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Mycophenolic Acid Production: Investigation of the Effects of Enzymatically Hydrolyzed Casein Concentration on Product Yield and Productivity

[1]*Fatemeh Ardestani and* [2]*Ghasem Najafpour*

[1]Department of Chemical Engineering, Islamic Azad University, Qaemshahr Branch, Qaemshahr, Iran
[2]Faculty of Chemical Engineering, Babol University of Technology, Babol, Iran

Abstract: Mycophenolic acid (MPA) is an antibiotic and immunosuppressive drug produced by *Penicillium* strains as a secondary extracellular metabolite. In this work, the effect of some various concentrations of enzymatically hydrolyzed casein on mycophenolic acid yield and process productivity was studied. The first, a base medium was applied for mycophenolic acid production by *Penicillium brevicompactum* MUCL 19011 in shake flask. The maximum MPA production, product yield and productivity of process were 1.379 g/L, 18.6 mg/g glucose and 4.9 mg/L. h, respectively. Then, in the next step, MPA production process was evaluated using various concentrations of enzymatically hydrolyzed casein. Maximum MPA production, product yield and productivity as 3.72 g/L, 50.3 mg/g glucose and 13.28 mg/L. h, respectively, were obtained using 26 g/L enzymatically hydrolyzed casein. These values show an enhanced MPA production, product yield and process productivity as 169.8%.

Key words: Enzymatically hydrolyzed casein · *Penicillium brevicompactum* · Mycophenolic acid · Shake flask fermentation · Product yield · Productivity

INTRODUCTION

Mycophenolic acid is a new antibiotic and immunosuppressive drug [1, 2].

MPA and some of its derivatives such as mycophenolate mofetil (MMF) and sodium mycophenolate have been approved by FDA as immunosuppressive drugs. These are applicable in decreasing the incidence of graft rejection after organ transplantation [3]. MPA has inhibitory effect on inosine monophosphate dehydrogenase enzyme "IMPDH". This is the rate-limiting enzyme in novo biosynthetic pathway of purine nucleotides. Then, MPA caused to stopping the biosynthesis of DNA and RNA and cell reproductivity [4].

MPA is produced by different species of *Penicillium* especially *P. brevicompactum*, *P. stoloniferum* and *P. roqueforti* and also by some other microbial strains such as *Byssochlamys nivea* in submerged and solid state fermentation processes as a secondary metabolite [5-11]. In the most cases *P. brevicompactum* was applied as a producer strain of mycophenolic acid [5, 6, 8, 9]. Different operational modes such as free cell in submerged culture [12], immobilized cells in submerged cultures [12], packed bed bioreactors [13] and solid state fermentation [5, 8, 9] were used for MPA production. Some researches have been focused on MPA biosynthetic metabolism in the yeast cells [14].

Casein is the predominant phosphoprotein (αS1, αS2, β, κ) that accounts for nearly 20% of proteins in cow milk and cheese. Enzymatically hydrolyzed casein is a good source of nitrogen and is used in fermentation processes for some antibiotic production [12].

In this study, the first, MPA was produced in a fermentative process by *Penicillium brevicompactum* MUCL 19011 in submerged culture in 250 ml shake flask. Then, in the next step, the effects of some various concentrations of enzymatically hydrolyzed casein on MPA production were evaluated. MPA production title, product yield and process productivity in each case were determined and compared.

Corresponding Author: Fatemeh Ardestani, Department of Chemical Engineering, Islamic Azad University, Qaemshahr Branch, Qaemshahr, Iran.
Tel: +98-1232253216-19.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation: Penicillium brevicompactum MUCL 19011 was obtained from the Belgian co-ordinated collection of micro-organism (BCCM). The stock culture was maintained on the potato dextrose agar (PDA) slants at 4°C. For inoculums preparation, spores were transferred to PDA and incubated at 27°C for 3 days. The cell suspension was made by collection of spores grown on Petri plates by shaving and extracting the spores with sterile water [5, 6]. The number of spores in suspension was counted by Thoma lam and adjusted to 10^7- 10^8 spores per ml. Spore suspension was used as the inoculums for shake flask. For fermentation process, 0.5 ml of a spore suspension (~ $5*10^7$/mL) was inoculated to each 250 ml shake flask containing 50 ml culture medium [12].

