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Comparison of Solid Substrate and Submerged Fermentation for Chitosan Production by *Aspergillus niger*

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Abstract. *Production yield of solid-state (SSF) and submerged fermentation (SMF) on chitosan from Aspergillus niger was investigated. A. niger BBRC 20004 was grown on soybean residue and Sabouraud Dextrose Broth medium (2% glucose). Chitosan was extracted from the fungal mycelia using hot alkaline and acid treatment. Soybean residue at a moisture content of 37%, and 8.4 ± 0.26% of nitrogen content produced the highest amount of chitosan, 17.053 ± 0.95 g/kg dry substrate after 12 days. Also, chitosan was extracted from A. niger on Sabouraud Dextrose Broth medium in submerged fermentation (0.8455 g/l after 12 days of cultivation). The yield of chitosan isolated in SSF was about 15-20 times more than in SMF (Submerged Fermentation).*

Keyword: *Chitosan; Soybean residue; Solid-State Fermentation (SSF); Submerged Fermentation (SMF); Sabouraud Dextrose Broth (SDB); Glucose; Aspergillus niger.*

INTRODUCTION

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It has a number of commercial and possible biomedical uses (Figure 1). Chitosan is produced commercially by deacetylation of chitin [1], which is the structural element in the exoskeleton of crustaceans (crabs, shrimp etc.). 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 carried out by chemical acidification with mineral or organic acid, such as hydrochloric or acetic acid [2]. The advantages of using fungi are: easy handling, harvesting and controlling in order to produce high quality chitosan. However, recent advances in fermentation technology suggest that many of these problems regarding conventional methods can be overcome by culturing chitosan-producing fungi [3]. Fungal biomass can be produced by Solid-Substrate Fermentation (SSF) and submerged substrate fermentation (SMF). Among various groups of microorganisms used in SSF, the

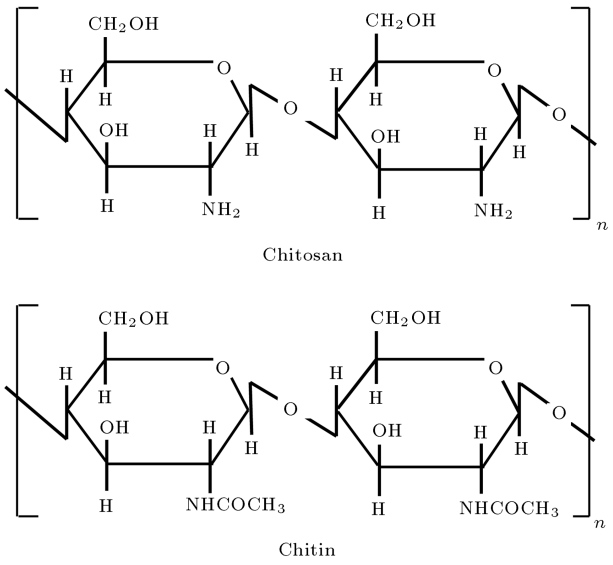

Figure 1. Schematic structures of chitosan and chitin.

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filamentous fungi are most exploited because of their ability to grow on complete solid substrate. It has been reported that the yield of chitosan using SSF (w/w) is higher than that of SMF (w/v) due to the low amount of mycelia produced in SMF, so there has been considerable interest in producing chitosan using the SSF process. These findings can be explained by the fact that the quality and amount of chitosan extracted from the fungal mycelia depend on fungal strain, fermentation type, fermentation medium composition and harvesting time. Since chitosan is a nitrogen containing biopolymer, fungi require an inorganic or organic nitrogen source as a nutrient to synthesize the chitin/chitosan for their cell wall [4].

It has, however, heterogeneous and inconsistent physiochemical properties. Since supplies of seafood waste are seasonable and variable, new research has been carried out on the use of alternative sources for chitosan [5].

The nitrogen source is one of the most important factors in the production of chitosan by fungi. Therefore, the purpose of the present work is to use nitrogen sources for the medium, such as soybean, which was selected as the culture medium and provided from the factory, to examine the effect of medium composition and harvesting time on chitosan production by fungal fermentation, *Aspergillus niger*.

MATERIALS AND METHODS

Chemicals and Micro Organism

Potato dextrose agar (PDA), NaOH and Acetic Acid were obtained from the Merk Company. The ethanol and acetone used in this study were of commercial grade. Other chemicals used for Macro Kjeldahl distillation (nitrogen test) and the Anthrone Colorimetric Method (carbohydrate test) were of analytical or higher grade.

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

Analytical Methods

The Anthrone Colorimetric and Kjeldahl methods were used to determine the carbohydrate and nitrogen content of organic and inorganic substances [6,7].

