

Influence of Different Nitrogen Sources on Amount of Chitosan Production by *Aspergillus niger* in Solid State Fermentation

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ABSTRACT: In this study the effect of different nitrogen source substrates on the amount of chitosan production by *Aspergillus niger* was investigated. *A. niger* PTCC 5012 from the Persian Type Culture Collection (PTCC) was grown on soy bean, corn seed and canola residues at 30 °C for specified cultivation days under sterilized conditions. Chitosan was extracted from the fungal mycelia using hot alkaline and acid treatment. The results were shown that soy bean residue at moisture of 37% and 8.4 ± 0.26 % of nitrogen content produced the highest amount of chitosan (17.053 ± 0.95 g/kg of dry substrate), after 12 days of incubation. Corn seed residue produced very low amount of chitosan (1.9 ± 0.4 % of nitrogen content). The chitosan was analyzed by FTIR spectroscopy and its spectrum was recorded.

KEY WORDS: Chitosan, Soybean residue, Canola residue, Corn seed residue, *Aspergillus niger*, Fungus, Solid-state fermentation (SSF).

INTRODUCTION

Chitosan, β -(1 \rightarrow 4) D-glucosamine, is a partially deacetylated form of Chitin, β -(1 \rightarrow 4) N-acetyl-D-glucosamine, by thermo-chemical deacetylation in concentrated sodium hydroxide. The enzymatic deacetylation by the enzyme chitin deacetylase (CDA) has been proposed as an environmental friendly alternative [13] (Fig. 1). Chitin is a substance found naturally in the exoskeletons of insects, shells of

crustaceans such as crab, shrimp, crawfish and fungal cell walls. Chitin and chitosan, as a biopolymer, are 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 (flocculant, chelating agent for metals), and medical applications (artificial skins, drug-delivery systems,

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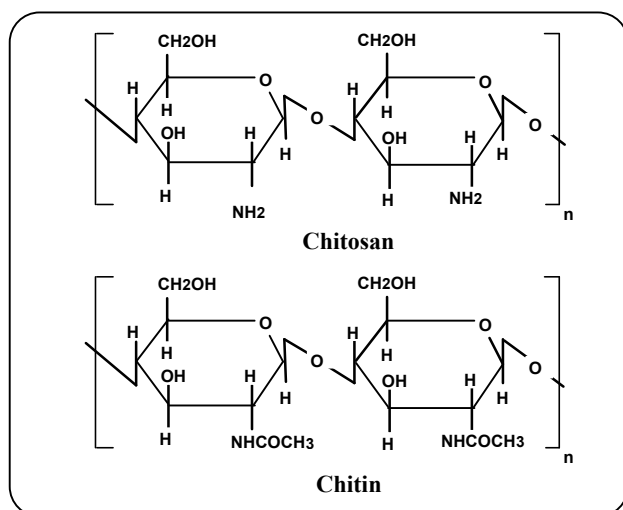


Fig. 1: Schematic structures of chitosan and chitin.

blood anticoagulant and recently in gene therapy). Since chitosan is usually insoluble in water, it is necessary to protonate its NH₂ 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 [3].

Chitosan from different animals are commercially available, mainly from shrimp, but also from squids, lobsters and crabs. Chitin is obtained from the shells by removing calcium carbonate, pigments, proteins and lipids immediately after peeling the shrimps [3,4]. However it has heterogeneous and inconsistent physicochemical properties, since supplies of the seafood wastes are seasonal and variable. New researches have been carried out on the use of alternative sources for chitosan [5,6]. The studies were focused mainly on chitosan from fungi. Production and purification of chitosan from the cell walls of fungi grown under controlled conditions, offer greater potential for more consistent products [6].

The advantage of using fungi is the easy handling, harvesting and controlling to produce high quality chitosan; however recent advances in fermentation technology suggest that many of these problems concerning conventional methods can be overcome by culturing chitosan-producing fungi. Fungal biomass can be produced by solid-state fermentation (SSF) and submerged fermentation (SMF). Among various groups of microorganisms used in SSF, the filamentous fungi are most exploited because of their ability to grow on complete solid state, where the yield of chitosan using

SSF (w/w) has been reported to be higher than that in SMF (w/v) due to low amount of mycelia produced in SMF [1,2], so, there has been considerable interest to produce chitosan in SSF processes.