Medium Composition: The base medium composition was included (g/L): glucose, 80; glycine, 9; enzymatically hydrolyzed casein, 15; methionine, 0.5; KH_2PO_4, 5; $MgSO_4.7H_2O$, 1; and 1 ml/L from trace element mixture including (g/L): $FeSO_4$•7H O, 2.2; CuSO •5H O, 0.3; $ZnSO_4$•$7H_2O$, 2.4; $MnSO_4$•$4H_2O$, 0.16; and $KMoO_4$, 0.2 [12].

In each experiment, the media components except for glycine, methionine and the trace element mixture were separately autoclaved at 121°C for 15 min. The pH values of the prepared media were adjusted to 6.0 with 2 N HCl or NaOH solutions. The glycine, methionine and trace element mixture were sterilized by a 0.2 µm filter (Millipore, USA).

Fermentation Process: A rotary shaker incubator (JAHL- JSH 20LUR, IRAN) was used for batch fermentation. After inoculation of 50 ml of culture medium with 0.5 ml of the spore suspension (~ 5×10^7 per ml), the 250 ml shake flasks containing the inoculated culture medium was incubated at 27°C with an agitation rate of 200 rpm on a rotary shaker for 300 h. Glucose consumption, biomass and MPA production profiles were investigated during cultivation. In the next step, the effects of some various concentrations of enzymatically hydrolyzed casein on MPA production were evaluated separately. In this step, the culture medium composition in each shake flask was the same base medium containing of different concentrations of enzymatically hydrolyzed casein. Finally, MPA concentration in each shake flask was measured after 280 hours.

Sampling and Sample Preparation for Analysis: In the shake flask experiments with base medium, one of the flasks was removed as a sample for analysis at appropriate time intervals. However, in the next step, to survey of enzymatically hydrolyzed casein impacts on MPA production, only one sample was removed at the end of cultivation time (after 280 h) in each medium. MPA concentration was measured after passing the samples through a 0.2 micron filter (Millipore, USA). The supernatants were stored at –20°C until analysis of MPA and glucose concentrations.

Analytical Methods: The glucose concentration was measured by a colorimetric method using the dinitrosalicylic acid (DNS) reagent and a spectrophotometer (Unico 2100, USA) at a wavelength of 540 nm. The biomass resulting from the above mentioned sampling procedure was dried at 60-65°C for at least 24 h until reaching constant weight. Cell dry weight was calculated based on the dried biomass weight [11]. Cell dry weight and glucose measurements were performed three times for each sample. The average value was considered as obtained data, if there was an acceptable error. The measurement of produced mycophenolic acid value in culture medium was performed through high performance liquid chromatography method (HPLC, Shimadzu, Japan) with a C_{18} column at 40°C [15]. The mobile phase was consisted of 0.1 M KH_2PO_4 solution and acetonitrile (50:50) at pH 3.0 and was used at a flow rate of 0.5 ml/min. The UV detector was set at a wavelength of 250 nm and an injection volume of 50 µL was used [16]. HPLC grade MPA (Biochemica, Germany) was used as standard for analysis. The stock solution of MPA (1 mg/ml) was prepared by dissolving in methanol. This stock solution was stored at -20°C. On the day of analysis, working standard solutions (2.5 –250 µg/ml) were prepared by serial dilutions of the 1 mg/ml stock solution with methanol. A calibration curve was then determined using the standard solutions.

