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Bioreactor studies of production of mycophenolic acid by *Penicillium brevicompactum*

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HIGHLIGHTS

- Mycophenolic acid (MPA) have a wider uses, mainly as a immunosuppressant.
- A batch fermentation attained a maximum MPA concentration of 1.26 g/L.
- MPA concentration in a fed-batch fermentation using glucose was 2.72 g/L.
- Replacement of glucose with sorbitol increased MPA production up-to 3.26 g/L.

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ABSTRACT

Batch and fed-batch fermentation processes for the production of mycophenolic acid (MPA) were optimized and compared. MPA was produced using the microfungus *Penicillium brevicompactum*. Different feeding strategies were assessed in fed-batch fermentations in a 14 L stirred bioreactor. A batch fermentation attained a maximum total (extracellular and intracellular) MPA concentration of 1.26 g/L after 288 h. The best fed-batch operation involving a constant-rate feeding of sorbitol (a feeding rate = 0.07 g/min, from 48 h to 240 h) and the pH controlled at 6, enhanced the total maximum MPA concentration to 3.26 g/L. The yield of MPA on substrate was 0.028 g/g, some 47% greater than in the aforementioned batch culture. The fermentation was highly aerobic. The fermentation temperature was 25 °C. Glucose could be used as an effective substrate instead of sorbitol, but the final total MPA concentration in a fed-batch fermentation using glucose was reduced to 2.72 g/L.

1. Introduction

Mycophenolic acid (MPA) is a secondary metaboliteproduced by many *Penicillium* species [1–7] and other fungi [8–10]. MPA and its derivatives have a wider uses, mainly as a immunosuppressant [11–13]. MPA is also useful in treating various autoimmune, cancer, fungal and viral diseases [14–20]. MPA is an anti-proliferative agent which suppressed the synthesis of lymphocytes in *de novo* pathway by inhibiting the enzyme inosine monophosphate dehydrogenase (IMPDH). Lymphocytes completely depend on IMPDH for synthesis of nucleotides; while other human cells use other pathways for this synthesis, and are less affected by the anti-proliferative effect of MPA. Due to this reason MPA is highly selective and have fewer side effects as compared to other immunosuppressants drug [21,22].

Although extensive work has been reported on development of MPA overproducing fungal strains through mutation and selection [7,23–26], the development of the fermentation process has received barely any attention [6,27–32]. Immobilized cell fungal fermentation in a rotating fibrous-bed bioreactor has provided a MPA concentration as high as 5.7 g/L in about 14 days [27], but such bioreactors are impractical for large scale industrial use. Metabolites of obligate aerobic microfungi are almost invariably produced using well-mixed aerated stirred tank bioreactors that have an established history of successful use in industrial fermentations. Here we report on batch and fed-batch production of MPA in stirred tank bioreactors. Various feeding strategies are explored to maximize the final concentration of the product.

MPA is a typical secondary metabolite which is made mostly during the stationary phase of fungal growth [28]. Production is generally dependent on the concentration of the producing cells. Therefore, the general production strategy is to attain a high concentration of the biomass by the time the stationary phase is reached and minimize cell autolysis. A suitable feeding strategy is important for achieving a high

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cell density fermentation [33–38]. Feeding methionine, a precursor of MPA, has been reported to enhance the final MPA concentration [1]. The culture pH is another factor that often plays a central role in microbial growth and metabolite accumulation [37,39] The effect of pH on growth and product accumulation depend on the microorganism, the culture medium and the other operating conditions [40]. Effects of pH and feeding methodologies are important for many fermentations [37,41].

In a previous study [28], we developed an optimal medium for the production of MPA using *Penicillium brevicompactum*. Using the same microorganism, here we report on the effects of different feeding strategies on MPA production in fed-batch operations in a 14 L stirred tank bioreactor. The different fed-batch operations are compared with a batch operation that used the earlier optimized medium. The effects of controlled pH in fed-batch fermentations are also reported.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The microbial culture of *Penicillium brevicompactum* MTCC 8010 was procured from Institute of Microbial Technology, Chandigarh, India. The culture was maintained on aseptic slants of potato dextrose agar (PDA). A spore suspension was prepared as previously reported [28] and used as inoculum.

2.2. Culture medium

In a previous study [28], we developed an optimal medium for the growth and production of MPA using *Penicillium brevicompactum*. Optimal medium contained the subsequent constituents (g/L): glucose 66, peptone 30, glycine 6.3, methionine 2, KH_2PO_4 6, $MgSO_4{\cdot}7H_2O$ 1, and 1 mL/L of a trace elements solution. The trace elements solution contained (g/L): $FeSO_4{\cdot}7H_2O$ 2.2, $CuSO_4{\cdot}5H_2O$ 0.3, $ZnSO_4{\cdot}7H_2O$ 2.4, $MnSO_4{\cdot}4H_2O$ 0.16, and $(NH_4)_2MoO_4$ 0.2.