Crestini *et al.* experienced that the yields of isolated chitosan were 120 mg/l of fermentation medium under liquid fermentation conditions and 6.18 g/kg of fermentation medium under solid-state fermentation. In their research *Lentinus edodes* was grown on wheat straw (the protein content was lower than 1%), used as the basic substrate for solid-state cultivation and the yield was more than 50 times higher with SSF than with SMF [8]. It is reported that the amount of chitosan from *A. niger* TISTR3245 grown on soybean residue with nitrogen content of 0.6% (moisture 69.9%, carbohydrate 18.4%) and mung bean with nitrogen content about 0.002% (moisture 77.5%, carbohydrate 21.4%), reached a maximum value of 2.1 g/kg and 1.5 g/kg at 12 days after incubation respectively [5]. These findings imply that the quality and amount of chitosan extracted from the fungal mycelia depends on fungal strain, fermentation type, fermentation medium composition and harvesting time [1,2]. Since chitosan is a nitrogen containing biopolymer, so does fungi require an inorganic or organic nitrogen source as nutrient to synthesize the chitin/chitosan for their cell wall [1].

The nitrogen source is one of the most important factors for the production of chitosan by fungi. Therefore, the purpose of this work is to use different nitrogen sources of medium, such as soybean, corn seed and canola residue which were selected as culture mediums.

MATERIALS AND METHODS

Chemicals

Potato dextrose agar (PDA), NaOH, and Acetic Acid obtained from Merck company. Ethanol and acetone used in this study were of commercial grade. Other chemicals used for macro Kjeldahl distillation (Nitrogen test) and Anthrone colorimetric method (Carbohydrate test) were of analytical or higher grade.

Microorganism

The fungus strain used in this study was *Aspergillus niger* PTCC 5012, obtained from the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST).

Determination of Nitrogen and Carbohydrate Content

The kjeldahl method [10] and Anthrone colorimetric method [11] were used to determine the amount of the nitrogen and carbohydrate content.

PDA Slants Preparation

Potato Dextrose Agar (PDA) slants were prepared in order to cultivate the selected *A.niger* PTCC 5012 [12].

Culture Media

A.niger PTCC 5012 was harvested on 3.9 % Potato Dextrose Agar (PDA) slants at 30 °C, an optimized temperature for growth of most fungi [5] for 3 days. After growth of micro organism on PDA, slants were stored at 4 °C in a refrigerator. Soybean, canola, and corn seed residues, as a nitrogen source, were supplied by a local factory, and also kept at 4 °C until used.

Spore Suspension Preparation

Spore suspension was used as an inoculum for residue culture mediums (canola, corn and soybean residues). Therefore 5-day-old culture of *A.niger* grown on PDA slant was used to prepare spore suspension for inoculation into the residue mediums. The sterilized sera (9 NaCl solutions) were poured into a prepared PDA slant, and after sufficient shaking, it was returned to the original serum tube. Then spores suspension in the serum tube was homogenized by means of tube shaker. The spore concentration was adjusted to about 3×10^6 spores/ml by 10 to 20 times dilutions.

Solid Substrate Preparation

Solid substrates with adequate nitrogen content were supplied for solid state fermentation. The substrates were examined for moisture, nitrogen, carbohydrate and ash content prior to solid-state fermentation, as shown in table 1.

With respect to heat transfer restrictions and in order to have a suitable porosity in solid substrate medium of fermentation, 30 g of dry substrate were weighted in 500 ml Erlenmeyer flasks. Since the prepared residues had moisture of 8-10 percent, which was not enough moisture for the growth of fungi; therefore, the moisture of the substrate was adjusted by adding distilled water. Then those flasks were hand shaken to homogenize the solid mediums and autoclaved (121 °C, 20 min). The aim of this

Table 1: Composition of different substrates used in this research study (^a Mean \pm SD, N=3).

Substrates	Composition (%)			
	Total Nitrogen	Carbohydrate	Ash	Moisture
Canola residue ^a	6.2 \pm 0.22	6.8 \pm 0.07	6 \pm 0.1	8 \pm 0.4
Corn seed residue ^a	1.9 \pm 0.4	8.6 \pm 0.18	5.8 \pm 0.2	8 \pm 0.4
Soy been residue ^a	8.4 \pm 0.26	8.9 \pm 0.08	6 \pm 0.1	8.2 \pm 0.7

research was to study the ability of *A.niger* PTCC 5012 to produce chitosan on different N-containing substrates, without adding any chemicals. The pH of the prepared culture media measured 5.9 for corn seed residue and 6.4 for canola, and soybean residue.

As inoculum, one ml of spore suspension (about 3×10^6 spores/ml) in sterile condition was inoculated into sterilized flasks and shaken to distribute the spores (the final moisture contents of the flasks before the incubation period were approximately 37 % & 50 %). 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 encountered with solid substrate beds. It can improve the accessibility of oxygen to the substrate surface by disrupting aerial fungal hyphae, which grow and fill up the inter-particle spaces. However, shaking also has deleterious effects, particular shear forces can damage conidiophores, leading to decreased spore production [14]. Regarding to thin layer bed and good oxygen accessibility, flasks remained static.

