The Effect of Methionine and Acetate Concentrations on Mycophenolic Acid Production by Penicillium bervicompactum MUCL 19011 in Submerged Culture

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Abstract—Mycophenolic acid "MPA" is a secondary metabolite of *Penicillium bervicompactum* with antibiotic and immunosuppressive properties. In this study, fermentation process was established for production of mycophenolic acid by *Penicillium bervicompactum* MUCL 19011 in shake flask. The maximum MPA production, product yield and productivity were 1.379 g/L, 18.6 mg/g glucose and 4.9 mg/L.h respectively. Glucose consumption, biomass and MPA production profiles were investigated during fermentation time. It was found that MPA production starts approximately after 180 hours and reaches to a maximum at 280 h. In the next step, the effects of methionine and acetate concentrations on MPA production were evaluated. Maximum 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. Further addition of methionine had not more positive effect on MPA production. Finally, results showed that the addition of acetate to the culture medium had not any observable effect on MPA production.   .

Keywords—Penicillium bervicompactum, Methionine, Mycophenolic acid, Submerged culture.

I. INTRODUCTION

MYCOPHENOLIC acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid) is a relatively new antibiotic and immunosuppressive drug [1]. MPA and its derivatives such as mycophenolate mofetile (MMF) and sodium mycophenolate are immunosuppressive agents which have been approved by FDA and used for

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decreasing the incidence of graft rejection in organ transplant patients. Since MPA has special function on B,T lymphocytes and low toxicity for other cells in human body, in comparison to other similar drugs such as cyclosporine A, it goes to be the main alternative of all of them [2].

MPA has inhibitory effect on inosine monophosphate dehydrogenase enzyme "IMPDH" that is the rate-controller enzyme in novo biosynthetic pathway of purine nucleotides. This effect caused to stopping the conversion of inosine monophosphate to adenosine and guanosine monophosphate and then forming the adenosine triphosphate, deoxy adenosine triphosphate, guanosine triphosphate and deoxy guanosine triphosphate and finally stopping the biosynthesis of DNA and RNA and cell reproductivity [3].

MPA can be produced by different species of *Penicillium* especially *P. bervicompactum*, *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], [10], [16], [17]. Filamentous fungi such as *Penicillium* strains are morphologically complex microorganisms, exhibiting different structural forms throughout their life cycles [8]. The life cycle of filamentous fungi starts and ends in the form of spores. In submerged cultures, these fungi have different morphological forms, ranging from dispersed mycelial filaments to densely mycelial masses as pellets [9]. Methionine and acetate are two important precursors of mycophenolic acid biosynthesis pathway in *P. bervicompactum*. 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 [11]. Methionine, the first, converts to S-adenosyl-methionine and then inters to MPA biosynthesis pathway. On the other hand, acetate is appears in form of acetyl-CoA that go to MPA special biosynthesis pathway and also other metabolic pathways such as TCA cycle, mevalonate and amino acid biosynthesis pathways [11], [13].

In this study, fermentative process of MPA production by *Penicillium bervicompactum* MUCL 19011 in submerged

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culture in 250 mL shake flask was performed and the effects of methionine and acetate on MPA production were evaluated.

II. MATERIALS AND METHODS

A. Microorganism and Inocula Preparation

P. bervicompactum MUCL 19011 was obtained from BELGIUM Culture Collection. The stock culture was maintained on the potato dextrose agar (PDA) slants at 4 ^{0}C and subcultured every month. For inocula 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 [9].

B. Medium Composition

The base medium composition was included (g/L): glucose, 80; glycine, 9; casein hydrolysate enzymatic, 15; methionine, 0.5; KH_2PO_4, 5; $MgSO_4.7H_2O$, 1; and 1 ml/L from trace element mixture including (g/L): $FeSO_4·7H_2O$, 2.2; $CuSO_4·5H_2O$, 0.3; $ZnSO_4·7H_2O$, 2.4; $MnSO_4·4H_2O$, 0.16; and $KMoO_4$, 0.2 [9], [11].

Each of the medium compositions except glycine, methionine and trace element solution was autoclaved separately at 121^{0}C for 15 minutes after adjusting the pH to 6.0 with 2 N HCl or NaOH solutions. The glycine, methionine and trace element mixture were sterilized by a 0.2 micron filter (Millipore).