2.3. Fermentations

Fermentations were carried out in a 14 L bioreactor (New Brunswick, Bioflo and Celligen 310 fermentors) with a working volume of 11 L. The specified medium was inoculated with 1% by volume of the earlier specified spore suspension. The bioreactor was equipped with two, six-bladed disc turbine impellers. The agitation speed was controlled in the range of 200 to 1000 rpm and the aeration rate was fixed at 1 vvm. The temperature was controlled at 25 ± 0.2 °C. The initial pH was adjusted to 5. In pH controlled fermentations, the pH was measured with an installed pH electrode and automatically adjusted at the set point value by feeding NaOH and HCl solutions, as needed. Samples were taken periodically and analyzed for biomass concentration, MPA concentration, and the residual substrate concentration. Fermentations were continued for up to 12 days. It was practically not possible to run the fermenter beyond 12 days due to heavy fungal growth and difficulties of taking out sample from the fermenter. Due to severe volume reduction on daily sampling up to 12 days and difficulties of running it for long, fermentations were carried out up to 12 days.

2.4. Analytical methods

2.4.1. Mycophenolic acid concentration

The concentration of MPA was measured in the both culture supernatant and biomass. As reported in our previous study, reverse phase high performance liquid chromatography was used at a wavelength of 220 nm for the measurement of MPA concentration [28,29].

2.4.2. Glucose concentration

Glucose was measured in the culture supernatant as total reducing sugars, using the dinitrosalicylic acid (DNS) method [42]. A suitably diluted sample (1 mL) was mixed with 1 mL DNS reagent, held at 90 °C for 10 min, and cooled to room temperature. The absorbance was measured at 540 nm against a blank of deionized water treated in the same way as the sample. The measured absorbance and the dilution factor were used to estimate the reducing sugar concentration by comparing with a calibration curve made using dilutions of a standard aqueous solution of glucose.

2.4.3. Sorbitol concentration

Sorbitol concentration was measured in the culture supernatant using a modification of the method described by Lambert and Neish [43]. The modified method replaced the toxic sodium arsenite with stannous chloride as an alternative reducing agent. In this method, sorbitol is oxidized by periodic acid to formic acid and formaldehyde. The conditions of the oxidation are such that little formaldehyde is produced from any glucose in the sample. The iodate and periodic acid are reduced to iodide by stannous chloride. Formaldehyde was determined directly in the oxidation mixture by color formation with chromotropic acid.

A 20 mL sample was pipetted into a 100 mL volumetric flask. Sulfuric acid (1 mL, 5 M) and 5 mL sodium periodate (0.1 M) were added to the flask and mixed. After 5 min, 5 mL stannous chloride (0.125 M) solution was added and mixed. A few seconds after addition of the stannous chloride, iodine appeared in solution and then faded away. After 5 to 10 min, 1 mL was pipetted from this mixture into a test tube and 10 mL chromotropic acid reagent was added. The tube was mixed and heated in a boiling water bath for 30 min. After cooling, the absorbance was measured at 570 nm against a blank of deionized water treated in the same way as the sample. The measured absorbance and the dilution factor were used to estimate the sorbitol concentration by comparing with a calibration curve made using dilutions of a standard aqueous solution of sorbitol.

2.4.4. Biomass concentration

The wet cell mass (WCM) method was used for the quantification of fungal biomass in the fermentation broth. A 50 mL sample of the fermentation broth was filtered through Grade 50 cheese cloth purchased at a local market. The biomass was washed with 50 mL water and the resulting cake was weighed [3,6].

All the analyses were carried out in triplicate and average values were reported except the values of constant parameters such pH, agitation rate, temperature and dissolved oxygen concentration.

3. Results and discussion

3.1. Batch fermentation

The complete medium as specified earlier was used in a batch fermentation. The results are shown in Fig. 1. Glucose was consumed rapidly as the biomass grew. Because of rapid growth and glucose consumption, the dissolved oxygen concentration fell to almost nil around 75 h despite the agitation speed being increased in attempts to improve oxygen supply. The pH reduced for the first 96 h during the phase of rapid glucose consumption. Afterwards, with slow consumption of glucose and almost no dissolved oxygen present, the culture pH rose until it had exceeded 8 near the end of the fermentation. MPA concentration began to increase only after growth had ceased, confirming it to be a secondary metabolite [27,28]. The MPA biosynthesis occurred mostly from 120 h onwards (Fig. 1). By around 220 h, essentially all glucose had been consumed and, therefore, oxygen demand reduced and the dissolved oxygen concentration in the bioreactor began to rise. The maximum concentration of MPA (= 1067 mg/L) was achieved at the end of the fermentation. At the end of the fermentation, the entire