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A sulfuric–lactic acid process for efficient purification of fungal chitosan with intact molecular weight

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ABSTRACT

The most recent method of fungal chitosan purification, i.e., two steps of dilute sulfuric acid treatment, pretreatment of cell wall at room temperature for phosphate removal and extraction of chitosan from the phosphate free cell wall at high temperature, significantly reduces the chitosan molecular weight. This study was aimed at improvement of this method. In the pretreatment step, to choose the best conditions, cell wall of *Rhizopus oryzae*, containing 9% phosphate, 10% glucosamine, and 21% N-acetyl glucosamine, was treated with sulfuric, lactic, acetic, nitric, or hydrochloric acid, at room temperature. Sulfuric acid showed the best performance in phosphate removal (90%) and cell wall recovery (89%). To avoid depolymerisation of chitosan, hot sulfuric acid extraction was replaced with lactic acid treatment at room temperature, and a pure fungal chitosan was obtained (0.12 g/g cell wall). Similar pretreatment and extraction processes were conducted on pure shrimp chitosan and resulted in a chitosan recovery of higher than 87% while the reduction of chitosan viscosity was less than 15%. Therefore, the sulfuric–lactic acid method purified the fungal chitosan without significant molecular weight manipulation.

1. Introduction

Chitosan, the deacetylated derivative of chitin, is used in a variety of applications, e.g., in wastewater treatment, food and medical industries, biotechnology, agriculture, cosmetics, pulp and paper industry, and membrane technology. Nowadays, chitosan is commercially produced through chemical deacetylation of chitin obtained from shellfish wastes. This production route suffers from seasonal supply and inconsistent quality of the raw materials [1].

The zygomycetes fungi are alternative sources for chitosan production. Cell wall of this class of fungi contains significant amounts of chitosan together with other biopolymers such as chitin, proteins, polyphosphates, and polyglucoronic acids [2]. Different methods have been suggested for extraction and purification of chitosan from fungal cell wall. Most recently, the fungal cell wall skeleton i.e., alkali insoluble material (AIM) of biomass, is subjected into a two-step treatment with dilute sulfuric acid solutions. In the first step, which is performed at room temperature, a major fraction of polyphosphates are eliminated from the cell wall. In the next step, the phosphate free cell wall derivative is subjected to extraction with hot sulfuric acid (120 °C) to extract the chitosan. Finally, the dissolved chitosan is precipitated and recovered at lowered temperatures [3–9].

One of the most important benefits of the sulfuric acid method, over the traditional methods, e.g., acetic acid extraction, is high purity of the product because of removal of the major impurity of chitosan, i.e., phosphates, prior to the extraction. Although, cold sulfuric acid has shown great potential in removal of phosphates from the cell wall, performance of this acid has not been compared to that of other acids. Additionally, conduction of the extraction process at high temperature facilitates separation of chitosan from its complex in the cell wall and consequently results in high chitosan yields. However, at the same time, chitosan is readily depolymerized in hot acid solutions and therefore, the product of the sulfuric acid method is generally expected to have a low molecular weight [2]. Since high molecular weight chitosans are preferred for some applications, an improvement in the sulfuric acid method is necessary to avoid molecular weight reduction during the course of separation.

The primary objective of the current work was to compare the performance of different acids in phosphate removal from the cell wall of filamentous fungus *Rhizopus oryzae*. The alkali insoluble material (AIM) of the fungal biomass was treated with 1 N solutions of sulfuric, nitric, lactic, acetic, and hydrochloric acids, and phosphate removal from the cell wall was investigated. Additionally, in order to prevent depolymerization of chitosan during the course of extraction by sulfuric acid, this acid was replaced with

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lactic acid. The main obstacle of the previous method was depolymerization of the chitosan. Finally, a new method was developed in which pure fungal chitosan can be obtained without significant depolymerization.

2. Materials and methods

2.1. Microorganism and growth

R. oryzae CCUG 28958 was obtained from Culture Collection of University of Gothenburg, Sweden, and grown on agar slants containing D-glucose (40 g/L), soy peptone (10 g/L), and agar (20 g/L), at pH 5.5 and 32 °C for 5 days.

2.2. Hydrolysis of wheat flour

Wheat hydrolysate was produced by adding 5 mL of alpha-amylase (Liquizyme, Novozymes A/S, Denmark) and 5 mL of gluco-amylase (Dextrozyme GA, Novozymes A/S, Denmark) to a 1.5 L suspension of 625 g dry wheat flour in distilled water. This mixture was heated to 65 °C and kept at this temperature for 2 h while mixing with a glass rod. Afterwards, the mixture was heated to 100 °C [10]. The product was stored at 4 °C until use.

