World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:5, No:1, 2011

Enhanced Mycophenolic Acid Production by *Penicillium brevicompactum* with Enzymatically Hydrolyzed Casein

F. Ardestani, S. S. A. Fatemi, B. Yakhchali

***Abstract*—**Mycophenolic acid (MPA) is a secondary metabolite produced by *Penicillium brevicompactum*, which has antibiotic and immunosuppressive properties. In this study, the first, mycophenolic acid was produced in a fermentation process by *Penicillium brevicompactum* MUCL 19011 in shake flask using a base medium. 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. Also the glucose consumption, biomass and MPA production profiles were investigated during batch cultivation. Obtained results showed that MPA production starts approximately after 180 hours and reaches to a maximum at 280 h. In the next step, the effects of some various concentrations of enzymatically hydrolyzed casein on MPA production were evaluated. Maximum MPA production, product yield and productivity as 3.63 g/L, 49 mg/g glucose and 12.96 mg/L.h, respectively were obtained with using 30 g/L enzymatically hydrolyzed casein in culture medium. These values show an enhanced MPA production, product yield and process productivity pr as 116.8%, 132.8% and 163.2%, respectively.

Keywords*—Penicillium brevicompactum*, Enzymatically hydrolyzed casein, Mycophenolic acid, Submerged culture

I. Introduction

MYCOPHENOLIC acid is a new antibiotic and immunosuppressive drug [1].

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 [2]. 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 [3].

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 [4]- [7], [8], [9], [10]. In the most cases *P. brevicompactum* was applies as a producer strain of mycophenolic acid [4, 5, 7, 8]. Different operational modes such as free cell in submerged culture [11], Immobilized cells in submerged cultures [11], packed bed bioreactors [12] and solid state fermentation [4, 7, 8] were used for MPA production. 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 [11].

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, at the next step, the effects of enzymatically hydrolyzed casein concentrations on MPA production were evaluated.

F. Ardestani is with Faculty of Chemical Engineering, Islamic Azad University, Qaemshahr Branch, Qaemshahr, Iran (corresponding author to provide phone: +981232253216, Fax: +981232240090, e-mail: ardestani_fatemeh@yahoo.com)

S. S. A. Fatemi is with Industrial and Environmental Biotechnology Department, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran (sfatemi@nigeb.ac.ir)

B. Yakhchali is with Industrial and Environmental Biotechnology Department, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran (byakhcha@nigeb.ac.ir)

II. Materials and Methods

A. 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 ^{0}C. For inoculums preparation, spores were transferred to PDA and incubated at 27 ^{0}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 [4], [5].

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 inoculum 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 [11].

B. Medium composition

The base medium composition was included (g/L): glucose, 80; glycine, 9; enzymatically hydrolysate 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): $FeSO_4 \cdot 7H_2O$, 2.2; $CuSO_4 \cdot 5H_2O$, 0.3; $ZnSO_4 \cdot 7H_2O$, 2.4; $MnSO_4 \cdot 4H_2O$, 0.16; and $KMoO_4$, 0.2 [11], [13].

In each experiment, the media components except 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).

C. 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 mL^{-1}), the 250 mL shake flask containing the inoculated culture medium was incubated at 27 ^oC 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 enzymatically hydrolyzed casein concentrations on MPA production were evaluated separately. In this step, the culture medium composition in each shake flask was the base medium containing of different concentrations of enzymatically hydrolyzed casein. Finally, MPA concentration in each shake flask was measured after 280 hours.

D. Sampling and sample preparation for analysis

In the shake flask experiments, one of the flasks was removed as a sample for analysis at appropriate time intervals. However, in the next step, for investigation 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 ^oC until analysis of MPA and glucose concentrations.

E. Analytical methods

The biomass resulting from the abovementioned 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 [10]. 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. 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. Mycophenolic acid was analyzed by high performance liquid chromatography (HPLC, Shimadzu, Japan) with a C_{18} column at 40°C [13]. The mobile phase 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^{-1}. The UV detector was set at a wavelength of 250 nm and an injection volume of 50 µL was used [14]. HPLC grade MPA (Biochemica, Germany) was used as standard for analysis. The stock solution of MPA (1 mg mL^{-1}) was prepared in methanol and stored at −20°C. On the day of analysis, working standard solutions (2.5 –250 µg mL^{-1}) were prepared by serial dilutions of the 1 mg mL^{-1} stock solution with methanol. A calibration curve was then determined using the standard solutions.

III. RESULTS AND DISCUSSION

A. Evaluation of MPA production in shake flask with base medium

MPA was produced in 250 mL shake flask fermentation process using the base medium. The production process was performed for 300 hours. Fig. 1 represents the concentration profiles for glucose, cell dry weight and MPA during the fermentation time. Process observation showed that *P. brevicompactum* grows as pellets after about 24 hours from incubation. These pellets served during the fermentation process until 250 h (the end of stationary phase) and then broke and lysed.

