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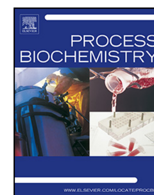

Production of β-glucosidase from wheat bran and glycerol by *Aspergillus niger* in stirred tank and rotating fibrous bed bioreactors

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ABSTRACT

Aspergillus niger NRRL 3112 can produce considerable amounts of β-1,4-glucosidase (BGL) when grown on wheat bran and glycerol as co-substrates. BGL production was first investigated in a stirred-tank bioreactor (STR) at 450 rpm and 2 vvm. About 5.4 U/mL BGL was obtained using spore suspension as inoculum, whereas a higher production of 9.3 U/mL was obtained using precultured cell pellets. The production of BGL in batch, fed-batch and repeated batch modes in a rotating fibrous bed bioreactor (RFBB) was then studied and compared to the STR. The highest BGL productivity of 1.78 U/mL/day was obtained in the RFBB operated at a repeated batch mode, which was slightly higher than that (1.65 U/mL/day) obtained in the STR with preformed cell pellets and about 1.75-fold of that (1.02 U/mL/day) for the free-cell fermentation in STR inoculated with spores. This work demonstrated that the RFBB could provide an efficient process for β-glucosidase production from low-cost wheat bran and glycerol.

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1. Introduction

The abundant and renewable lignocellulosic biomass is promising feedstock for sustainable production of biofuels and chemicals [1,2]. However, lignocellulosic materials usually must be first converted to fermentable sugars, mainly glucose, by enzymatic hydrolysis involving synergistic actions of three different cellulases: *endo*-β-1,4-glucanase (endoglucanase, EG), cellobiohydrolase (CBH), and β-1,4-glucosidase (BGL) or cellobiase [3,4]. EG randomly cut the β-1,4-glucosidic linkages of cellulose to expose the polysaccharide chain ends, which CBH acts upon to release cellobiose and cellodextrins (cellotriose, cellotetraose, etc.). BGL then hydrolyzes cellobiose and cellodextrins to produce glucose. BGL increases the efficiency of cellulose hydrolysis by reducing cellobiose inhibition of CBH, which hinders the complete hydrolysis of cellulose [5–8]. Commercial cellulases produced by *Trichoderma reesei* and other filamentous fungi are highly potent in EG and CBH, but low in BGL activity [9]. New enzyme cocktails developed for cellulose hydrolysis are thus often supplemented with BGL produced by, for example, *Aspergillus niger* [9]. BGL also has many other industrial applications [10], including uses in the hydrolysis of glucosides of isoflavones for aglycones production in soy [11,12] and for hydroxytyrosol release from olive byproduct [13].

There have been extensive efforts to find and develop suitable microorganisms for BGL, and cellulases in general, production through strain screening and engineering [3,9,10,14–16]. For economical production of enzymes, it is important to develop an efficient fermentation process with high product yields and desirable activities from low-cost feedstocks [17–22]. Various fungal fermentation processes, including solid state fermentation and submerged fermentation in stirred tanks and air-lift bioreactors have been studied for cellulases production [9,23–26]. Current large-scale enzyme production is primarily carried out in conventional stirred-tank reactors (STR), which have high shear stress causing mycelial rupture and enzyme deactivation [27]. In addition, it is difficult to control the morphology of filamentous fungi in STR [28], and undesirable fungal morphology could lead to poor mass transfer and product secretion during fermentation [28–30]. Cell immobilization on or in solid supports, including polyurethane foam, alginate beads, loofah sponge, polyester fabrics, and cotton towel, has been widely studied and effectively used for fungal morphology control in submerged fermentation [31–35]. In particular, immobilization of fungal spores and mycelia in a rotating fibrous bed bioreactor (RFBB) has been shown to have many advantages in culturing filamentous fungi for chemicals and enzymes production, including increased product titers, productivities, and operational stability [34–37].

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The objective of this research was to develop an economical process for BGL production from low-cost substrates. Our previous screening and medium optimization studies in batch culture experiments have shown that *A. niger* NRRL 3122 was capable of producing large amounts of BGL with wheat bran and glycerol as substrates [38]. Wheat bran was a superior substrate and inducer for BGL production, while glycerol was used as a low-cost co-substrate for its availability from the biodiesel industry. In the present work, we further studied and compared BGL production by *A. niger* in STR and RFBB operated at fed-batch and repeated batch modes. Our results showed that stable BGL production by *A. niger* at high titer and productivity could be achieved from wheat bran and glycerol in the RFBB, offering a potentially more efficient process for BGL production.

2. Materials and methods

2.1. Cultures and media

A. niger NRRL 3122, obtained from Agricultural Research Service (ARS) Culture Collection (Peoria, Illinois USA), was used in this study. The stock culture was maintained on potato-dextrose-agar (PDA) slants at 4 °C and sub-cultured every 4 weeks. Spores were harvested by scraping from PDA slants (7 days incubated at 30 °C) with 10 mL distilled water to give a spore suspension of approximately 2×10^7 spores/mL. Unless otherwise noted, the fermentation medium contained (g/L): 5 glycerol (analytical grade), 3.5 wheat bran, 7.5 corn steep liquor (CSL, 50% dry matters), 1 $NaNO_3$, 0.3 K_2HPO_4, 0.1 KCl, $MgSO_4·7H_2O$, and 0.01 $FeSO_4·7H_2O$. This medium composition was chosen based on our previous medium optimization study [38]. Wheat bran, obtained from a local supermarket and used un-altered, contained (wt%) ~15 starch, ~50 fiber (cellulose and hemicellulose), ~16 protein, and some vitamins and minerals. CSL containing (wt%) crude protein and amino acids (25–45), lactic acid (10–25), reducing sugars (2.5), and ash (17) was obtained from Cargill (Eddyville, IA). All media were adjusted to pH 5.6 with 1 N NaOH and sterilized by autoclaving at 121 °C, 15 psig for 30 min.

