0.99 ± 0.03	0.21 ± 0.01	43.98 ± 3.05	4.43	20.72
Rhizopus arrhizus	0.97 ± 0.02	0.26 ± 0.02	46.80 ± 8.05	4.78	18.27
Rhizopus microsporus	0.88 ± 0.04	0.19 ± 0.01	29.98 ± 1.77	3.40	15.94
Rhizopusstolonifer	0.91 ± 0.01	0.12 ± 0.01	20.48 ± 7.82	2.25	16.68
Rhizopus oligosporus	0.93 ± 0.01	0.22 ± 0.01	29.98 ± 5.81	3.21	13.94
Absidia glauca	1.21 ± 0.02	0.28 ± 0.04	65.24 ± 10.81	5.37	23.59
Mucor sp	1.17 ± 0.03	0.29 ± 0.02	50.40 ± 4.46	4.31	17.51
Mucor hiemalis	0.89 ± 0.04	0.26 ± 0.01	51.22 ± 4.63	5.77	19.99
Zygorhyncus moelleri	1.15 ± 0.10	0.26 ± 0.04	47.72 ± 13.09	4.14	18.69
Cunninghamella echinulata	1.12 ± 0.02	0.35 ± 0.02	79.73 ± 6.04	7.14	22.50
Gongronella butleri 0489	1.62 ± 0.06	0.24 ± 0.02	76.63 ± 9.00	4.73	31.68
Gongronella butleri 0201	1.63 ± 0.05	0.41 ± 0.04	93.38 ± 6.69	5.74	22.56

extractable chitosan ranged from 20.5 mg to 55.7 mg per 200 ml substrate. Among all strains, *G. butleri* USDB 0201 produced the highest amount of extractable chitosan (93.4 mg/200 ml substrate) followed by *C. echinulata* (79.3 mg/200 ml substrate) and *G. butleri* USDB 0489 (76.6 mg/200 ml substrate). Therefore, *G.butleri* USDB 0201 was the best strain for chitosan production in terms of chitosan yield per 200 ml of substrate. However, the ratio of extractable chitosan to the mycelia mass yield of chitosan was the highest in *C. echinulata* (7.14). In addition, its absolute extractable chitosan was the second highest after *G. butleri* USDB 0201. Therefore, besides *G. butleri* USDB 0201, *C. echinulata* may also be considered as a good fungus candidate for chitosan production.

The molecular weight and degree of acetylation of chitosan are important criteria for its use in applications. Therefore, the extracted chitosan from *G. butleri* USDB 0201 and *C. echinulata* would have to be characterized further to optimize their yield and physicochemical properties.

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