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Solid-State Fermentation Production of Chitosanase by *Streptomyces* with Waste Mycelia of *Aspergillus niger*

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Abstract

Solid-state fermentation was carried out using mycelium powder of *Aspergillus niger* as substrate for the production of chitosanase of *Streptomyces*. Results of the experiments indicated that the optimal medium consisted of wheat bran and mycelium powder of *Aspergillus niger* with initial moisture content of 60% - 70%. The enzyme activity reached 41.33 U per gram dry medium after cultured for 5 days at 28˚C - 30˚C and an initial pH 6.5. Chitosanase was detected on the second day of incubation and had maximal activity at 5 days and decreased gradually within a 1 month period. Solid-state fermentation is maybe an economic alternative in the production.

Keywords

Streptomyces, Solid State Fermentation, Chitosanase, Waste Mycelia

1. Introduction

Chitosan is a cationic amino polysaccharide with a linear structure of b-1,4-linked D-glucosamine (Glc N) residues [1]. It is widely found in fungi, crustaceans, insects and some algae. A large number of applications in food and nutrition, cosmetics, packaging and preservation, textile, waste water treatment, immobilization and agricultural industry of chitin and chitosan have been well documented [2] [3]. Chitosan can be degraded into chitosan oligosaccharides (COSs) by chemical or enzymatic methods [4]. The functional properties of COSs depend on their molecular weight. COSs with degrees of polymerization 2 - 20 exhibit promising biological activities including antitumor, antifungal and antimicrobial activity [5] [6] [7] [8]. Chitosanase (EC 3.2.1.132) is characterized as a kind of

enzyme, which catalyzes the cleavage of chitosan to form COSs. Chitosanases are usually divided into six glycoside hydrolase (GH) families including GH-5, GH-7, GH-8, GH-46, GH-75 and GH-80 in the CAZy (Carbohydrate Active Enzymes) database. Chitosanase is mainly found in bacterial [9] and fungal cells [10] [11]. But it has some drawbacks to be commercialized due to chitosanase fermentation levels in the microbial system are too low to be separated and used for industrial preparations. And the cost is another key factor.

Streptomyces albolongus ATCC 27414 is a bacterium species from the genus of *Streptomyces* sp., which has been found to produce chitosanase, which was in GH-46 chitosanase [12]. *Streptomyces* is the largest antibiotic-producing genus in the microbial world so far. Solid state fermentation (SSF) is a traditional fermentation technology facing new challenges. It has the advantages of high production efficiency, simple process, extensive operation, less energy consumption, less waste liquid, easy product separation, etc. Compared with liquid fermentation (SMF), the production cost is lower. With people's attention to energy saving and environmental pollution reduction, SSF technology has been paid more and more attention. Using solid state fermentation technology to produce chitosanase has better economic and environmental benefits [13] [14].

In recent years, there have been many reports on the production of chitosanase by the fermentation of bacteria and fungi, mainly using chitosan or chitin in liquid fermentation to induce the production of chitosanase. When chitosanase is used, the substrate needs to be dissolved in an acidic solution in advance to make a gel. In order to reduce costs, chitosanase usually needs to be freeze-dried first, and a lot of water and energy are wasted and expensive inducer increases production costs [15] [16] [17]. Solid-state fermentation (SSF) is close to the natural environment to which the selected microorganisms, especially fungi, are naturally acculturated. It can be considered to be a "closed system" and takes place in the absence or near absence of free water. Owing to much reduced production cost and direct applicability, SSF enzyme without downstream processing (cheap in situ enzyme, crude enzyme) may be an excellent candidate for some applications [18] [19] [20] [21].

In this study, waste mycelia of *Aspergillus niger*, which is commonly used in a citric acid industry, was used as the induction material to produce chitosanase by solid-state fermentation of *Streptomyces albolongus*. The low-cost medium for chitosanase production may facilitate process optimization for the economical production of microbial chitosanase at an industrial scale.

2. Materials and Methods

2.1. Microorganism and Medium

The mycelium powder was prepared according to the following methods. Waste mycelia of *Aspergillus niger* from a citric acid production plant are simply treated with boiling 30% - 40% NaOH aqueous solutions for 4 - 6 hr [22]. The mycelium powder was obtained after drying and grinding at 121˚C, and the chi-

tosan content was 13.62% determined by ninhydrin [23].