2.2. Preparation of cell pellets

Cell pellets for seeding submerged fermentation in stirred tanks were prepared by inoculating spores (3.6×10^8) in a shake-flask containing 150 mL of the preculture medium, which contained only glucose (10 g/L) as the carbon source (no glycerol and wheat bran) and all other medium components as the fermentation medium. After inoculation, the flask was incubated at 30 °C on a rotatory shaker at 200 rpm for 48 h.

2.3. Fermentation kinetics studies in stirred-tank bioreactor

Batch fermentation kinetics was first studied in a 3-L stirred-tank reactor (STR) containing 1.5 L of the fermentation medium. After autoclaving at 121 °C for 30 min, the bioreactor was inoculated with 0.9 mL of spore suspension (4×10^8) or 150 mL of preformed cell pellets and operated at 30 °C with aeration at 1–2 vvm and agitation at 450 rpm for 10 days, unless otherwise noted. The medium pH was not controlled during the fermentation. Silicone antifoam 204 (Sigma-Aldrich) was added as needed to control foaming. For fed-batch fermentation, the bioreactor initially contained 1 L of the medium and was pulse-fed with 500 mL of the medium containing 1.5 g/L wheat bran and 7.5 g/L glycerol at 120 h. For the repeated-batch culture, 66% of the fermentation broth was removed and replaced with the same volume of fresh medium containing 3.5 g/L wheat bran and 7.5 g/L glycerol at the end (~240 h) of each batch. Unless otherwise noted, samples were taken once per day, centrifuged at 13000 rpm for 10 min, and stored at −20 °C for further analysis.

2.4. Fermentation kinetics studies in rotating fibrous bed bioreactor

The rotating fibrous bed bioreactor (RFBB) was made of a 3-L stirred-tank bioreactor with a perforated stainless steel cylinder affixed with polypropylene cloth for cell immobilization mounted on the impeller shaft in the bioreactor (Fig. 1). The bioreactor with 1.5 L of the fermentation medium was autoclaved at 121 °C for 30 min. After cooling, the bioreactor was inoculated with 17 mL of spore suspension (2×10^7/mL) and then operated at 30 °C with aeration at 2 vvm and agitation initially at 90 rpm and then increased to 150 rpm when wheat bran and mycelia had been adsorbed on the polypropylene cloth. The medium pH was not controlled during the fermentation. For the fed-batch culture, 500 mL of the medium containing 1.5 g/L wheat bran and 7.5 g/L glycerol was pulse-fed when enzyme production had leveled off at 288 h. For repeated batch culture, 66% of the fermentation broth was removed and replaced with the same volume of fresh medium containing 3.5 g/L wheat bran and 7.5 g/L glycerol at the end of the batch fermentation (~288 h) as indicated by the cessation of enzyme production.

2.5. Analytical methods

The activity of β-glucosidase was assayed by the method described elsewhere [39]. Briefly, 0.5 mL of the substrate (0.4% cellobiose in 0.05 M citrate phosphate buffer, pH 4.8) was added to 0.5 mL of appropriately diluted enzyme solution (fermentation broth filtrate) and incubated at 50 °C for 30 min. The reaction mixture was placed in boiling water for 5 min to stop the reaction and then immediately cooled in an ice bath. Standard β-glucosidase (4 U/mg, Sigma) was used as positive control under the same assay conditions. One unit of β-glucosidase activity is defined as the amount of enzyme producing 1 μmol glucose per min from cellobiose. The glucose concentration in the mixture was determined by using a glucose analyzer (YSI Biochemical Analyzer, Yellow Spring, Ohio) or high performance liquid chromatograph (HPLC) with an Aminex HPX–87H column (BioRad) at 45 °C and 0.005 M H_2SO_4 as the mobile phase at 0.6 mL/min [40]. The total reducing sugar concentration was determined by the DNS method [41]. The concentration of soluble protein present in the fermentation broth was assayed using the commercial protein assay kit (BioRad Laboratories, USA) with bovine serum albumin as the standard. All assays were performed in triplicate.

3. Results and discussion

3.1. Free cell fermentation in stirred-tank bioreactor

Batch fermentation kinetics was first studied in STR. Fig. 2 shows the kinetics for batch fermentation inoculated with either spores or preformed cell pellets. The pH was not controlled, and it decreased from the initial value of ~5.5 to ~2.8 for the one inoculated with spores (Fig. 2A) and more rapidly to ~1.6 with cell pellets (Fig. 2B). The pH then went back up to ~3.2 in both cases. A similar trend was observed with the total reducing sugars, which decreased from the initial value of ~2.5 g/L to ~0.22 g/L at 120 h and then increased slightly to ~0.46 g/L due to the continuous saccharification of wheat bran [34,38]. Meanwhile, protein and β-glucosidase production started after 24 h and increased to reach the maximum values of 0.76 mg/mL and 5.4 U/mL, respectively, for the one inoculated with spores (Fig. 2A) and 1.63 mg/mL and 9.3 U/mL, respectively, with cell pellets (Fig. 2B). The specific BGL enzyme activity was lower

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