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Optimization of Culture Conditions and Continuous Production of Chitosan by the Fungi, *Absidia coerulea*

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Abstract The production of chitosan from the mycelia of *Absidia coerulea* was studied to improve cell growth and chitosan productivity. Culture conditions were optimized in batch cultivation (pH 4.5, agitator speed of 250 rpm, and aeration rate of 2 vvm) and the maximum chitosan concentration achieved was 2.3 g/L under optimized conditions. Continuous culture was carried out successfully by the formation of new growth spots under optimized conditions, with a chitosan productivity of 0.052 g $L^{-1}h^{-1}$, which is the highest value to date, and was obtained at a dilution rate of 0.05 h^{-1}. Cell chitosan concentrations reached about 14% in the steady state, which is similar to that achieved in batch culture. This study shows that for the continuous culture of *Absidia coerulea* it is vital to control the medium composition.

Keywords: chitosan production, *Absidia coerulea*, continuous culture, optimum conditions, fungal morphology

INTRODUCTION

Chitosan is a novel biopolymer with numerous applications, for example, as a viscosity control agent, an adhesive, and a paper-strengthening agent in the food and biomedical industries, and is commercially produced from shellfish wastes by extraction and deacetylation processes [1,2]. However, this method has several drawbacks, that have limited its potential industrial acceptance, because supplies of raw materials are variable and seasonal, and the production costs are high. Recently, the production of chitosan using fungi has received much attention due to the need for an alternative source of chitin to solve these problems. It is well known from earlier studies that certain fungi contain chitosan as a cell wall component [3]. Using microorganisms the control of physicochemical properties is possible, which is very important for medical applications. Recently, a number of studies were carried out to identify fungi with significant amounts of chitin in their cell walls, and upon the extraction of chitosan from these cells [4-8]. Despite the need for the development of a process for the economical production of chitosan from fungi, little has been done due to experimental difficulties [9,10].

In this study, the characteristics of fermentation and optimum culture conditions were investigated to improve cell growth and chitosan productivity through batch and continuous cultures of *Absidia coerulea*, which is reported to contain the highest chitosan levels.

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MATERIALS AND METHODS

Microorganism

The fungi, *Absidia coerulea* ATCC 14076, used in this study was obtained from the American Type Culture Collection, USA. The strain was maintained on agar slants containing YM medium (Difco, USA) at 4°C.

Culture Medium

The medium was optimized in flask cultivation (unpublished data) and consisted of five components: A. 20 g/L glucose, B. 15 g/L yeast extract, 1 g/L NaCl, C. 5 g/L $(NH_4)_2SO_4$, D. 1 g/L K_2HPO_4, E. 5 g/L $MgSO_4 \cdot 7H_2O$, F. 0.1 g/L $CaCl_2$. The medium constituents were sterilized separately to prevent precipitation and caramelization, by autoclaving at 121°C for 15 min, and were mixed thoroughly before inoculation. The chemicals used in this study were of reagent grade.

Cultivation

Batch cultures were carried out with a working volume of 2 L in a 2.5-L fermentor (Korea Fermentor Co., Korea). The stirrer shaft was fitted with two six-blade turbine impellers. Each batch fermentation was inoculated with solutions of 2.4×10^8 spores. pH was controlled using 2 N HCl and 2 N NaOH, and an antifoamer (silicone oil, Sigma Co.) was added to control

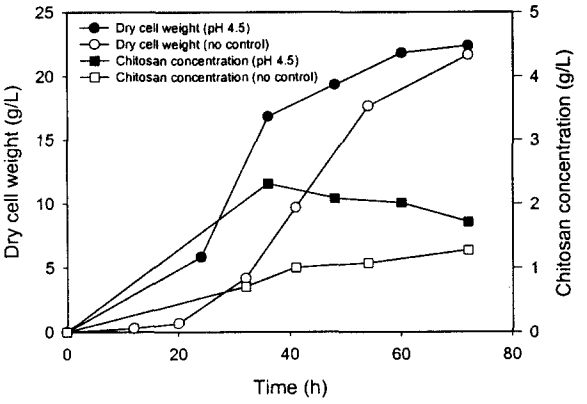

Fig. 1. Effect of pH on cell growth and chitosan production.