Preparation of Spore Suspension

Potato Dextrose Agar (PDA) slants were prepared in order to cultivate the selected *A. niger* strain. Therefore, 3.9 gr of PDA powder were solved in 100 ml of distilled water by continuous shaking of the erlenmeyer flask solution over a mild heat until the solution foamed by reaching its boiling point. Afterwards, 10 ml of prepared PDA solution were distributed into ten tubes; the tubes were plugged with cotton wool and then covered with aluminium foil. Finally, the tubes were autoclaved at 121°C for 20 minutes [8].

A. niger was harvested on 3.9% Potato Dextrose Agar (PDA) slants at 30°C for 3 days. After the growth of micro organisms on the PDA, the slants were stored at 4°C in a refrigerator. Soybean, as a nitrogen source, was supplied by a local factory (for solid substrate fermentation medium) and kept at 4°C until used. Sabouraud Dextrose Broth (2% glucose) was used for submerged fermentation as a medium. The content of glucose in the substrate was also changed up to 10%.

Spore suspension was used as an inoculum for residue culture mediums. Therefore, a 5-day-old culture of *A. niger* grown on a PDA slant was used to prepare spore suspension for inoculation into the residue medium.

The sterilized serum (9 gr/lit NaCl solution), which was steam sterilized at 121°C for 20 minutes previously, was poured into a prepared PDA slant and after sufficient shaking, was poured back into the original serum tube. Then, spore suspension in the serum tube was completely homogenized by means of a tube shaker. Finally, the number of spores in suspension was examined for a microscopic count with a Neubauer. The spore concentration was adjusted to about 3×10^6 spores/ml by 10 to 20 times dilution.

Solid Substrate Fermentation

Solid substrates with adequate nitrogen content were supplied for a solid culture medium of fermentation. Chemical Analysis of the substrate was carried out prior to solid-substrate fermentation and the results are: moisture (82 ± 0.7; Mean $\pm$ SD, N=3), nitrogen (8.4 ± 0.26), carbohydrate (89 ± 0.08), and ash (6 ± 0.1). With respect to heat transfer restrictions, and in order to have a suitable porosity in the solid substrate medium of fermentation, 30 gr of dry substrate were weighted in 500 ml Erlenmeyer flasks. Since the prepared residues had a moisture content of 8-10 percent, which is not enough for the growth of fungi, the moisture of the substrate was adjusted by adding distilled water. Then, those flasks were hand shaken to homogenize the solid medium and autoclaved (121°C, 20 min). One ml of spore suspension (about 3×10^6 spores/ml) under sterile conditions was inoculated into sterilized flasks and shaken to distribute the spores (The final moisture content of the flasks before the incubation period was approximately 37% and 50%) [9]. The flasks were cotton-plugged and remained static during incubation for 4, 8, 12 and 16 days at 30°C.

Shaking can alleviate some of the problems en-

countered with solid substrate beds. It can improve the accessibility of oxygen to the substrate surface by disrupting aerial fungal hyphae, which grow into and fill up the inter-particle spaces. However, shaking also has deleterious effects. In particular, shear forces can damage conidiophores leading to decreased spore production [10]; regarding thin layer beds and good oxygen accessibility, the flasks remained static. Samples were taken after 4, 8, 12 and 16 days. The cell mass was separated from the grown medium by washing four times with distilled water and centrifuged at 6000 rpm for 30 min, and the collected cell wall material was dried at 45°C for 24 h.

Submerged Fermentation

One ml of spore suspension (about 3×10^6 spores/ml) under sterile conditions was inoculated into sterilized flasks containing Sabouraud Dextrose Broth medium (2% glucose) and shaken to distribute the spores[11]. The flasks incubated at 30°C for 4, 8, 12 and 16 days. After the desired incubation period, the grown biomass was washed with distilled water and the cell mass was separated from the grown medium by centrifugation at 6000 rpm for 30 min. The residue was then washed several times with distilled water, recentrifuged until all grown medium was removed from the biomass and, then, the cell wall material was dried at 45°C for 24 h.

