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Effect of pellet size and stimulating factor on the glucosamine production using *Aspergillus* sp. BCRC 31742

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ABSTRACT

The higher GlcN production using a wild-type fungi, *Aspergillus* sp. BCRC 31742 cultivated under submerged fermentation was investigated. Several fermentation aspects were studied, such as pellet size, working volume, agitation rate and stimulating factor. Culture cultivation with conditions, such as pellet diameter of 2.15 mm, 50 mL working volume (250 mL T-flask), incubation at 30 °C, 200 rpm and pH 7.0 for 5 days yielded highest biomass concentration which was 33.82 g/L, with a GlcN concentration of 7.05 g/L. Methanol was found to give the best stimulatory effect in terms of GlcN concentration as compared to glutamic acid, cycloheximide and ethanol. Addition of methanol (1.5% v/v) into fermentation medium could increase GlcN content from 0.21 (control) to 0.26 g/gdw cells and led to maximum GlcN concentration of 7.48 g/L obtained.

1. Introduction

Filamentous fungal fermentation is widely used to produce useful products commercially, such as organic acids, enzymes, antibiotics, cholesterol lowering statins, and other fine chemicals. There are three extreme morphologies of filamentous fungi reported, such as suspended mycelia, clump and pellet morphology (Metz and Kossen, 1977). Specifically, when grown in submerged fermentation, fungi will exhibit different morphological forms, starting from dispersed mycelia filaments to densely interwoven mycelia masses or spherical masses of hyphae referred to pellet form (Papagianni, 2004). Focusing on pellet morphology, long transport path in big pellet can cause a decrease in substrate consumption rate and will lead to a lower biomass concentration as compared to small pellet morphology (Siedenberg et al., 1998). Hence, the influence of suitable pellet size on nutrition consumption rate is expected in order to give either maximum product formation rate or biomass concentration.

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) and its acetylated form, *N*-acetylglucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose) are building units of biopolymer chitin and chitosan. Generally, these compounds are synthesized almost in all organisms, including bacteria, yeast, fungi, plants and animals including those from marine sources (Deng et al., 2005; Subramanyam and Rao, 1987). GlcN and GlcNAc have been produced as supplements in their salt forms because they have been proved to have positive clinical benefits for human in a variety of conditions, such as providing a good regeneration for human joints cartilage as GlcN or GlcNAc acts as precursors for cartilage synthesis and body fluids of vitreous humour of the eye (Anderson et al., 2005; Reginster and Deroisy, 2001).

More than several decades, most of GlcN preparations were derived from the exoskeletons of shellfish or other marine resources. GlcN was usually produced using acid hydrolysis of chitosan or chitin (a linear polymer of GlcNAc) extracted from crab and shrimp shells. Concentrated hydrochloric acid would degrade and deacetylate either chitinous and chitosanous materials to form GlcNAc and GlcN under specific reaction conditions (Abdou et al., 2008; Mojarrad et al., 2007; Trung et al., 2006). The GlcN production from these sources may be limited as the demand of raw material continues to grow. Moreover, GlcN from shellfish may not be suitable for people or consumers with shellfish allergies.

As mentioned above, both chitin and chitosan can be found in fungi as building blocks of fungal cell walls. Therefore, recently more and more studies have been carrying out to investigate GlcN production using fungal fermentation regarding to those limitations of marine sources derived GlcN mentioned above. Several studies have been reported on GlcN production using fungal fermentations (Carter et al., 2004; Hsieh et al., 2007; Sparringa and Owens, 1999; Yu et al., 2005). Latter, Chang (2008) employed statistical method of response surface methodology for optimizing

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Table 1
Fermentation profiles as affected by different pellet sizes.

No.	Medium	Conc. (g/L)	Diameter (mm)	Circularity max = 1.0	Biomass (g/L)	GlcN conc (g/L)	Content (g GlcN/gdw cells)	Productivity (mg/L h)
1	PDB (control)	20	2.45	0.83	19.45 ± 1.28	3.70 ± 0.18	0.19 ± 0.008	30.85 ± 1.03
2	PDB $CaCO_3$	20 1.5	4.35	0.85	19.61 ± 0.89	3.61 ± 0.17	0.18 ± 0.006	30.10 ± 1.86
3	PDB $CaCO_3$ Peptone	20 1.5 8	2.15	0.82	20.50 + 0.09	4.40 ± 0.19	0.22 ± 0.006	38.58 ± 1.28
4	WF CaCO Peptone	25 1.5 8	1.40	0.85	16.93 ± 0.61	3.07 ± 0.10	0.18 ± 0.003	25.61 ± 0.59

Note: The Biomass, GlcN concentration, content and productivity values were obtained in triplicate with mean standard deviations. Diameter size and circularity value were evaluated using software of ImageJ 1.42 bundled with 32-bit Java 1.6.0-14.

GlcN fermentation medium and resulted in higher GlcN concentration that could be up to 5.48 g/L as compared to previous study which used same strain of *Aspergillus* sp. BCRC 31742 (Hsieh et al., 2007).