2.3. Fungal biomass production

The fungus was cultivated in wheat hydrolysate supplemented with urea (3 g/L), $(NH_4)_2HPO_4$ (1 g/L), and $MgSO_4{\cdot}7H_2O$ (0.5 g/L) in a 15 L fermenter at 36–38 °C and pH 5.5–6.0 for 24 h. The obtained fungal mycelium was harvested on a screen, washed with water, and freeze-dried.

2.4. Preparation of alkali insoluble material (AIM) of biomass

Alkali insoluble material (AIM) of biomass was prepared according to the method of Zamani et al. [11]. Dried mycelium of *R. oryzae* was treated with 0.5 M NaOH solution (30 mL/g) at 121 °C for 20 min. AIM was separated from the mixture by centrifugation (15 min, 4000 × *g*), washed with distilled water until neutral pH, and stored at 4 °C until use.

2.5. Phosphate removal from biomass

The obtained AIM was treated with 0.1 N solutions of sulfuric, hydrochloric, nitric, acetic, and lactic acids (100 mL acid per g of dry AIM), at room temperature for 30 min to remove phosphates from the cell wall. Then, the solid phase was separated by centrifugation (10 min, 4000 × *g*) and washed three times with distilled water. This was freeze-dried and stored at room temperature until use.

2.6. Extraction of chitosan from phosphate free AIM

Due to extensive depolymerization of chitosan during dissolution in hot sulfuric acid and insolubility of chitosan in sulfuric acid at low temperatures, in the current study, sulfuric acid was replaced with lactic acid in the chitosan extraction step. To extract the chitosan from cell wall, phosphate free AIM was mixed with 0.1 N lactic acid either at room temperature or 50 °C. Then, the insoluble fraction was separated by centrifugation (10 min, 4000 × *g*), and chitosan was precipitated from the liquid phase at pH 8–10, washed with distilled water until neutral pH, and freeze dried. To check the molecular weight reduction during the process, pure shrimp chitosan with viscosity of 120 MPa s (1% solution in acetic acid) and degree of deacetylation of 85% (BioLog, Germany) was also treated at the same conditions. Briefly, the shrimp chitosan was pretreated with sulfuric acid, washed once with 1% NaOH solution followed by three times with distilled water. Then, the chitosan was mixed with 0.1 N lactic acid either at room temperature or 50 °C and mixed until complete dissolution which happened after 60 and 30 min, respectively. Afterwards, the dissolved chitosan was precipitated by raising the pH to 8–10, washed with distilled water until neutral pH, and freeze dried. The viscosity of the obtained chitosan was measured after freeze-drying.

2.7. Determination of glucosamine and N-acetyl glucosamine content

Glucosamine and N-acetyl glucosamine content of AIM and its derivatives were measured according to the method developed by Mohammadi et al. [12]. In brief, AIM and its derivatives were subjected into three steps of hydrolysis using concentrated sulfuric acid at room temperature, dilute sulfuric acid at 120 °C, and nitrous acid at room temperature to convert chitin and chitosan to acetic acid and anhydromannose. The products were analyzed by HPLC and concentrations of glucosamine and N-acetyl glucosamine were calculated accordingly. An ion exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA) at 60 °C with 0.6 mL/min eluent of 5 mM sulfuric acid with UV–vis detector (Waters 2486, Waters, MA) was employed for measurement of acetic acid and anhydromannose concentrations [13].

2.8. Determination of phosphate contents in cell wall materials

Phosphate content of AIM and concentration of released phosphate during the pretreatment steps were measured using ammonium molybdate spectrometric method according to the European standard ISO6878 [11]. The samples were mixed with ascorbic acid and acid molybdate reagents, and their absorbances were recorded at 880 nm. Different concentrations of potassium dihydrogen phosphate were used as standards.

2.9. Determination of chitosan viscosity

The viscosity of shrimp chitosan before and after the purification process was measured using a thermo scientific Haake rotational viscometer (389-001). A solution of 0.3 g chitosan in 400 mL 1% (v/v) acetic acid was prepared, and the viscosity was measured at 25 °C and 200 rpm using an L_1 type spindle.

2.10. Scanning electron microscopy (SEM)

The morphologies of biomass, AIM, and acid-treated AIM derivatives were investigated using scanning electron microscopy (Philips Xl30, Philips, Netherlands). The samples were freeze-dried and subjected to a high resolution imaging with ultra-stable electron source. The images were taken using an Everhart Thornley secondary detector at 15 kV under vacuum mode.

All experiments were conducted at least in duplicates and the results are presented as averages.

3. Results

3.1. Cultivation of the fungus and preparation of alkali insoluble material (AIM)

The fungus *R. oryzae* was cultivated on wheat hydrolysate for 24 h. The glucose content of the hydrolysate was 207 g/L as measured by high performance liquid chromatography (HPLC). The solution was diluted to obtain glucose concentration of 30 g/L. This solution was employed for cultivation of the fungus. After 24 h, the