RESULTS AND DISCUSSION

Evaluation of MPA Production in Shake Flask with Base Medium: In the first stage, MPA was produced in a fermentation process in 250 ml shake flasks using the base medium. The total incubation time in this process was considered as 300 hours. Fig. 1 represents the concentration profiles of glucose, cell dry weight and MPA during the fermentation time. Process observation

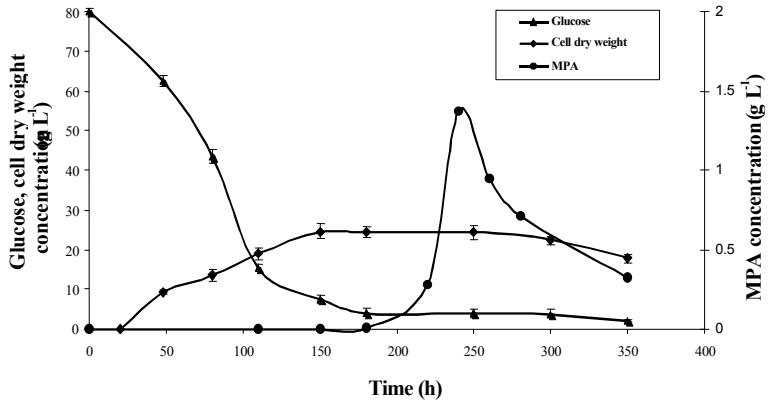

Fig. 1: Glucose, cell dry weight and MPA concentration profiles in MPA production process by *Penicillium brevicompactum* MUCL 19011 in 250 ml shake flask using base medium. Process conditions were set as Temperature 27°C, agitation rate 200 rpm, incubation time 300 h and pH 6.

Table 1: The effects of various concentrations of enzymatically hydrolyzed casein on MPA production in 250 ml shake flask. Process conditions were set as Temperature 27°C, agitation rate 200 rpm, incubation time 300 h and pH 6

		Enzymatically hydrolyzed casein concentration (g/L)									
	Base medium	16	18	20	22	24	25	26	28	30	32
MPA Con. (g/L)	1.379	1.50	2.14	2.99	3.11	3.43	3.21	3.72	3.69	3.63	3.46
Product yield (g/ g glucose)	0.0186	0.0203	0.0289	0.0403	0.0420	0.0457	0.0433	0.0503	0.0499	0.0490	0.0467
Productivity (mg/L. h)	4.92	5.36	7.64	10.67	11.11	12.25	11.46	13.28	13.18	12.96	12.36
Enhanced MPA production (%)	-	8.8	55.2	116.8	125.5	148.7	132.8	169.8	167.6	163.2	150.9

showed *P. brevicompactum* grows as pellets after about 24 hours from incubation. These pellets served during the fermentation process until 250 h (the end of the stationary phase) and then broke and lysed.

Obtained results showed that the major amount of glucose was consumed in the early 180 h of incubation. This period actually is the trophophase stage of *P. brevicompactum* growth. Then, glucose consumption rate was decreased and reached to an approximate constant value (in the idiophase stage of cell growth).

Cell dry weight was increased in the early 180 h of incubation, then reached to the stationary phase and finally decreased after about 280 h. In other word, the stationary phase of *P. brevicompactum* in this fermentation process, with 80 g/L initial glucose concentration, was happened between 180 to 280 h. MPA production also was occurred in the same period.

MPA is a secondary metabolite of *P. brevicompactum* and its production was started from 180 h and reached to maximum (1.379 g/L) in 280 h and then decreased. Obtained MPA yield and productivity were 18.6 mg/g glucose and 4.9 mg/L. h, respectively.

Effect of Enzymatically Hydrolyzed Casein on MPA Production: Shake flask fermentation processes using base medium enriched by different concentrations of enzymatically hydrolyzed casein were performed separately. MPA concentration in each flask was measured after 280 hours (Table 1). Results showed that maximum MPA production, 3.72 g/L, was obtained when the culture medium was contained 26 g/L of enzymatically hydrolyzed casein. In this condition, MPA yield and productivity were 50.3 mg/g glucose and 13.28 mg/L. h, respectively. These values were higher than MPA production in base medium containing of 15 g/L enzymatically hydrolyzed casein as 169.8% (Fig. 2).

