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Research Note

Production of Chitosan by Submerged Fermentation from *Aspergillus niger*

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Abstract. *The effect of glucose concentration in submerged fermentation (SMF) on chitosan production by Aspergillus niger was investigated. A. niger, BBRC, 20004, from the Biochemical and Bioenvironmental Research Centre at Sharif University of Technology, Tehran, Iran, was grown in a Sabouro Dextrose media. Chitosan was extracted from the fungal mycelia using hot alkaline and acid treatment and after 12 days of cultivation, 0.8455 g chitosan /l of the fermentation medium was obtained. The content of glucose in the Sobouro Dextrose Broth media was also changed and the highest yield of chitosan 0.9121g/l was obtained in Sobouro Dextrose Broth media containing 8% glucose.*

Keywords: *Chitosan; Aspergillus niger; Submerged fermentation; Sabouro Dextrose Broth media; Glucose.*

INTRODUCTION

Chitin and chitosan are copolymers of N-acetyl-D-glucosamine and D-glucosamine linked by $\beta-(1\rightarrow 4)$ glycosidic bonds (Figure 1). Chitin is a substance found naturally in the exoskeletons of insects, in the shells of crustaceans, such as crab, shrimp and crawfish and in fungal cell walls. Chitin is obtained from the shells by removing calcium carbonate, pigments, proteins and lipids immediately after peeling the shrimps [1,2]. Chitosan is a partially deacetylated form of chitin by thermo-chemical deacetylation in concentrated sodium hydroxide. The enzymatic deacetylation by the enzyme chitin deacetylase (CDA) has been proposed as an environmentally friendly alternative [3]. From the different animals mentioned, chitosan is mainly commercially available from shrimp, but also from squid, lobster and crab. Chitosan is a natural and biodegradable biopolymer, but is very scarce. It can be used in a wide range of fields, such as biotechnology (enzyme immobilization), food and nutrition (emulsifying, thickening and stabilizing agent, packaging membrane, antioxidant and dietary supplement), water engineering (flocculants, chelating agent for metals) and in medical applications (artificial skin, drug-delivery systems, blood anticoagulants and, recently, in gene therapy too) [4].

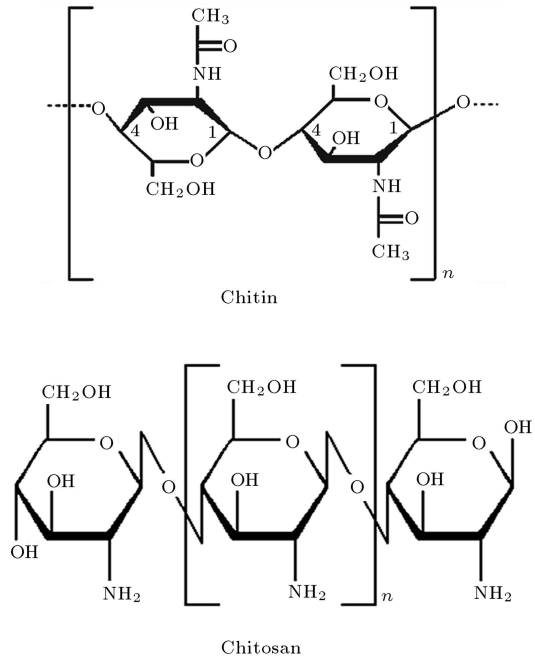

Figure 1. Schematic structures of chitin and chitosan.

Since chitosan is usually insoluble in water, it is necessary to protonate its NH_2 groups to obtain the soluble acidic form. Chitosan solubilization is usually

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carried out by chemical acidification with a mineral or organic acid, such as hydrochloric or acetic acid [1]. Since supplies of seafood waste are seasonable and variable, new research has been carried out on the use of alternative sources for chitosan [2]. The studies were focused mainly on chitosan from fungi. The production and purification of chitosan, from the cell walls of fungi grown under controlled conditions, offer a greater potential for more consistent products [4].

The purpose of the present work is to use different sugar concentrations in a medium to produce chitosan by fungal fermentation using *Aspergillus niger*. (We have already worked on SSF and have made a comparison between these two methods in a previous paper) [5].