Chitosan Extraction

After cultivation for every 4 days, NaOH of 1 N, (1:30 w/v) was added gently into the culture medium and the alkaline suspension was homogenized. Then the flask containing homogenized black fungal alkali suspension, was sterilized at 121 °C for 20 minutes (alkali treatment). After that, the alkali insoluble materials (AIM) centrifuged at 6000 rpm, and they were washed with distilled water several times and centrifuged until neutral condition obtained (pH 7). 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 condition 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 clear yellowish colour, the pH was adjusted with 2N NaOH, and then flocculated chitosan was centrifuged at 6000 rpm, for 15 min. Isolated chitosan was washed four to five times with distilled water to neutralise. At the same time ethanol (96 %) and acetone were employed to rinse chitosan and then it was dried in vacuum oven dryer at 60 °C [5-7, 9, 13].

Infrared spectrum of fungal chitosan was recorded using Unicam Mattson 1000 FTIR spectrophotometer to monitor the chitosan extraction (Fig. 1).

RESULTS AND DISCUSSION

Production of chitosan from *A.niger* PTCC 5012 grown under solid state fermentation using soybean residue with moisture content 50 % and 37 % and pH 6.4 (incubation temperature 30 °C) is depicted in Fig. 2 and Fig. 4. Chitosan production in both cases reached the highest value in 12th day of incubation, but a decline was observed. This might be due to consumption of the chitin and chitosan biopolymers by microorganism as nutrient and increase in biomass concentration, including diffusion of enzymes, hydrolysis of polymers by hydrolytic enzymes and the diffusion of the hydrolysis products [15]. As it is shown in Fig. 3, AIM (Alkali Insoluble Material) is increased, but chitosan is decreased. Corn, canola and soybean residues with high moisture content did not produce efficient amounts of chitosan and caused reduced porosity, loss of particle structure, development of stickiness and made it impossible for the microorganism to access oxygen and nutrient (Fig. 2, Fig. 4).

Chitosan extraction by fungus *A.niger* PTCC 5012 growth on canola substrate with moisture content of 37 % (pH 6.4) incubated for 4 days and 22 days, at 30 °C is shown in Fig. 5.

As depicted in Fig. 4 and Fig. 5, fungal chitosan yield extracted from fermented soybean residue was higher than yield from canola residue at the same moisture content, which is 17.053 ± 0.95 and 12.73 ± 1.22 chitosan (g/kg dry substrate) respectively (12th day). (All the results presented are the mean value of two replicates \pm standard deviation, N=2).

Meanwhile, corn seed residue was tested for fungal chitosan production, after adding distilled water the substrate was sticky and agglomerated which would have restricted the diffusion of oxygen within the substrate bed and made it troublesome for fungus to have good access to all nutrient materials. And after acid extraction the

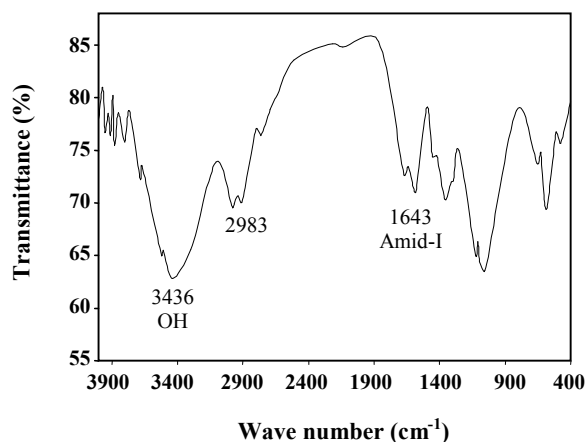


Fig. 1: FTIR spectrum of isolated chitosan.

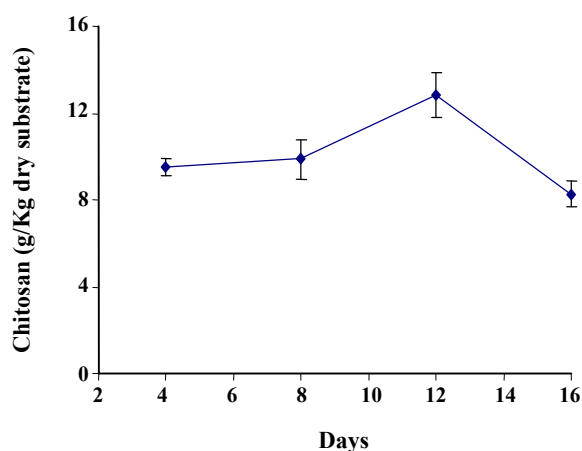


Fig. 2: The amount of chitosan for soybean residue at 50 % moisture at different days after inoculation, N=2, values are the mean of two replicates \pm standard deviation.