C. Fermentation Process

After inoculation with spore suspension, 250 mL shake flasks containing of 50 mL culture medium were kept on a rotary shaker incubator (JAHL- JSH 20LUR, IRAN) at 200 rpm and 27 ^{0}C for 300 hours. At first, shake flask fermentation by using base medium was performed and glucose consumption, biomass and MPA production profiles were investigated during fermentation time. In the next step, the effects of methionine and acetate 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 methionine or acetate. Finally, MPA concentration in each shake flask was measured after 280 hours.

D. Sampling and Sample Preparation for Analysis

In shake flask process with base medium, at proper time intervals, one of the flasks was outed as a sample for analysis of glucose, biomass and MPA concentrations. However, for investigation of methionine and acetate concentrations effects on MPA production, for each medium, only one sample was removed at the end of process (after 280 h) for measurement of MPA concentration. All samples were passed through a 0.2 micron filter (Millipore). The supernatants were stored at –20 ^{0}C until analysis of MPA and glucose concentrations.

E. Analytical Methods

Cell dry weight was measured by drying the centrifuged or filtrated biomass at 60-65 ^{0}C for at least 24 h until constant weight [17]. The glucose concentration was measured with DNS (Dinitro salicylic acid) method (spectrophotometer at 540 nm wavelength) [12]. MPA was analyzed by high performance liquid chromatography (HPLC, Shimadzu, pump: LC-20 AD, detector: SPD-20 A, auto sampler: SIL-20A, degasser: DGU-20 A) with C_{18} column at 40 ^{0}C. The mobile phase was included 0.1 M KH_2PO_4 solution and acetonitrile (50:50) at pH 3.0 with 0.5 mL/min flow rate. The UV detection was at 250 nm wavelength and injection volume was 50 μL [9], [12].

III. RESULTS AND DISCUSSION

A. Evaluation of MPA Production in Shake Flask with Base Media

Fermentation process for MPA production in 250 mL shake flask with base medium was performed and evaluated during 300 hours. Glucose, cell dry weight and MPA concentration profiles during the fermentation time were presented in Fig. 1. It was observed that *P. bervicompactum* grow as pellets after about 24 hours from inoculation of culture media with spore suspension. These pellets served during the fermentation process until 250 h (the end of stationary phase) and then broke and lysed.

Results showed that, the major amount of glucose was consumed in the early 180 h of process that is the trophophase of *P. bervicompactum* and 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 fermentation, then reached to the stationary phase and finally decreased after about 280 h. In other word, the stationary phase of *P. bervicompactum* 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. bervicompactum* 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. In comparison to the previous research [15] (presented in Table II) on submerged culture, mycophenolic acid production by *P. bervicompactum* ATCC 16024 in 250 mL shake flask with 1.7 g/L and 5.012 mg/Lh productivity, it was concluded that the MPA production of *P. bervicompactum* MUCL 19011 is approximately the same and existing about 0.3 g/L difference between these two strains may be a normal phenomena. However by using a genetically modified derivative of *P. bervicompactum* ATCC 16024, MPA production has been increased to 5.3 g/L (more than 3 fold) [15].

A few studies have been performed on MPA production in solid-state fermentation process (Table II) using of *P. bervicompactum* ATCC 9056 with no satisfactory results

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International Journal of Chemical and Molecular Engineering
Vol:3, No:8, 2009

(0.38 g/Kg MPA production and 3.811 mg/Kg.h productivity) [4], but the same process by *P. bervicompactum* ATCC 16024 resulted in 3.29 g/Kg MPA production and 23.01 mg/Kg.h productivity [14]. However in solid-state fermentation by *P. roqueforti* 603-A, MPA production was higher (4.06 g/Kg) but the longer fermentation time, decreased the process productivity to 16.92 mg/Kg.h [7].
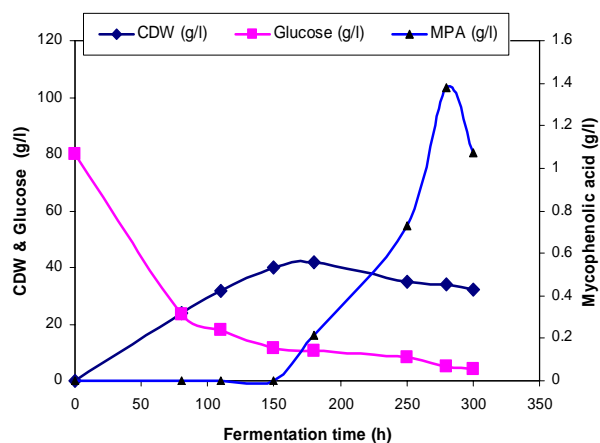