MATERIALS AND METHODS

Chemicals

Potato Dextrose Agar (PDA), NaOH, and acetic acid were obtained from the Merk Company. The ethanol and acetone used in this study were of a commercial grade. Chitosan was from the Sigma Company.

Micro Organism

The fungus strain used in this study was *A. niger*, BBRC, 20004, from the Biochemical and Bioenvironmental Research Centre at Sharif University of Technology, Tehran, Iran.

PDA Slants Preparation

Potato Dextrose Agar (PDA) slants were prepared according to the manufacturer's instructions in order to cultivate the selected *A. niger*, BBRC, 20004 strain.

Inoculum Preparation

The spore of *A. niger*, BBRC, 20004, was inoculated on PDA slants and incubated at 30°C. After 3 days, the fungus growth on the PDA slants was stored at 4°C in a refrigerator. The sterile serum (9 gr/lit NaCl solutions) was poured into a tube and mixed well in order to bring the spore into a solution. Spores in the suspension were counted and the number was adjusted to 3×10^6 spores/ml [6].

Submerged Fermentation

Sabouro Dextrose Broth (2% glucose) media were used for submerged fermentation as a medium. The content of glucose in the SDB was changed up to 12%. 3×10^6 spores were inoculated into 250 ml sterilized flasks containing 50 ml of Sabouro Dextrose Broth (2% glucose). The culture flasks were incubated at 30°C for 2, 4, 6, 8, 10, 12 and 16 days at 150 rpm.

Chitosan Extraction

The fungal mycelia were harvested and 50 ml of 1 N NaOH solution were added per gm (wet weight) of mycelia and homogenized. The content was sterilized at 121°C for 20 minutes (alkali treatment). The alkali Insoluble Materials (AIM) were collected by centrifugation at 6000 rpm for 20 min, and then washed several times with distilled water to neutralise them (pH 7). AIMs were dried in an oven at 40°C. They were then treated with acetic acid 2% (v/v), as a chitosan solvent, under a reflux condition for 6 hours at 95°C (1:30 w/v). The acid insoluble fraction was separated by centrifugation at 6000 rpm for 15-20 min and the supernatant containing the chitosan was isolated.

The pH was adjusted with a 2N NaOH solution in order to precipitate the fungal chitosan. The flocculated chitosan was centrifuged at 6000 rpm, for 15 min. Isolated chitosan was washed four to five times with distilled water to neutralise it. At the same time, ethanol (96%) and acetone were employed to rinse the chitosan and then it was dried in a vacuum oven dryer at 60°C [7,8]. The IR spectra of chitosan was carried out using the KBr disc method in a Unicam Mattson 1000 FTIR spectrophotometer. Based on the infra-red spectrum, the Degree of Acetylation (DA) is determined, using the absorbance ratio A_{1655}/A_{3450} [9] and is calculated by the following equation:

$$A(\%) = (A_{1655}/A_{3450}) \times 100/1.33. \quad (1)$$

In this research, the FTIR spectrum of three isolated samples of chitosan were compared with chitosan from Sigma company. In general, chitosan shows bands at 3000-3500 cm^{-1} (NH bond) at 1400-1650 cm^{-1} (C=O bond) [8]. In Figure 2, at 3423, a peak can be observed which is attributed to the NH bond; the C=O, NH bond was obtained at 1650-1557 cm^{-1}. The degree of acetylation has not been studied in this research and this information was presented in previous work [1].

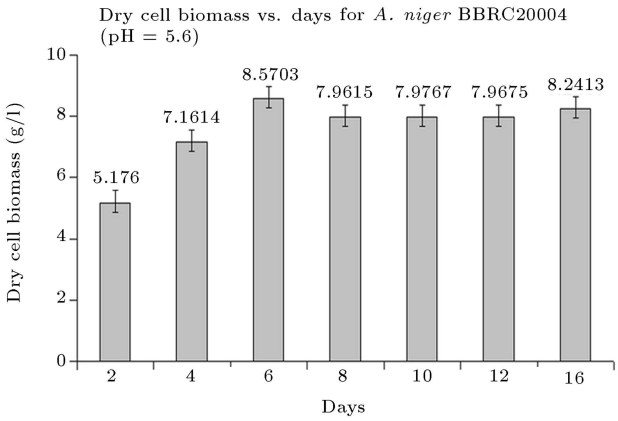

Figure 2. Dry biomass from *A. niger* in submerged fermentation at different days. Values are the mean of two replicates ± standard deviation.

