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preliminary communication

Alternative Carbon Sources from Sugar Cane Process for Submerged Cultivation of *Cunninghamella bertholletiae* to Produce Chitosan

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Summary

A mucoralean strain of *Cunninghamella bertholletiae* was used to evaluate the influence of culture medium on chitosan production. In the traditional medium for the growth of mucoralean strains, constituted of yeast extract, peptone, and D-glucose as carbon source, the highest chitosan yield found was 55 mg/g of dry mycelia in a 72-hour submerged culture. Regional substrates from sugar cane process in Northeast Brazil, as sugar cane juice and molasses, which were supplemented with 0.3 % yeast extract, were used as economic substrates to produce chitosan. The optimal production of chitosan was found in sugar cane juice medium, yielding 128 mg/g of dry mycelia in batch flasks at 28 °C. This condition did not need high concentration of sugar cane and gave a good yield of chitosan produced within 48 h (580 mg per L of medium). Molasses did not show to be a good carbon source for chitosan production.

Key words: chitosan, *Cunninghamella bertholletiae*, submerged culture, sugar cane juice, molasses

Introduction

Chitosan, a cationic biopolymer consisting of (1,4)-linked 2-amino-deoxy-β-D-glucan, is derived from chitin, a polysaccharide found in the exoskeleton of shellfish, like shrimps and crabs. Chitosan, besides chitin, occurs in fungal cell walls, particularly of Zygomycetes. Both compounds have been isolated from *Mucor rouxii* (*1*), *Absidia coerulea* (2) and other Mucoraceae strains (*3*,*4*).

Industrially, chitosan is derived from the chemical deacetylation of chitin, using strong alkali, a waste product of the crustacean exoskeletons obtained after industrial processing of seafood. This process is subject to seasonal, limited supply and it pollutes the environment,

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due to the large amount of waste of concentrated alkaline solution. An alternative to solve these problems is the fungal production of chitosan, whose main advantage is that it is environmentally friendly. Therefore, physicochemical properties and yields of chitosan isolated directly from a fungus may be optimised by controlling fermentation and processing parameters. Fungal biomass can be produced by solid-state/substrate fermentation (SSF) and submerged fermentation (SmF). SmF has specific advantage as fermentation method providing easier control of fermentation parameters, such as pH and nutrient concentration in the fermentation medium (*5*).

In general, the media reported for chitosan production from fungi usually contain yeast extract, D-glucose, and peptone. Few studies have been performed on the effect of changes in the ratio of individual components of the medium, especially carbon sources, and use of inexpensive nutrients for chitosan production.

The development of applications for chitosan has expanded rapidly in recent years. Chitosan, being polycationic, nontoxic, biodegradable as well as antimicrobial, has many applications especially in the agriculture, food and pharmaceutical industries. It has been used in enzyme immobilization, wastewater treatment, as food additive and anticholesterolemic, for wound healing, and in pharmaceuticals in several drug delivery systems (*4,6*).

The aim of this work was to investigate inexpensive carbon sources from sugar cane process for fungal growth and chitosan production from a promising fungus, *Cunninghamella bertholletiae* IFM 46.114 in submerged culture and to analyze its quality.

Materials and Methods

Microorganism, medium and culture conditions

Cunninghamella bertholletiae IFM 46.114, obtained from the Institute for Food Microbiology, Chiba University (Japan), was maintained on potato dextrose agar (PDA) slants at 4 °C. We used a well-known YPD medium with pH adjusted to 4.5 (*7*) and two alternative media, sugar cane juice and molasses, as carbon sources with a range of sucrose concentration from 11.5 to 105.5 g/L and from 16 to 160 g/L, respectively, both of them supplemented with 0.3 % yeast extract at pH=4.5. The media were inoculated with spore suspension harvested from the culture on PDA plates with final concentration of 10^4 spores/mL and incubated in a shaking incubator set at 28 °C and 150 rpm, throughout 7 days. At the end of the desired incubation period mycelia were harvested by filtration, washed with approximately 0.5 L of distilled water, dried by lyophilization and used for biomass determination and chitosan extraction. All culture experiments were performed using three replicates.