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

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.

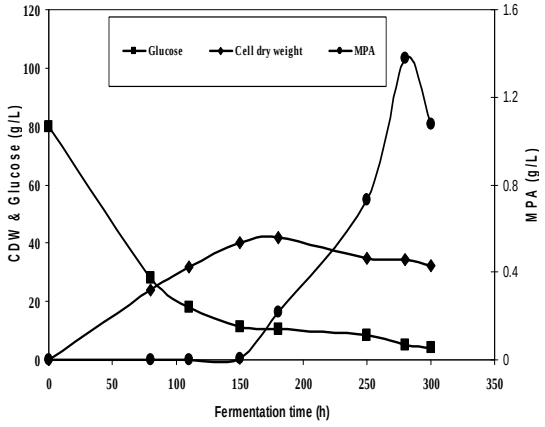

Fig. 1. Glucose, cell dry weight and MPA concentration profiles in MPA production by Penicillium brevicompactum MUCL 19011 in shake flask using base medium.

MPA is a secondary metabolite of *P. brevicompactum* and its production was started from180 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.

B. Effect of enzymatically hydrolyzed casein on MPA production in shake flask

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 I). Results showed that maximum MPA production, 3.63 g/L, was obtained when the medium containing 30 g/L enzymatically hydrolyzed casein was applied. In this condition, MPA yield and productivity were 49 mg/g glucose and 12.96 mg/L. h, respectively. These values were higher than MPA production in base medium containing of 15 g/L enzymatically hydrolyzed casein as 163.2% (Fig. 2).

Thus, Enzymatically hydrolyzed casein (30 g/L) had a positive impact on process productivity as 163.2%. These could be related to some unknown effects of these compound with regard to activation or inhibition of certain enzymes involved in the MPA biosynthetic pathway of *P. brevicompactum*.

Enzymatically hydrolyzed casein has an inhibitory effect on homocitrate synthase that is responsible for the conversion of glutamate to lysine [15]. The inhibitory effect of enzymatically hydrolyzed casein on homocitrarte synthase has been reported in *P.chrysogenum* in previous studies [16]. Thus, 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 [17]. 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 [17, 18]. Methionine, the first, converts to S-adenosyl-methionine and then inters to MPA biosynthesis pathway [19]. 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. MPA production, product yield and productivity (1.763 g/L, 23.8 mg/g glucose and 6.30 mg/L. h, respectively) were obtained with using 2.5 g/L methionine in culture medium of *P. brevicompactum* MUCL 19011 [20]. Also the positive impact of methionine on other antibiotics in *Acremonium chrysogenum* was reported [21].

IV. CONCLUSIONS

MPA production by *P. brevicompactum* MUCL 19011 in shake flask using the base medium was observed at 180 h after incubation. The base medium was involved of 80 g/L glucose. 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 30 g/L of enzymatically hydrolyzed casein. In this case, maximum MPA production, product yield and productivity were 3.63 g/L, 49 mg/g glucose and 12.96 mg/L.h, respectively. These values show an enhanced MPA production, product yield and process productivity pr as 116.8%, 132.8% and 163.2%, respectively.

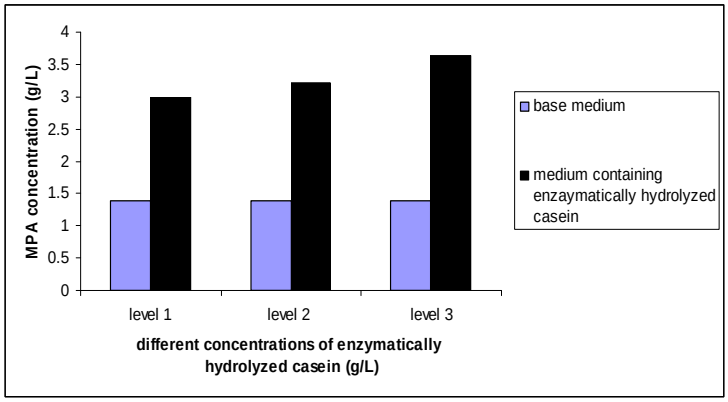

Fig. 2. a copmparison of MPA production between the base medium and the media containing different concentrations of enzymatically hydrolyzed casein.

TABLE. I
THE EFFECTS OF ENZYMATICALLY HYDROLYZED CASEIN CONCENTRATIONS ON MPA PRODUCTION IN SHAKE FLASK FERMENTATION

	base medium	enzymatically hydrolyzed casein conc. (g/L)		
		20	**25**	**30**
MPA Conc.	1.379	2.99	3.21	3.63
Yeild (g/g glucose)	0.0186	0.0403	0.0433	0.0490
Productivity (mg/L.h)	4.92	10.67	11.46	12.96
Enhanced (%)	-	116.8%	132.8%	163.2%

ACKNOWLEDGMENT

We would like to thank Dr M.A Asadollahi faculty member of Isfahan University for his good points of views and also the Iran Antibiotic Sazi Company for experimental and analytical support of this research project.