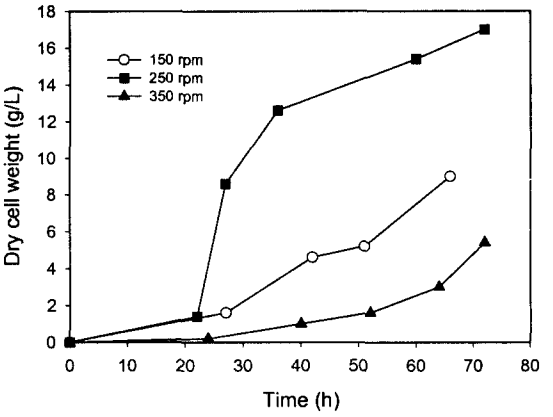

Fig. 2. Effect of agitator speed on cell growth.

the form produced during the fermentation. 50-mL samples were withdrawn at designated times during the fermentation.

For chemostat culture, the fermentor was operated batchwise untill a moderate cell concentration was obtained and then operated continuously at a dilution rate of 0.05 h^{-1}. The liquid level in the fermentor was controlled at a fixed value so that the inlet flow rate was exactly equal to the outlet flow rate. Medium feeding rate and outlet flow rate were adjusted by a peristaltic pump (Cole Parmer Co., USA).

Assays

Glucose was measured by an enzymatic (GOD/POD-glucose oxidase/peroxidase) method (Glucose-E kit, Yeoungdong Pharm. Inc., Korea) at 505 nm using a spectrophotometer (Spectronics 21, Milton Roy Co.). Spore number was counted using a hemocytometer (American Opticals Inc., USA) and cell dry weight was determined after filtering the cell suspension using a vacuum pump, through Toyo No. 5A filter paper, washing the cells in distilled water, and drying with a vacuum dryer. Chitosan was extracted according to the method described by Rane and Hoover [11].

RESULTS AND DISCUSSION

Effect of pH

Culture conditions for batch fermentation were determined using the medium optimized in flask culture, which contained 20 g/L glucose, 15 g/L yeast extract, and 5 g/L $(NH_4)_2SO_4$ (Figs. 1-3). Fig. 1 shows the effect of pH on cell growth and chitosan production by *Absidia coerulea* ATCC 14076. pH decreased from 6.3 to 4.8 during cell growth under these conditions, which were without pH control. The initial rate of cell growth and the maximum growth rate were improved by controlling the pH at 4.5. However, the growth rate at pH 4.5 drastically decreased after 50 h and the dry cell weight after 72 h was 20 g/L, which was similar to that without pH control and was due to substrate depletion. pH affected fungal morphology and smaller pellets were formed at pH 4.5 as compared to those formed without pH control. Reducing autolysis improved the growth rate.

With pH controlled at 4.5 the maximum chitosan concentration was 2.3 g/L and the percentage of chitosan in the cell reached 13.7 on dry weight basis after 36 hours of cultivation. Whereas without pH control, the chitosan concentration only reached 1.3 g/L after 72 hours of cultivation. When the cells were cultivated for 72 h at a controlled pH of 4.5, the chitosan concentration and yield decreased, though there was an increase in the cell concentration. From these results it is evident that the pH and cell harvest time must be controlled to maximize the chitosan concentration.

Effect of Agitator Speed

Different strains have their own specific requirements to develop a morphology capable of a high growth rate. The determination of strain morphology is intrinsic but can be highly influenced by shear forces in the fermentor [9,12]. The effect of agitator speed on growth and morphology was studied in batch cultivation (Fig. 2, Table 1). Sterile air was added at a rate of 1 vvm to provide adequate air for cell growth. The spores of *Absidia coerulea* germinated at about 18 h, and cells then grew at an exponential rate, which later declined during the course of cultivation. At an agitator speed of 250 rpm, ca. 17 g/L (dry cell weight) was obtained at 72 h and growth was maximal. Whereas the growth rate was very low and a cell concentration was only ca. 5 g/L at 350 rpm (Fig. 2). Optimum agitator speed for cell growth was 250 rpm.