Sugar analyses

Sucrose measurement from culture filtrate was carried out by acid hydrolysis to sugar monosaccharides by the estimation of reducing sugars (*8*).

Chitosan extraction and analyses

Chitosan from freeze-dried mycelia was extracted by using an alkali-acid treatment described by Synowiecki and Al-Khateeb (*9*). This involves deproteinization with NaOH, extraction of chitosan from alkali-insoluble fraction with acetic acid, and separation of crude chitin by centrifugation and precipitation of the chitosan at pH=9, adjusted with NaOH solution. Chitosan was washed three times by using sequentially water, ethanol, and acetone, and then air-dried at 25 °C.

The IR spectra of chitosan preparations of *C. bertholletiae* cultured from different media and commercial chitosan Sigma (C-0792) were measured by FTIR spectrophotometer (Bruker IFS 66) with KBr tablets. The degree of acetylation (DA) was determined according to Roberts (*10*), using the absorbance ratio A_{1655}/A_{3450} and calculated applying the following equation:

$$\mathrm{DA}/\% = (A_{1655}/A_{3450}) \cdot 100/1.33.$$

Thermogravimetric analyses were carried out using a Shimadzu TGA-50 analyser, under nitrogen flux and a 10 °C/min temperature gradient.

Statistical analysis

ANOVA was applied using Statistica software (StatSoft, Inc., Tulsa, USA) and the effects were evaluated by F-test. The standard error was estimated and the comparisons of the mean values of treatments were evaluated ($p \leq 0.05$) using Tukey's HSD test.

Results

The fungal growth in YPD medium totalled about 9 g/L, and chitosan production in 2 days was 40 mg/g of dry mycelia, growing closely with the biomass up to 3 days (55 mg/g of dry mycelia) and, beyond this, the chitosan decreased (Fig. 1). The kinetic parameters for this fungus showed a generation time of 4.3 h with a μ_{max} of 0.16/h. The calculation of 4.3 h generation time was made as follows: $t_g = (\ln 2)/\mu_{max}$.

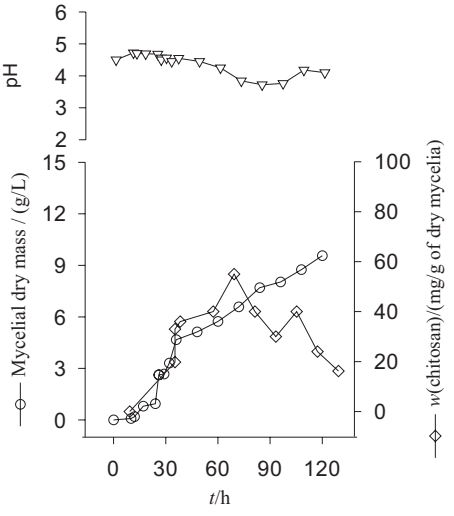

Fig. 1. Growth of *C. bertholletiae* and chitosan production in submerged culture at 28 °C, and 150 rpm using YPD medium, with related pH of the culture

In this work two inexpensive carbon sources (sugar cane juice and molasses) supplemented with 0.3 % yeast extract were used to grow *C. bertholletiae* for chitosan production. Cultivation of this fungus by using only these carbon sources without nitrogen supplements resulted in inexpressive growth and chitosan production (data not shown). The dry mass of *C. bertholletiae* and extractable chitosan in the medium with sugar cane juice plus 0.3 % yeast extract in a 2-day-old culture are shown in Fig. 2. The fungal biomass increased in proportion with increasing sucrose concentration up to 105.5 g/L, reaching 7.7 g/L and the highest yields of chitosan (128 mg/g of dry mycelia) were found with 10.5 g/L of sucrose, with total sucrose consumption. This yield was over 3-fold higher than those observed in YPD medium at the same harvest time.

