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Production of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. TS1-1: Optimization of carbon and nitrogen concentration in the feed medium using central composite design

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Abstract

Optimisation of nutrient feeding was developed to overcome the limitation in batch fermentation and to increase the CGTase production from *Bacillus* sp. TS1-1 in fed batch fermentation. Optimisation of the C/N ratio in the feed stream was conducted in a 5 l fermenter, where feeding was initiated at constant rate of 0.02 h^{-1}. In our initial screening process, the addition of nitrogen source boosted the growth of the microbes, but on the other hand reduced the CGTase production. The amount of tapioca starch and yeast extract was optimised in order to obtain a sufficient growth and thus, increased the CGTase production. Results were analysed using three-dimensional response surface plot, and the optimised values of carbon and nitrogen concentration of 3.30% (w/v) and 0.13% (w/v) were obtained, respectively. CGTase activity increased up to 80.12 U/ml, which is 13.94% higher as compared to batch fermentation (70.32 U/ml). This also led to 14.54% increment of CGTase production in fed batch culture as compared to the production before the optimisation. The CGTase activity obtained was close to the predicted value, which is 78.05 U/ml.

Keywords: Cyclodextrin glucanotransferase; C/N ratio; Fed batch; Central composite design

1. Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an extracellular enzyme, a member of the amylolytic glucosylase family. CGTase differs from its family members, possesses a capability to catalyse multiple reactions [1]. It has both strong hydrolytic and synthetic capabilities as well as having multiple product specificity [2]. The ability of CGTase to convert starch into favoured industrial substance called cyclodextrin through cyclization process is of great interest to researchers [2]. Cyclodextrins are able to form an inclusion complex with various kinds of organic compounds inside the cavity of the ring structure [3].

Most production of the CGTase were carried out as batch processes [4,5]. However, CGTase production in batch processes show many limitations caused by substrates suppression, catabolite repression and limiting of some essential nutrients. Therefore, applying fed batch fermentation was constructive to overcome all the limitations in batch fermentation. Unfortunately, limited information was obtained regarding CGTase production using fed batch culture [6–8]. The main purpose of employing fed batch culture was to remove the repressive effects of rapidly utilized carbon sources, to reduce the viscosity of the medium, to reduce the effect of toxic medium constituents or simply to extend the product formation stage of the process for as long as possible. Besides CGTase, fed batch method had been proven to increase the production of proteases by *Bacillus sphaericus* [9] and production of enzyme β-1,4-endoglucanase from recombinant *Bacillus subtilis* DN18859 (pCH7) [10].

Media optimisation for feed stream using statistical experimental design for production of novel CGTase by fed batch fermentation had not been reported before. On the other hand, the optimising of media formulation in batch fermentation was read-

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ily available. Optimisation of either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites in batch fermentation has been cited by many authors such as Lee and Chen [11], Dey et al. [12], Liu et al. [13], Khairizal et al. [14], Chen et al. [15], Li et al. [16] and Roshanida et al. [17].

Only few reports on the effect of C/N ratio on CGTase production by batch fermentation were available [4]. In the present study, optimisation of C/N ratio in the feed stream was conducted using central composite design in order to enhance the production of CGTase from *Bacillus* sp. TS1-1 by fed batch fermentation.

2. Materials and methods

2.1. Preparation of bacteria inoculum

Bacillus sp. TS1-1 is provided by our own laboratory [14]. The bacteria was isolated from the soil and grown in an optimised medium with the composition of 2% (w/v) sago starch (food grade), 1% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4{\cdot}7H_2O$ [14]. The medium was added with 10% (w/v) Na_2CO_3 stock solution, separately depending on the pH of the medium. *Bacillus* sp. TS1-1 was cultured in 250 ml conical flasks and incubated in an orbital shaker at of 37 °C and 200 rpm for 18 h. The cells were then centrifuged at 5000 rpm for 5 min, washed once with normal saline solution 0.85% (w/v) NaCl, to give an optical density (OD) reading of 0.5 at 600 nm.