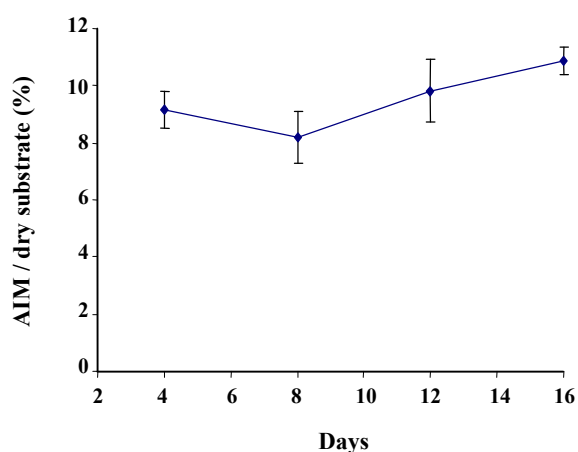


Fig. 3: Alkali Insoluble Materials (AIM) vs. cultivation time for soybean residue at 50 % moisture, N=2, values are the mean of two replicates \pm standard deviation.

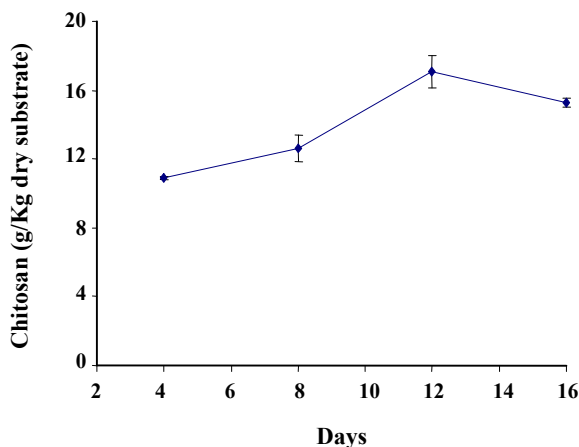


Fig. 4: The amount of chitosan for soybean residue at 37 % moisture at different days after inoculation, $N=2$, values are the mean of two replicates \pm standard deviation.

supernatant appeared highly turbid. Therefore the results obtained revealed that production was very low with an unsatisfactory quality.

In the present work, good mediums, as food processing waste with high protein content, were used for fungal chitosan production. There are no reports about the potential of high nitrogen content substrate, but it can be concluded that if the substrate has not got enough nitrogen content, no production can occur. In the literature chitosan extraction based on the dry weight of the substrate was around 4.3 (g/kg dry substrate) for soybean residue and 1.6 (g/kg dry substrate) for mung bean residue [5], showing that the moisture content of the medium is a critical factor that affects microbial growth and product yield, also nitrogen content and the type of microorganism play a major role in the production. In the solid state fermentation, the solid bed temperature was a primary aim to control the production, because 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. Appropriate temperature and water content of the solid bed has been recognized as a good factor to optimize the conditions.

Finally, with respect to valuable applications of chitosan in industry, this promising method of growth of fungi on solid state medium could be a new method to produce chitosan.

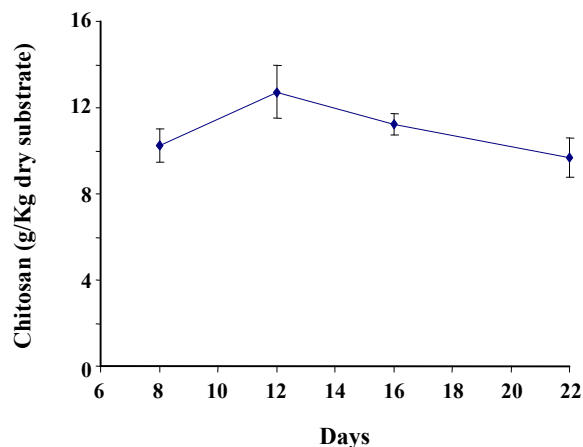


Fig. 5: The amount of chitosan for canola residue at 37 % moisture at different days after inoculation, $N=2$, values are the mean of two replicates \pm standard deviation.

CONCLUSIONS

In this study, it was shown that *A.niger* can be used effectively to produce chitosan 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.

After extraction of fungal chitosan, the yield of precipitation was the highest (17.053 ± 0.95 g/kg dry substrate) for soybean at 8.4 % total nitrogen content indicating that the suitable solid medium in terms of nitrogen nutrient and moisture could result in higher production.

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