Thus, Enzymatically hydrolyzed casein (26 g/L) had a positive impact on process productivity as 169.8%. These could be related to some unknown effects of this compound with regard to activation or inhibition of certain enzymes involved in the MPA biosynthetic pathway of *P. brevicompactum*.

As reported in some previous studies, enzymatically hydrolyzed casein has an inhibitory effect on homocitrate synthase. Homocitrate synthase is the enzyme responsible for the conversion of glutamate to lysine in the amino acid biosynthesis pathway of most yeast cells [17]. For example, the inhibitory effect of enzymatically hydrolyzed casein on homocitrarte synthase has been reported in *P.chrysogenum* in previous studies [18]. As a

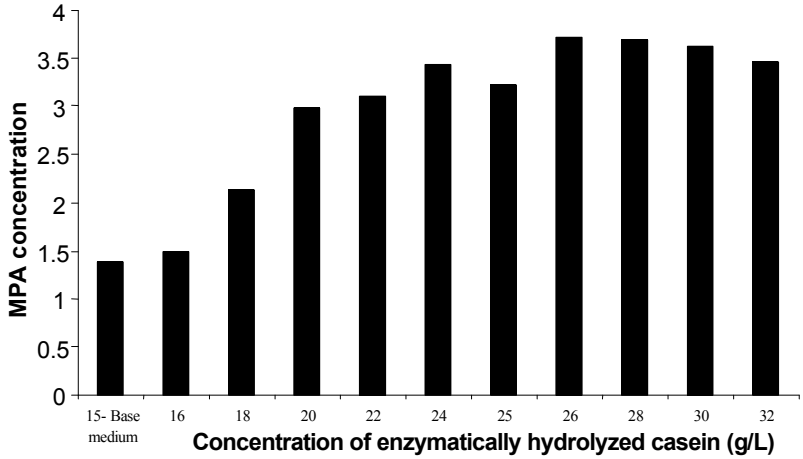

Fig. 2: A comparison of MPA production between the base medium and the media containing different concentrations of enzymatically hydrolyzed casein.

result of this inhibitory effect, glutamate may be converted to aspartate instead of lysine. In the next step, aspartate will be converted to methionine, which is the main source of methyl groups present in the MPA structure [19]. The basic skeleton of mycophenolic acid molecule is acetate-derived and methionine and mevalonic acid serve as precursors of the methyl group and acidic side chain attached to the aromatic nucleus as discussed in previous studies [19, 20]. Methionine, the first, converts to S-adenosyl-methionine and then inters to MPA biosynthesis pathway [21]. On the other hand, the addition of enzymatically hydrolyzed casein could lead to greater amounts of nitrogen becoming available in the medium, thus culminating in increased MPA production.

The positive impact of methionine and acetate on MPA production in submerged culture of *P. brevicompactum* was reported in some previous researches [22, 23]. MPA production, product yield and productivity (1.763 g/L, 23.8 mg/g glucose and 6.30 mg/L. h, respectively) were obtained by using 2.5 g/L methionine in culture medium of *P. brevicompactum* MUCL 19011 [22]. Also the positive impact of methionine on other antibiotics in *Acremonium chrysogenum* was reported [23].

CONCLUSIONS

MPA production by *P. brevicompactum* MUCL 19011 in shake flask using the base medium, involved of 80 g/L glucose, was observed at 180 h after incubation. The maximum concentration of MPA in base medium was 1.379 g/L after 280 hours. Results showed that *P. brevicompactum* MUCL 19011 has a good efficiency for MPA production. MPA yield and productivity was 18.6 mg/g glucose and 4.9 mg/L. h, respectively. Maximum MPA concentration, product yield and productivity were obtained after using 26 g/L of enzymatically hydrolyzed casein. In this case, maximum MPA production, product yield and productivity were 3.72 g/L, 50.3 mg/g glucose and 13.28 mg/L. h, respectively. These values show an enhanced MPA production, product yield and process productivity as 169.8%.

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