2.2. CGTase activity assay

CGTase assay was carried out according to the method of Kaneko et al. [18]. The reaction mixtures containing 40 mg of soluble starch in 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 0.5 ml supernatant was incubated at 60 °C for 10 min. The reaction was stopped by adding 3.5 ml of 30 mM NaOH, followed by 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution, and left for 15 min at room temperature. The colour intensity of the samples was measured at 550 nm. A blank solution (fresh medium) was prepared for each batch of assay. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-CD/min.

2.3. Biomass determination

Two methods were carried out for the determination of biomass concentration, with regards to presence or absence of starch in the culture broth [19]. If the culture contains traces of starch, 0.1 ml enzyme α-amylase (Novo Nordisk) was added to 1 ml of culture to hydrolyse the residual starch to soluble sugars. The mixture was incubated at 100 °C for 20 min and centrifuged at 3000 rpm for 30 min. The cells were filtered onto pre-weighed 0.2 μm cellulose nitrate filter (Whatman), washed twice with distilled water and dried in an oven at 95 °C for 24 h. Fresh medium was treated similarly and used as blank. If starch was totally depleted from the sample: 1 ml of culture was centrifuged at 5000 rpm for 3 min. The cell pellet was washed twice with saline solution and dried in a vacuum dryer, followed by drying in the oven at 80 °C.

2.4. Starch concentration

Determination of starch concentration was carried out according to the method of Kitahata et al. [20] 1.0 ml of supernatant was mixed with 4 ml of 0.01 M iodine in 0.25 M potassium iodide (KI) and diluted with 15 ml of distilled water. The colour intensity was measured at 465 nm against blank of distilled water treated in the same manner.

2.5. Protein content

Protein assay was carried out according to Lowry [21] 0.2 ml of each standard or crude enzyme was mixed with 1.0 ml of Lowry reagent and left at room temperature for 10 min. At the end of the incubation period, 0.1 ml of the diluted reagent Folin–Ciocalteu (1 distilled water:1 folin solution) was immediately mixed to the reaction samples, and left at room temperature for another 30 min. The absorbancy of the sample was measured at 750 nm.

2.6. Calculation of C/N ratio

CN ratio was calculated on molar basis. One gram of starch/l is converted to 1.1 g of glucose/l. The formula used for the conversion of mass concentration of starch to (g/l) to mM concentration of carbon was [starch (g/l) × 36.7 = mM carbon]. The total nitrogen content in the yeast extract used is 11.4%. The conversion of yeast extract mass concentration (g/l) to mM concentration of nitrogen is [yeast extract (g/l) × 11.4 = mM nitrogen].

2.7. Fermentor set-up

All fermentations processes were carried out in a 5 l borosilicate glass fermenter (with working volume of 4 l, Biostat® B, Germany), with an internal concave bottom of height and diameter ratio of about 2:1 (dimension $W \times H \times D = 400$ mm × 685 mm × 330 mm). The fermenter was equipped with a peristaltic pump, temperature, pH and dissolved oxygen (DO) controllers. A polagraphic dissolved oxygen probe (Ingold, Switzerland) was used to measure the DO levels, and a glass pH electrode (Ingold) was used to measure the culture pH. The fermenter was fitted with three six bladed Rushton turbine impellers (diameter, $d = 40$ mm) on the agitator shaft and the impeller speed was fixed at 200 rpm.

The initial cultivation medium that contained (w/v): 2% starch, 1% yeast extract, 0.1% K_2HPO_4 and 0.02% $MgSO_4{\cdot}7H_2O$ was added with 10% of sterilised Na_2CO_3 separately to give a final pH of 10.32. The media was sterilized at 121 °C for 15 min and sterilization of the fermenter was carried out by autoclaving for 20 min. About 10% (v/v) of bacterial inoculums were inoculated into the cultivation medium for both batch and fed batch fermentation. Cultivation was carried out at 29.6 °C for 48 h at 200 rpm, with dissolved oxygen levels (DO) con-