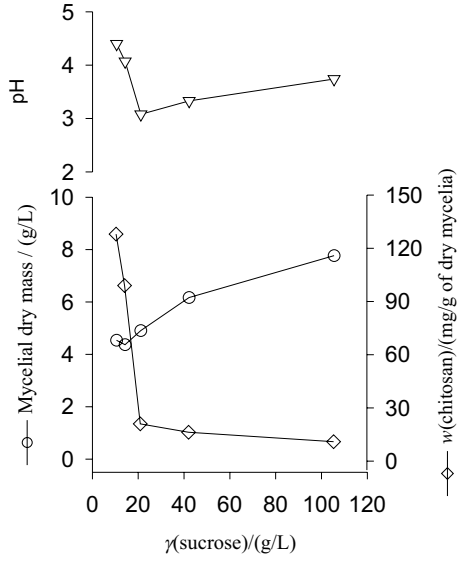

Fig. 2. Effect of initial sucrose concentration on *C. bertholletiae* final biomass and chitosan production at 48 h in submerged culture at 28 °C, and 150 rpm using sugar cane juice with 0.3 % yeast extract

The *C. bertholletiae* growth and chitosan yields in a 2-day-old culture using molasses as carbon source plus 0.3 % yeast extract are shown in Fig. 3. With sucrose concentration of 16 g/L the highest fungal biomass (6.1 g/L) was obtained, as opposed to sugar cane juice used as medium, where the highest fungal growth needed the highest sucrose concentration. The best yield of chitosan of 27 mg/g of dry mycelia was obtained in molasses with sucrose concentration of 79 g/L, which was 75 % less than with sugar cane juice containing sucrose concentration of 10.5 g/L.

The highest concentration of sucrose, up to 20 g/L in sugar cane juice medium, inhibited the strain from producing chitosan, showing that the culture medium yield was not improved in a well-balanced nutrient content aiming at the production of this biopolymer. Thus, the concentration of sugar should not have been the only factor that caused the absence of chitosan in molasses medium. This could be explained by the presence of other substances, generated probably during their different production processes. Such substances would inhibit the enzyme that produces chitosan, as shown from the highest quantities of inorganic impurities detected by thermogravimetric analyses in chitosan preparations obtained from the *C. bertholletiae* cell growth in molasses. The impurities detected in molasses medium were significantly larger ($p \leq 0.05$) than in YPD and sugar cane juice media (Table 1).

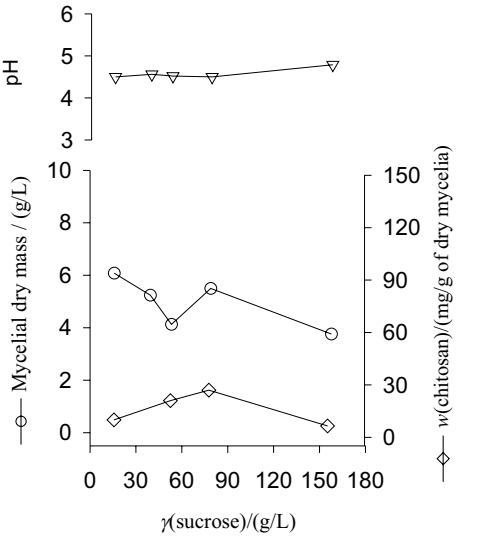

Fig. 3. Effect of initial sucrose concentration on *C. bertholletiae* final biomass and chitosan production at 48 h in submerged culture at 28 °C, and 150 rpm using molasses with 0.3 % yeast extract

Table 1. Effect of carbon sources on the properties of chitosan extracted from *Cunninghamella bertholletiae* cultured in YPD, sugar cane juice, and molasses media in a shaking incubator

Chitosan from *C. bertholletiae*	**DA/%**	***w*(ash)/%**
YPD	11.80 AB	1.70 C
Sugar cane juice	10.30 B	3.80 B
Molasses	13.55 A	9.20 A

Results are the mean values of three replicates. Values in each row with the same letter are not significantly different ($p \leq 0.05$) by Tukey's HSD test

The infrared spectrum profile of the chitosan extracted from *C. bertholletiae* grown in molasses and mainly in sugar cane juice media was quite similar to that shown by commercial chitosan C-0792 with the following characteristic bands: hydroxyl at 3450 cm^{-1}, amide from 1655 to 1550 cm^{-1}, amine from 1630 to 1550 cm^{-1} and C-H at 3250 cm^{-1}. The results of the acetylation degree of chitosan are shown in Table 1.