RESULTS AND DISCUSSION

Figure 2 shows the dry cell biomass of *A. niger*. The maximum value was obtained at 6 days after inoculation under submerged fermentation (8.5703 g/l). After 6 days of incubation, the amount of biomass decreased as the amount of nutrients decreased. Chitosan was extracted from the fungal mycelia using a hot alkaline and acid treatment and, after 12 days of fermentation, 0.8455 g/l of chitosan was obtained (Figure 3).

Different amounts of chitosan production have been reported. Muzzarelli et al. [10] obtained about 1.8 g/l of chitosan with *Abidia coerulea* using a PGY medium, while Davoust and Persson [10] reported a 2.8 g/l yield using glucose, yeast and mineral media. The yield of chitosan produced in this work was lower than the production of chitosan which was obtained by Muzzarelli et al. and Davoust and Persson [10,11] due to the nature of the native fungus and the type of medium. We also obtained higher amounts of chitosan than a yield of 0.47 g/l, which was obtained by Tan et al. [12] from *G. butleri* USDB0201. Crestini et al. [13] experienced that the yields of isolated chitosan were 0.12 g/l of fermentation medium under liquid fermentation conditions. Ke-Jin Hu et al. [14] reported a 78.3 mg/l yield using PGY salt broth for *A. niger*.

Glucose concentrations were changed in the medium and the results showed that, at the 12th day of inoculation, the chitosan yield increased to 0.9121 g/l in the medium containing 8% glucose. From Figure 4, it can be found that by increasing the concentration of glucose from 2 to 8 gr, an increase of chitosan by 7.88% could be obtained. No reports have been found regarding different sources of sugar in media.

CONCLUSION

Aspergillus niger was a good candidate and its production by submerged fermentation seems to be economical. Since supplies of seafood waste are seasonable and variable, new research has been carried out on the use of alternative sources for chitosan, mainly from fungi. The effects of incubation time and sugar content were observed in submerged mediums. The yield of precipitated chitosan was 0.8455 g/l after 12 days of incubation. The content of glucose in the substrate SD medium also was changed and the highest yield of chitosan was obtained with 8% glucose.

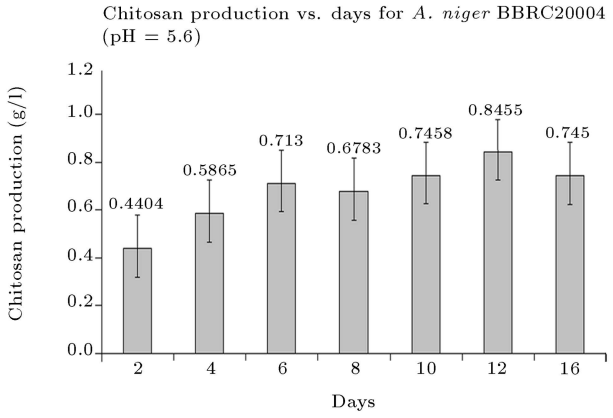

Figure 3. Chitosan production from *A. niger* in submerged fermentation at different days. Values are the mean of two replicates ± standard deviation.

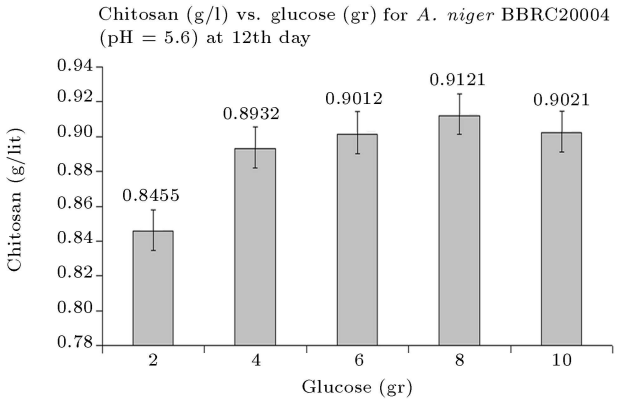

Figure 4. Chitosan production from *A. niger* in different concentration of glucose at 12th day of inoculation. Values are the mean of two replicates ± standard deviation.

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