Chitosan Extraction

Both washed mycelia (free from grown medium) from SMF and SSF were autoclaved at 121°C for 15 min after homogenizing in a blender with 1 N NaOH solution, (1:30 w/v). Then, the flask containing homogenized black fungal alkali suspension was sterilized at 121°C for 20 minutes (alkali treatment). The Alkali Insoluble Material (AIM) fractions were recovered after centrifugation at 6000 rpm, washed with distilled water several times and centrifuged until a neutral condition was obtained (pH 7). In both SSF and SMF, the amount of AIMs was about 10.8 g/kg and 8.5 g/l of mycelia, respectively. The AIMs were dried in an oven at 40°C. Dried Alkali Insoluble materials were treated with acetic acid 2% (v/v) as a chitosan solvent under reflux conditions for 6 hours at 95°C (1:30 w/v). Afterwards, by centrifugation the acid insoluble fraction was precipitated at 6000 rpm for 15-20 min and the supernatant containing the chitosan was isolated. To precipitate fungal chitosan with a clear yellowish color, the pH was adjusted with a 2N NaOH solution, and the flocculated chitosan was centrifuged at 6000 rpm for 15 min. The isolated chitosan was washed four to five times with distilled water to neutralize. At the same time, ethanol (96%) and acetone were employed to rinse the chitosan, which was then dried in a vacuum oven dryer at 60°C [3,12,13].

The IR spectra of chitosan was carried out using the KBr disc method in a Unicam Mattson 1000 FTIR spectrophotometer (Figure 2), similar to that of the Sigma crab chitosan (the figure is not presented here). Based on the infra-red spectrum, the degree of acetylation (DA) is determined according to [14], using the absorbance ratio, A_{1655}/ A_{3450}, and calculated according to the following equation:

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

RESULTS AND DISCUSSION

Chitosan production yield from *A. niger* BBRC 20004 under solid substrate fermentation (SSF), using soybean residue with a moisture content of 50% and 37%, at pH 6.4 (incubation temperature 30°C) is shown in Figures 3 and 4. The maximum amount of chitosan in both cases was observed after 12 days of incubation,

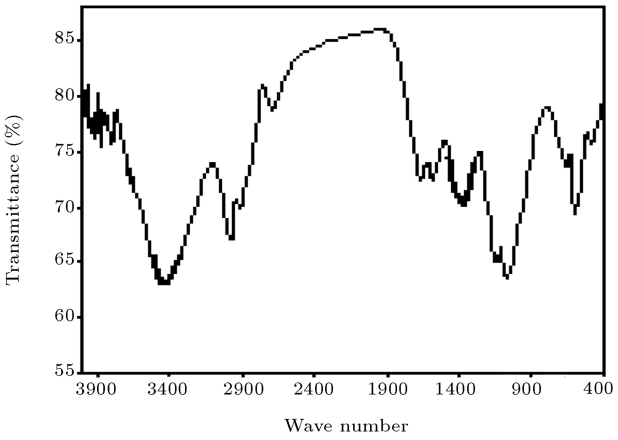

Figure 2. FTIR spectrum of isolated chitosan in this research. The thick black corners are at wavenumber ~ 1655 cm^{-1} amid band and ~ 3450 cm^{-1} hydroxy group absorption band.

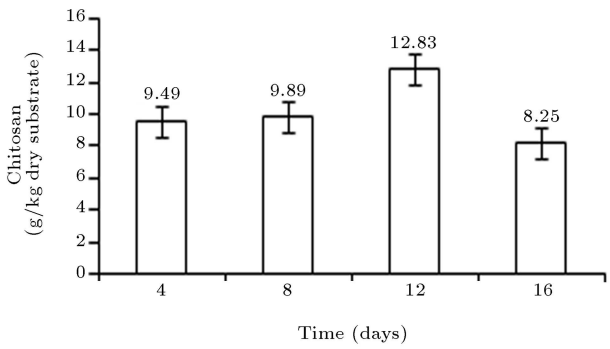

Figure 3. Production of chitosan from soybean residue at 50% moisture at different days after inoculation (*A. niger* BBRC20004). Values are the mean of two replicates ± standard deviation.

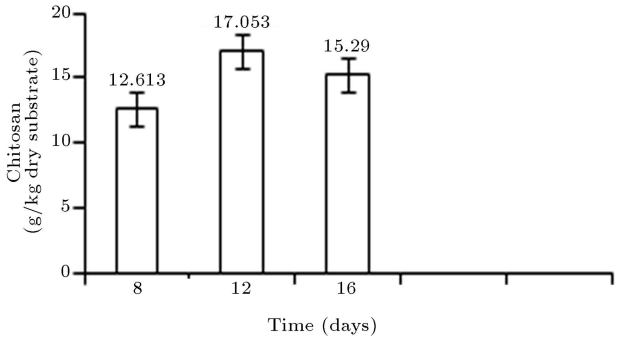

Figure 4. Production of chitosan from soybean residue at 37% moisture at different days after inoculation (*A. niger* BBRC20004). Values are the mean of two replicates ± standard deviation.