Discussion

The highest chitosan yield in YPD medium was 55 mg/g of dry mycelia in a 72-hour culture, which is in agreement with the highest chitin deacetylase activity

related to the same strain of *C. bertholletiae* and cultural conditions (*11*). The decline of extractable chitosan seen in the late exponential growth phase might be due to physiological changes in the fungal cell wall. Once the culture enters the stationary phase, more chitosan is anchored to the cell wall of the Zygomycetes by binding to chitin and other polysaccharides, and the extraction becomes more difficult (*2*).

M. rouxii, a well studied fungus for chitosan production, shows chitosan yields ranging from 6 to 9 % of the dry cell mass (*1*). Tan *et al.* (*12*), in a screen of different Zygomycetes strains, reported *C. echinulata* as a good fungus for chitosan production, yielding 7.14 % of biopolymer per mycelial mass using different culture conditions and chitosan extraction method.

The effect of media on chitosan production from *Absidia coerulea* was studied by Rane and Hoover (*13*), who showed that media with greater amounts of glucose and protein supplemented with minerals gave higher chitosan yields and lower degrees of *N*-acetylation of chitosan as compared to the cultures grown in minimal medium, with no added minerals. Arcidiacono and Kaplan (*5*) reported that removal of peptone or yeast extract reduced biomass to around 30 and 40 %, respectively.

We have been studying a new strain of *Syncephalastrum racemosum*, isolated from dung of herbivores of Northeast Brazil, for chitosan production in media with sugar cane juice and molasses as carbon sources. The largest yields of chitosan (74 mg/g of dry mycelia) were obtained from sugar cane juice with a sucrose concentration of 21 g/L and the increase in sucrose concentration did not improve the chitosan yield. However, molasses did not show to be a good carbon source for chitosan production with that fungus (*14*), confirming the results of the present work.

An alternative medium was studied by Hang (*15*) with *Rhizopus oryzae* mycelia using rice and corn as carbon sources, yielding 601 mg/L of extractable chitosan in rice medium for over 48 h of cultivation. In our results with *C. bertholletiae* the highest yield of chitosan was 580 mg/L, obtained in a culture with sugar cane juice containing 10.5 g/L of sugar, maintained in shake flask for the same harvest time.

Yokoi *et al.* (*16*) used alternative media, barley/buckwheat and sweet potato, from shochu distillery wastewater for the production of chitosan with *Gongronella butleri* IFO 8081, and the highest level of chitosan yields occurred in sweet potato medium, approx. 730 mg/L in a 5-day-old culture. However, with the same microorganism and a different strain (*Gongronella butleri* USDB 0201) under different culture conditions, using a complex medium, Tan *et al.* (*12*) obtained 470 mg/L. Nwe *et al.* (*17*) studying the influence of nitrogen source and urea in the media with pieces of sweet potato in solid substrate fermentation, in order to optimize the chitosan production, obtained a chitosan yield of approx. 11 % of the dry cell mass.

Suntornsuk *et al.* (*18*) produced chitosan from four fungal strains cultivated in solid-state fermentation with soybean and mungbean residues that were used in food processing, containing limited nitrogen. They found that *R. oryzae* TISTR3189 produced the highest yields of chitosan on soybean residue (4.3 g/kg of substrate), as opposed to chitosan yielded on mungbean residue (1.6 g/kg substrate).

The effect of the rare sugar D-psicose on chitosan production by *Rhizopus oryzae* has recently been studied, and D-psicose supplementation in a medium containing a low amount of D-glucose resulted in 17.6 % increase in the fungal chitosan productivity (*19*).

The *N*-acetylation degree was measured from the IR spectra by the method of Roberts (*10*), which yielded approx. 10 and 13 % of chitosan from *C. bertholletiae* grown in sugar cane juice and molasses, respectively. This low acetylation degree resulted in a high degree of deacetylation of the chitosan and caused a large positive charge density due to free amine groups, making fungal chitosan unique for biological applications.

Conclusion

Sugar cane juice, an inexpensive carbon source, showed to be a potentially economic alternative for chitosan production from *C. bertholletiae*, especially because it does not need high concentration of sugar and can reach a good yield of chitosan within two days of culture with chemical properties that enable it to be used in biological applications. Therefore, chitosan production from this fungus can be further optimised to improve yield in a large-scale production.

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