Before starting the SSF, the *Streptomyces albolongus* ATCC 27414 was pre-cultured in the solid medium. The solid medium was composed of 1% soluble starch, 0.2% $(NH4)_2SO_4$, 0.2%$CaCO_3$, 0.1% NaCl, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ and 2% agar. Spores were washed with 5 ml 0.05% Tween-80 in sterile water and harvested by centrifugation at 3000 × g for 10 min. Each ml of the suspension contained about 10^8 spores. The solid state medium contained (g): mycelium powder 10, wheat bran20, $(NH_4)2SO_4$ 1, $CaCO_3$ 0.5, NaCl 0.2, KH_2P0_4 0.2 and $MgSO_4{\cdot}7H_2O$ 0.1. The medium was mixed thoroughly with seed culture and the initial moisture content was then adjusted to 60% with distilled water. Incubation was at 28˚C for 2 - 5 days.

2.2. Preparation of the Chitosanase

A bottle of solid culture medium was taken, and 100 mL acetic acid buffer (pH 5.6, 20 mmoL/L) was added. After being broken, the medium was mechanically stirred for 1 h. Four layers of gauze were used for filtration. The filtrate was collected and freeze-dried to obtain chitosanase solution for determination.

2.3. Enzyme Activity Assay

Chitosanase activity was determined at 40˚C by estimating the amount of the reducing ends of sugars using a modified dinitrosalicyclic acid (DNS) method with glucosamine·HCl as the calibration standard. One unit of chitosanase was defined as the amount of enzyme required to liberate 1 mol reducing sugar per min under the conditions described above. Three replicates were performed per analysis.

2.4. Solid-State Fermentation and Conditions Optimization

The effects of different initial moisture contents on the production of chitosanase were investigated. The moisture contents tested were between 50% and 80%. The medium was sterilized at 121˚C for 20 min. The sterile medium was inoculated and the appropriate volume of sterile distilled water was added to make up the desired moisture content. These were then incubated statically in flasks at 30˚C for 2 - 5 days with stirring once a day.

The effects of suitable carbon source were investigated. Wheat bran, molasses meal, corn flour, and straw were chosen. The additive amount of carbon sources was 20 g per 10 g mycelium powder. And the temperature and cycle of SSF were 30˚C and 2 - 5 days respectively.

After optimizing the SSF medium, the fermentation conditions such as initial inoculums size, fermentation temperature, initial pH and fermentation time were optimized step by step. The initial inoculums size varied from 5% to 15% (5%, 8%, 10%, 12% and 15%). The fermentation temperature varied from 25˚C to 38˚C (25˚C, 28˚C, 30˚C, 35˚C, and 38˚C). The initial p H included varied from 5 to 7.5 (5, 5.5, 6, 6.5, 7 and 7.5). The effects of fermentation time were

monitored from 3 d to 7 d.

2.5. Properties of Chitosanase Products

Chitosanase solution was taken to determine its enzyme activity under different temperature, pH, substrate concentration and other conditions, and its optimal temperature, optimal pH, temperature stability, acid-base stability and other properties were studied.

3. Results and Discussion

3.1. Effects of Initial Moisture Content on Chitosanase Production by Solid-State Fermentation

SSF is more favourable over submerged fermentation in terms of biological, processing, environmental and economic aspects. In general, designing a solid-state bioreactor should be focused on moisture content control, because it affects O_2 transfer, CO_2 evolved and monitoring temperature, further changed the spore germination efficiency and enzyme production [18]. The moisture content of materials plays a key role in the growth of bacteria and the accumulation of products during the solid-state fermentation process. This could be due to that high moisture content may lead to the reduction of oxygen transfer because of low substrate porosity, whereas low moisture content may result in poor diffusion of nutrients [24] [25] [26].

This study investigated the effect of initial water content on solid state fermentation to produce chitosanase. During fermentation, activity was first detected on day 3, reached a maximum yield on day 5 and gradually decreased. As shown in Figure 1, chitosanase production increased with initial moisture content of 60% - 70%, having its maximum at 67% (27.32 U/g). When the initial moisture content was less than 50%, chitosanase production was low as the substrate was too dry for cell growth. While at initial moisture content of 80%, total

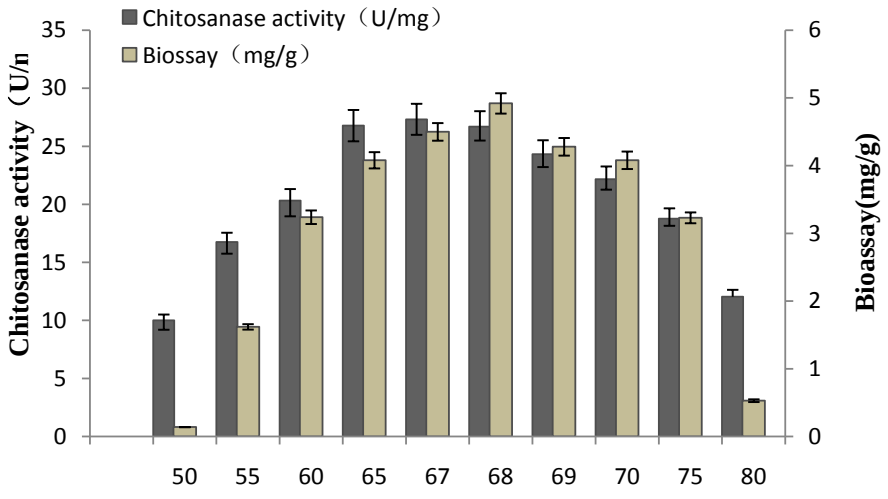

Figure 1. Effects of initial moisture content on chitosanase production by solid-state fermentation.

bioassay was only 0.61 mg/g, since the togetherness of the substrate prevented gas exchange.