A lower agitator speed of 150 rpm caused the formation of smooth hollow pellets of 2-3 mm diameter due to autolysis caused by of oxygen and mass transfer difficulties into the pellet (Table 1) [13]. More compact pellets of size 0.3-0.5 mm were formed at 250 rpm. However, pellets were not formed and cell morphology

Table 1. Effect of agitator speed on cell morphology

Agitator Speed (rpm)	Morphology
150	Pellet Type: Smooth and Hollow Diameter: 2-3 mm
250	Pellet Type: Compact Diameter: 0.3-0.5 mm
350	Granular

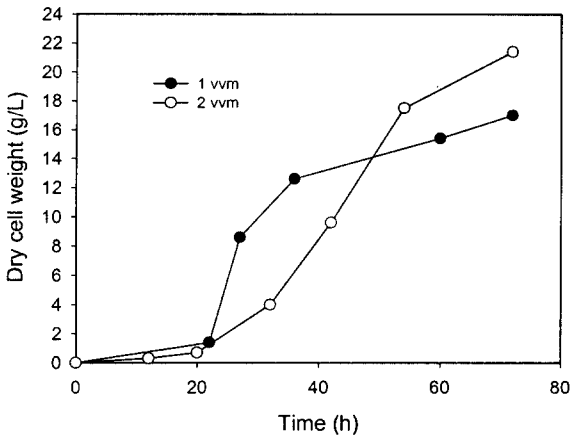

Fig. 3. Effect of aeration on cell growth.

was granular at speeds higher than 350 rpm due to the higher shear rates. From these results, it is vital to control agitator speed to form compact pellets and obtain high cell concentrations.

Effect of Aeration

Autolysis also took place in the mycelial pellets due to difficulties in oxygen transfer, and therefore, the effect of aeration on cell growth was studied (Fig. 3). The fermentation was carried out at 250 rpm, which gave good cell growths. Initial growth rate was slow up to 20 h of cultivation for both 1 vvm and 2 vvm. Due to a high rate of shear, pellet formation was initially slow when the aeration rate was 2 vvm. However, at this aeration rate, the growth rate increased after 20 h and the final cell concentration reached 10% more than that obtained at 1 vvm. Aeration at 2 vvm could also have prevented cell agglomeration and adhesion of mycelia to baffles, impellers, and the fermentor wall.

The optimum conditions for cell growth and chitosan production in the batch cultivation of *Absidia coerulea* ATCC 14076 were pH 4.5, agitator speed of 250 rpm, and an aeration rate of 2 vvm. The maximum specific growth rate obtained was 0.08 h^{-1} under these optimum conditions. Maximum chitosan concentration and chitosan yield based on glucose were 2.3 g/L and 11.5% respectively at a cell harvest time of 36 h. This chitosan concentration is about 5 times of that obtained by Rane and Hoover [10] in batch culture using the same strain.

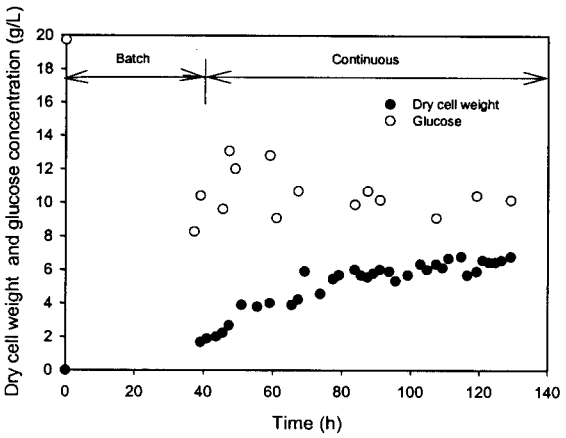

Fig. 4. Continuous culture of *Abisidia coerulea* at a dilution rate of 0.05 h^{-1} (pH 4.5, agitator speed of 250 rpm, and aeration rate of 2 vvm).

Continuous Culture

Continuous fermentation was carried out to obtain high productivity and produce chitosan with unique properties. However, Davoust and Hansson [9] reported that continuous cultivation was difficult to establish due to the lack of new growth spots and accreted growth. To prevent washout and the formation of new pellets in the continuous culture, the spores need to be released from pellets or the mycelia may rupture. However, *Absidia coerulea*, which belongs to *Zygomycetes*, could not form septa and the rupture of mycelia was not easy [13]. The pellet structure is very important for continuous culture.