REFERENCES

[1] T.Rainiene, Immunosuppression in the past and today, Acta Medica Lituanica. 2005, pp. 10-17.
[2] S. Tian, P. Mele, F. Halloran, The use of mycophenolate mofetil in transplant recipients, J. Immunopharma. 2000, pp. 215-245.
[3] A.Charlotte, J. Hans Charleston, Mycophenolic acid inhibits inosine 5-monophosphate dehydrogenase and suppresses immunoglobulin and cytokine production of B cells, Int. Immunopharma. 2003, pp. 31-37.
[4] C. D. BARTMAN, et al, Mycophenolic Acid Production by *Penicillium brevicompactum* on Solid Media, Appl. Environ. Microbiol. 1981, pp. 729-736.
[5] D. L. Doerfler, C. D. Bartman, L. M. Campbell, Mycophenolic acid production by *Penicillium brevicompactum* in two media. Can. J. Microbiol. 1979, pp. 940-943.

[6] Vinokurova, NG., Ivanushkina, NE., Kochkina, GA., Arinbasarov, MU., Ozer-skaya, SM., Production of mycophenolic acid by fungi of the genus *Penicillium* link, Appl Biochem Microbiol., 2005, Pp 83–86.
[7] Bartman, C. D.; Doerfler, D. L.; Bird, B. A.; Remaley, A. T.; Peace, J. N.; Campbell, I. M. mycophenolic acid production by *Penicillium brevicompactum* on solid media, App. Environ. Microb, 1981, Pp 729-736. .
[8] A. Sircar, et al. Manufacture and purification of mycophenolic acid, United States Patent. PCT No.: PCT/INOO/00017 , 2003.
[9] O. Puel, S. Tadrist, et al, *Byssochlamys nivea* as a source of mycophenolic acid, Appl. Environ. Microbiol. 2005, pp. 550–553.
[10] P. Lafont, J. Debeaupuis, M. Gaillardia, J. Payen, Production of mycophenolic acid by *Penicillium roqueforti* strains, Appl. Environ. Microbiol. 1978, pp. 365-368.
[11] Z. Xu, S. Yang, Production of mycophenolic acid by *Penicillium brevicompactum* immobilized in a rotating fibrous-bed bioreactor, Enzyme Microb. Technol. 2007, pp. 623–628.
[12] Alania, F.; Grove, J. A.; Anderson, W. A.; Young , M. M. Mycophenolic acid production in solid-state fermentation using a packed-bed bioreactor, Biochem. Eng, 2009, 44, 106–110.
[13] Bahrami, G.; Mohammadi, B. An isocratic high performance liquid chromatographic method for quantification of mycophenolic acid and its glucuronide metabolite in human serum using liquid liquid extraction: Application to human pharmacokinetic studies, Clinica Chimica Acta, 2006, 370, Pp 185-190.
[14] A. Fawzy, A. Elbarbry, Simple high performance liquid chromatographic assay for mycophenolic acid in renal transplant patients, J. Pharma. Biomedica. Ana. 2007, pp. 788–792
[15] www.brenda.com
[16] Masurekar, P. S., Demain, A. Insensitivity of homocitrate synthase in extracts of *Penicillium chyrosogenum* to feedback inhibition by lysine, Appl. Microbiol, 1974, 28 (2), Pp 265-270
[17] C. T. Bedford, C. Fairlipe, Sequence Studies in Biosynthesis; Mycophenolic Acid, Chem. Com. 1971, pp. 323-324.
[18] W. L. Muth, I. Nash, Biosynthesis of mycophenolic acid: purification and characterization of S-adenosyl-L-methionine: demethylmycophenolic acid o-methyltransferase, Antimicrob. Chemo. 1975, pp. 321-327.
[19] H. Ozaki, M. Ishihara, T. Kida, S. Yamanaka, H. Shibai, Mycophenolic acid production by drug-resistant and methionine or glutamic-acid requiring mutants of *Penicillium brevicopactum*, Agric Biol Chem. 1987, pp. 2509–2514.
[20] F. Ardestani, S.A. Fatemi, B. Yakhchali, M. Hosseyni, G. Najafpour, The effects of methionine and acetate concentrations on mycophenolic acid production by *Penicillium bervicompactum* MUCL 19011 in submerged culture, Int. J. of Natural Sciences and Engineering, 2009 (2:2), pp. 83-86.
[21] Karaffa, L.; Sandor, E.; Kozma, J.; Szentirmai, A. Methionine enhances sugar consumption, fragmentation, vacuolation and cephalosporin-C production in *Acremonium chrysogenum*, Process Biochem, 1997, 32 (6), 495-499.