Fig. 1 Glucose, cell dry weight (CDW) and MPA concentration profiles in shake flask fermentation process with base medium at 200 rpm shaker, 27 ^{0}C and pH 6, for 300 hours

B. Effect of Methionine and Acetate on MPA Production in Shake Flask

Shake flask fermentation processes with base medium enriched by different methionine and acetate concentrations were performed separately and MPA concentration in each flask was measured after 280 hours (Table I). Results showed that maximum MPA production, 1.763 g/L, was obtained after addition of 2 g/L methionine to the base medium (final concentration of methionine was 2.5 g/L). In this condition, MPA yield and productivity were 23.8 mg/g glucose and 6.3 mg/L. h respectively that were higher than MPA production by using base medium containing of 0.5 g/L methionine. Further methionine concentrations had not any more positive effect on MPA production. As the mentioned, methionine is the main source of MPA methyl groups and inters directly to the special MPA biosynthesis pathway. Thus increased MPA production with addition of methionine to base medium could be accepted. Addition of different amounts of acetate had not effect on MPA production as shown in Table I. One probable reason for this phenomenon could be the consumption of this additional concentration range of acetate in other metabolic pathways in the cell metabolism. Thus, acetate may be gone to other pathways such as TCA cycle, amino acid biosynthesis, biomass formation and mevalonate pathway.

TABLE I
THE EFFECT OF METHIONINE AND ACETATE CONCENTRATIONS ON MPA PRODUCTION IN SHAKE FLASK

	base media	methionine conc. (g/L)						acetate conc. (g/L)				
		1	**1.5**	**2**	**2.5**	**3**	**4**	**1**	**2**	**3**	**4**	**5**
MPA Conc. (g/l)	1.379	1.383	1.416	1.428	1.763	1.698	1.695	1.382	1.380	1.378	1.381	1.380
Yield	0.018 6	0.018 7	0.019 1	0.019 3	0.023 8	0.022 9	0.022 9	0.018 7	0.018 6	0.018 6	0.018 7	0.018 6
Productivity (mg/ L.h)	4.92	4.94	5.06	5.1	6.30	6.06	6.05	4.93	4.93	4.92	4.93	4.93

TABLE II
COMPARISON OF MPA PRODUCTION IN VARIOUS FERMENTATION PROCESSES

Process	Microbial strain	Time (h)	MPA titer	Yield (g/g glucose)	Productivity (mg/L h)	Ref
Solid-state in Petri dish	*P. b*[a] ATCC 9056	100	0.38 g/kg	-	3.8 11	[4]
Solid-state in Petri dish	*P. roqueforti* 603-A	240	4.06 g/Kg	-	16.92	[7]
Solid-state in shake flask	*P. b* ATCC 16024	144	3.29 g/kg	-	23.01	[14]
Submerged in shake flask	*P. b* ATCC 16024	336	1.7 g/L	-	5.0 12	[15]
Submerged in shake flask	*P. b* No. 5-1[b]	336	5.3 g/L	-	15.8	[15]
Submerged in shake flask	*Byssochlamys nivea*	670	0.02 g/L	-	0.03	[16]
Submerged in shake flask [c]	*P. b* MUCL 19011	280	1.379 g/L	0.0186	4.9	this work
Submerged in shake flask [d]	*P. b* MUCL 19011	280	1.763 g/L	0.0238	6.3	this work

a   *P. brevicompactum*
b   *P. brevicompactum* No. 5-1 was a genetically modified high-yield mutant derived from ATCC 16024
c   base medium
d   base medium including 2.5 g/L methionine

IV. CONCLUSION

MPA production by *P. bervicompactum* MUCL 19011 in shake flask with base medium containing of 80 g/L initial glucose concentration was started at 180 hours after inoculation and reached to maximum concentration of 1.379 g/L after 280 hours and then decreased. MPA yield and productivity was 18.6 mg/g glucose and 4.9 mg/L.h respectively.

MPA production in submerged shake flask with base medium, using *P. bervicompactum* MUCL 19011 was approximately similar to *P. bervicompactum* ATCC 16024 of previous researches (Table II).

Maximum MPA concentration, product yield and productivity (1.763 g/L, 23.8 mg/g glucose and 6.3 mg/L. h respectively) were obtained after using 2.5 g/L methionine. Further methionine concentrations had not any more positive effect on MPA production. Also addition of acetate to culture medium had not effect on MPA production.

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