3.2. Effects of Different Carbon Sources on Chitosanase Production by Solid-State Fermentation

In addition, carbon resource was an important limiting factor when culturing the microorganism. The mycelium of *Aspergillus niger* can be used as an inducer. Of course, it also serves as a carbon source to provide growth necessary.

It was found that the best initial moisture was 60% - 70% while the moisture of fresh mycelium was about 80%. We decided to supplement some water-insoluble carbon sources into fresh mycelium, which could not only make up for the lack of carbon source but also decrease the moisture. Cellulolytic materials are abundantly available globally and can be used by a number of microorganisms, such as *Streptomyces* species [27] [28]. Agricultural byproducts are mainly utilized as substrates in SSF because its advantages of low production costs and environmental friendliness. For example, Zeng X *et al.* obtained natamycin with wheat bran, rapeseed cake, rice hull by *Streptomyces* gilvosporeus Z28 through solid-state fermentation, led to a 50.05% cost reduction of raw materials, less energy consumption and waste water discharge [29]. Solid substrates such as wheat bran, molasses bran, rice bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs and crushed wheat were studied for Amylase production [30].

As shown in **Figure 2**, Wheat bran, Molasses meal, Corn flour and Straw were chosen to be as carbon sources. The additive amount of carbon sources was 20 g per 10 g dry mycelium, with the moisture 68%. Among the four kinds of carbon sources, wheat bran did well in chitosanase accumulation, the yield of chitosanase reached to 30.05 U/g. In this study, wheat bran molasses meal was chosen as a supplementary carbon source for high production.

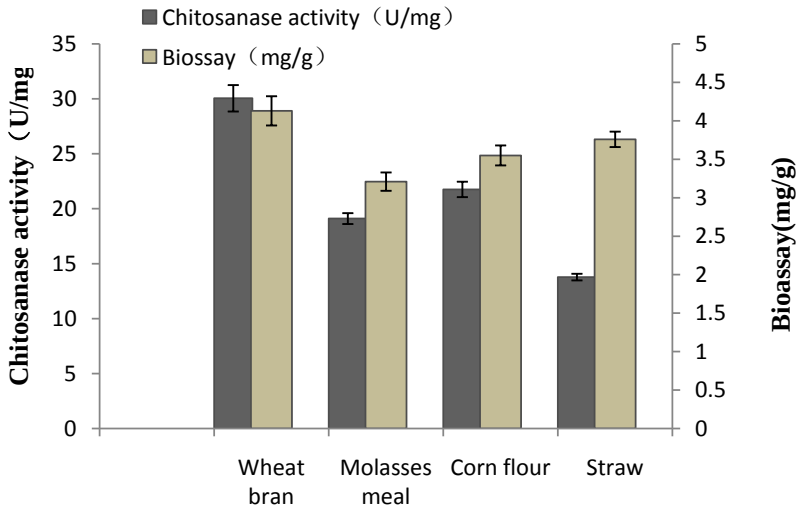

Figure 2. Effects of different carbon sources on chitosanase production by solid-state fermentation.

3.3. Optimizing Solid-State Fermentation Conditions for Chitosanase Production

In order to obtain the best fermentation parameters after selecting the optimal carbon source, also it was investigated the effects of initial inoculums, temperature, and initial pH on the yield of chitosanase. Initial inoculums size can influence the growth of bacteria and the yield of metabolites accumulation. A suitable initial inoculums size can help us get more production of chitosanase. If the level of initial inoculums size is too low, the growth of the bacteria will be in the period of lag phase for a long time. It is helpless for gaining the metabolites. On the other hand, a high level of initial inoculums size will cause the mass growth of the bacteria. It will reduce dissolved oxygen, mycelium is easy to fracture and the metabolites will be decreased. In our study (Figure 3(a)), the best yield of chitosanase was 35.47 U/g while the inoculum size was 10% (v/w).

After investigating the effect of initial inoculums size, the relationship between the temperature and the yield of chitosanase was studied. As shown in Figure 3(b), the best fermentation temperature was 28˚C - 30˚C and the yield of chitosanase increased up to 36.85 U/g.