According to Sparringa and Owens (1999), best pellet diameter range for producing GlcN was 16–35 mm, which its GlcN content could be up to 107 ± 4.3 g/kg dry biomass, cultivated in sabouraud dextrose broth for 72 h using *Rhizopus oligosporus* NRRL 2710. Furthermore, typical pellet size of fungi was used for co-production of fumaric acid and chitin using *Rhizopus oryzae* ATCC 20344 (Liao et al., 2008). Pellet size was found to affect GlcN content of *R. oryzae*. A maximum GlcN content that could be obtained was 0.19 g/gdw cells with pellet diameter of 5.0 mm. Reduction in pellet size from 5.0 mm to 1.2 mm would gradually decrease GlcN content to 0.15 g/gdw cells.

Considering all these conditions, pellet size may have a significant effect on growth of fungi in terms of GlcN production regarding to nutrient transport and oxygen uptake rate limitations. Therefore, this study aims to enhance GlcN production by employing different pellet sizes as inoculum to run fermentation in a flask system. Thus, addition of stimulating compounds, e.g., glutamic acid, cycloheximide, methanol, and ethanol are employed to increase GlcN production in the fermentation processes.

2. Methods

2.1. Microbial fermentation

Aspergillus sp. BCRC 31742 was purchased from Bioresource Collection and Research Center (BCRC) in Taiwan. The strain was cultured on potato dextrose agar (PDA, Difco) slants and incubated at 30 °C for 7 days. Thus, the spores were collected and suspended with sterile water, and transferred quantitatively to a potato dextrose broth (PDB, 24 g/L) and incubated at 30 °C, and 200 rpm for 7 days. The spores with mycelia, clumps and pellets isolated from broth were preserved by putting 0.5 mL culture suspension (mycelia, clumps and pellets) and 0.5 mL glycerol into an eppendorf and stored at −80 °C. Inoculum was prepared by activating preserved fungi using different pretreatment media (Table 1). The activation was carried out at 30 °C, 200 rpm, and pH 7.0 for 5 days. The fermentation was started by putting 0.23 ± 0.001 gdw seed/L of inoculum into a glucose peptone medium contained superior white fine granulated sugar (Taiwan Sugar Corporation, Taiwan; 33.9 g/L), mycological peptone (DIFCO, USA; 40.6 g/L), KH_2PO_4 (R.D.H, Germany; 0.5 g/L), $MgSO_4{\cdot}7H_2O$ (R.D.H, Germany; 0.5 g/L), and $CaCl_2{\cdot}2H_2O$ (Yakuri, Japan; 0.1 g/L). For studying effects of pellet size, fermentation conditions were similar to our previous studies (Hsieh et al., 2007; Chang, 2008), using shake flask (250 mL, working volume of 150 mL), incubated at 30 °C, shaken at 200 rpm (orbital shaker), pH 7.0 for 5 days, whereas for further studies, various working volumes and agitation rates would be employed.

2.2. Determination of GlcN

GlcN determination was adopted from our previous studies (Hsieh et al., 2007; Sitanggang et al., 2009), wherein hydrochlorination process used conventional thermal method. There were two main steps employed for sample preparation: (1) hydrochlorination process and (2) derivatization process. Biomass were separated from fermentation broth using a vacuum filter and then washed several times with distilled water. Hydrochlorination process was carried out with 6 N HCl at temperature of 100 °C for 4 h. Thus, 10 mL distilled water was added into solution to stop the reaction and then cooled to 30 °C. The pH of solution was adjusted to 7.0 using 12 N NaOH. Finally, the solution then filtered using 45 μm sieve. For derivatization procedure, a 0.1 mL sample solution and 0.3 mL of 40 mol/m^3 of 1-napthyl isothiocyanate in pyridine solution were mixed together using a constant bath shaker at 100 rpm, and 50 °C for 1 h. The derivatized solution then filtered using 45 μm sieve. The internal standard of HPLC (0.1 mL, 0.1 wt% of 3,5-dinitrobenzoacetonitrile in acetonitrile) was added into the filtered-derivatized solution and then mixed well. Finally, 10 μL sample was injected into HPLC to measure GlcN concentration.

The analytical HPLC column was a LiChrospher® 100 RP-18 endcapped (5 μm, 4 mm i.d. × 250 mm) column. This reversed phase column is shipped in acetonitrile–water. The detector was UV–Vis detector (Simadzu SPD-10 A, 0.0100 AUFS, Japan). Pressure was maintained at 130–150 kgf. The mobile phase was a mixture solution of HPLC-grade water and acetonitrile (99.8% in purity) which ratio was 87:13. The temperature of column was maintained at 40 °C with a mobile phase flow rate of 1.3 mL/min. Detection was performed at a wavelength (λ) of 230 nm with an analytical time was 40 min.

2.3. Data and morphological analysis

Fermentation profiles were evaluated specifically in terms of biomass (g/L) and GlcN concentration (g/L), content (g GlcN/gdw cells) and productivity (mg/L h). The software of ImageJ 1.42 bundled with 32-bit Java 1.6.0-14 and microscope Olympus IX71 (DP 70 for lighting, U-TVO, 5XC-2, Japan) were utilized for monitoring fungal morphology, diameter size and circularity. For statistical analysis, SPSS 11.5 for windows was utilized. Level of significancy was performed at 5% ($P < 0.05$) using Tukey's post hoc test. Both biomass and GlcN concentration were included as dependent variables.