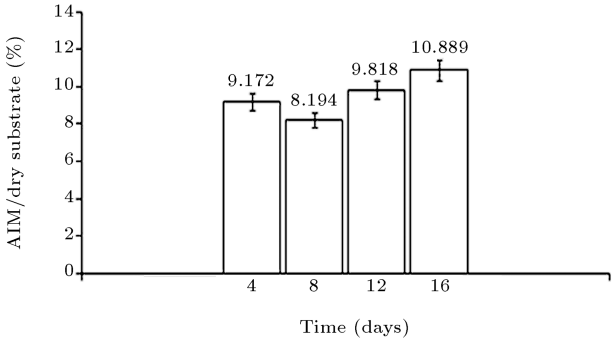

Figure 6. Alkali Insoluble Materials (AIM) versus cultivation time for soybean residue (*A. niger* BBRC20004). Values are the mean of two replicates ± standard deviation.

but a decline was observed. This might be due to consumption of the chitin and chitosan biopolymers by micro organisms as nutrients, and an increase in biomass concentration including the diffusion of enzymes, hydrolysis of polymers by hydrolytic enzymes, and the diffusion of the hydrolysis products [15]. Culture mediums with high moisture content did not produce efficient amounts of chitosan and caused reduced porosity, loss of particle structure, development of stickiness, and the micro organism access to oxygen and nutrients would be impossible [11].

As depicted in Figure 4, fungal chitosan yield extracted from fermented soybean residue on the 12th day of incubation and at 37% moisture content was 17.053 ± 0.95 g/kg dry substrate.

In submerged fermentation (SMF), the maximum value of the chitosan yield was obtained 12 days after inoculation, which is the same for solid substrate fermentation. However, the yield was more than 20 times higher with solid substrate fermentation than with submerged fermentation (0.8455 g/l) (Figure 5).

This finding can be explained that under solid state fermentation conditions, the biomass is more concentrated than under submerged fermentation, ranging from 9.81% (Figure 6) of alkali insoluble materials

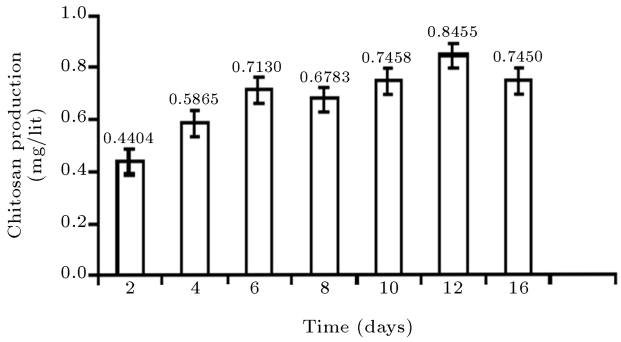

Figure 5. Chitosan production from *A. niger* BBRC20004 in submerged fermentation at different days. Values are the mean of two replicates ± standard deviation.

(AIM) in solid state fermentation to 0.03% of the biomass in submerged fermentation.

In SSF [5], chitosan extraction, based on the dry weight of the substrate, was around 4.3 (gr/kg dry substrate) for soybean residue and 1.6 (gr/kg dry substrate) for mung bean residue. In SMF, Muzzarelli et al. [16] obtained about 1.8 g/l of chitosan with *Absida coerulea* using YPG medium, while Davoust and Persson [17] reported a 2.8 g/l yield using glucose, yeast and a mineral medium. A yield of 0.47 g/l was obtained by Tan [18] from *G. butleri* USDB0201. Crestini et al. [19] experienced that the yields of isolated chitosan were 0.12 g/l of fermentation medium under liquid fermentation conditions and 6.18 g/kg of fermentation medium under solid-substrate fermentation. Amorim and et al. [20] described that under submerged cultures, for both fungi, the maximum chitosan yield from the mycelia mass was obtained early in the growth phase. Within 24 days, *Mucor racemosus* produced chitosan 35.1 mg/g dry mycelia weight and *Cunninghamella elegans* 20.5 mg/g dry mycelia weight.

CONCLUSION

In this study, it was shown that a microorganism can be effectively used as a medium to produce chitosan. *Aspergillus niger* was a good candidate and its production by solid-state fermentation seems to be economical. The effect of incubation time, nitrogen and moisture content were observed for different solid mediums. In solid state fermentation, the solid bed temperature was a primary aim in controlling production, as overheating could seriously impair process performance. Since any microorganism generates energy (metabolic heat) during its growth, this heat must be dissipated to avert undesirable solid bed temperature conditions. Hence, in this study, the solid bed was thin enough to solve this problem. The appropriate temperature and water content of the solid bed have been recognized as good factors in optimizing conditions. After the extraction

of fungal chitosan, the yield of precipitated chitosan was the highest (17.053 ± 0.95 g/kg dry substrate) for soybean at 8.4% total nitrogen content; 20 times higher than with submerged fermentation (0.8455 g/l).

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BIOGRAPHIES

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