In addition, fermentation pH is also an important factor affecting the growth

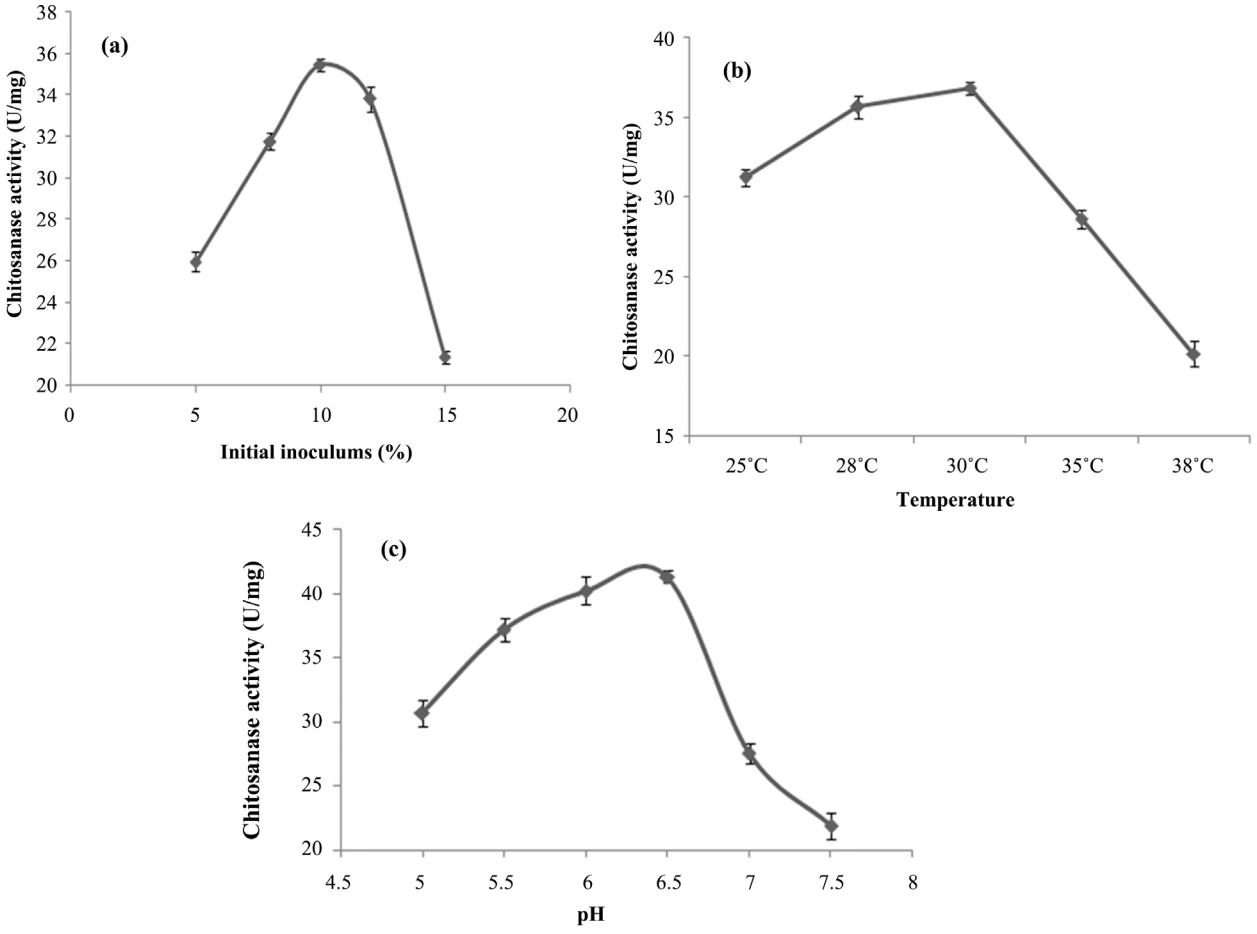

Figure 3. Optimization of SSF conditions. (a) Inoculum size, (b) Fermentation temperature, (c) Initial pH.

of bacteria and the synthesis of products. The effect of initial pH on chitosanase production was also investigated during the solid-state fermentation process. As shown in **Figure 3(c)**, the slightly acidic environment was favorable for the accumulation of chitosanase. The most suitable pH was 6.5 and the yield of chitosanase reached 41.33 U/g.

Chitosanase was detected on the second day of incubation and had maximal activity at 5 days and decreased gradually within a 1 month period. It was a secondary metabolite synthesized and secreted in the late lag phase or in the stationary phase. In submerged fermentation, Activity sharply decreased after prolonged incubation due to cell autolysis. Solid-state fermentation gave a product that was more stable than that from submerged culture, and also required less energy input [31]. It could be stored temporarily without significant loss of activity. It is concluded that solid-state fermentation may be an economic alternative in the production.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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