Generally, the medium composition does influence the pellet structure [12]. Table 2 shows two different media and culture conditions adopted in continuous culture that have been reported to date. We investigated the effect of medium composition on cell morphology (data not shown). High ammonium sulfate concentration and/or chelating agent like Fe-EDTA in the medium gave smaller and more compact pellets, as reported by Metz and Kossen [12], and probably made the release of spores impossible.

We examined the possibility of continuous cultivation using optimized culture conditions (pH 4.5, agitator speed of 250 rpm, and aeration rate of 2 vvm) and an optimized medium which probably created new growth spots (Fig. 4). Continuous culture with a dilution rate of 0.05 h^{-1} was started with an initial cell concentration of ca. 1 g/L; the cell concentration then increased until it reached steady state. At the steady state, the concentrations of cell and residual glucose were about 7 g/L and 10 g/L respectively. Chitosan productivity obtained in this study was 0.052 g L^{-1} h^{-1}, which is higher than that reported by Rane and Hoover [10]. The cell chitosan level was about 14% at the steady state, which was similar to that obtained in batch culture.

Table 2. Comparison of the results of two previous trials of continuous culture with the present work

	Davoust and Hansson [9]	Rane and Hoover [10]	This work
Strain	*Absidia coerulea*	*Absidia coerulea*	*Absidia coerulea*
Carbon Source	Glucose 20 g/L	Glucose 20 g/L	Glucose 20 g/L
Nitrogen Source	Yeast extract 6 g/L $(NH_4)_2SO_4$ 11 g/L	Yeast extract 1 g/L Peptone 10 g/L $(NH_4)_2SO_4$ 5 g/L	Yeast extract 15 g/L $(NH_4)_2SO_4$ 5 g/L
Salts	KH_2PO_4 3 g/L $MgSO_4$ 0.6 g/L $ZnSO_4 \cdot 7H_2O$ 1.8 mg/L $MnSO_4 \cdot H_2O$ 0.3 mg/L $CuSO_4 \cdot 5H_2O$ 0.4 mg/L	K_2HPO_4 1 g/L NaCl 1 g/L $MgSO_4 \cdot 7H_2O$ 5 g/L $CaCl_2$ 0.1 g/L	K_2HPO_4 1 g/L NaCl 1 g/L $MgSO_4 \cdot 7H_2O$ 5 g/L $CaCl_2$ 0.1 g/L
Additive	Fe-EDTA 1.3 g/L	-	-
Dilution Rate (h^{-1})	0.03-0.15	0.025	0.05
Agitator Speed (rpm)	300	150	250
Result	Failure	Success	Success
Chitosan Concentration (g/L)	-	1.36	1.04
Percentage of Chitosan in the Cell (%)	-	10.9	14.0
Chitosan Productivity ($g\ L^{-1}\ h^{-1}$)	-	0.034	0.052

Granule-like spores and mycelia were observed in the broth during continuous culture. This broth could be used as the seed, assured it formed pellets and its growth was like that obtained using a spore suspension. Our results emphasize that it is very important to control the medium composition for the continuous culture of *Absidia coerulea*, to enable the formation of new growth spots.

CONCLUSION

Cell growth and chitosan production are highly influenced by culture conditions such as pH, agitator speed, and aeration rate. Optimum conditions obtained for the batch cultivation of *Absidia coerulea* were a pH 4.5, an agitator speed of 250 rpm, and an aeration rate of 2 vvm. The maximum specific growth rate and chitosan concentration obtained under these conditions were 0.08 h^{-1} and 2.3 g/L, respectively.

Continuous culture was undertaken to increase productivity and produce chitosan with unique properties. The pellet structure was found to be very important for the formation of new spots in continuous culture and this was influenced by medium composition and culture conditions. Chitosan productivity obtained in this study was 0.052 $g\ L^{-1}\ h^{-1}$. Because few difficulties were encountered during the operation, continuous culture with *Absidia coerulea* ATCC 14076 was carried out successfully and proved to be effective at producing chitosan with high productivity.

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