

Production of Mycophenolic Acid by *Penicillium brevicompactum* in a Submerged Batch Culture: Optimization of Culture Conditions Using Taguchi Approach

Fatemeh Ardestani

Department of Chemical Engineering, Qaemshahr Branch,
Islamic Azad University, Qaemshahr, Iran

(Received: Feb. 24, 2013; Accepted in Revised Form: April 9, 2013)

Abstract: Mycophenolic acid (MPA) is an antibiotic and immunosuppressive agent, as a secondary metabolite of *Penicillium brevicompactum*. Parameter optimization of culture composition using Test plan L16, available in the form of an orthogonal array and software for automatic design and analysis of the experiments, both based on Taguchi approach was performed in MAP production process by *Penicillium brevicompactum* ATCC 16024 in a batch submerged culture. Optimal levels of key parameters including glucose, enzymatically hydrolyzed casein, methionine and glycine concentrations were determined. Optimum glucose, enzymatically hydrolyzed casein, methionine and glycine concentrations were obtained 80, 25, 1.2 and 20 g. L⁻¹, respectively. Theoretically expected and also experimentally actual obtained mycophenolic acid concentrations under the optimal conditions were 2.025 and 1.995 g. L⁻¹, respectively with a good consistency as 98.5%. Analysis showed the glucose concentration was found to be the most significant factor as well as glycine concentration was the less important factor on MPA production in the investigated process.

Key words: Mycophenolic acid • Optimization • *Penicillium brevicompactum* • Submerged culture • Taguchi method

INTRODUCTION

Mycophenolic acid [6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid MPA] as an antibiotic substance for bacteria, fungi and viruses [1-3] is produced by several species of *Penicillium* [4]. MPA also has been showed a high anti-HIV activity and was introduced as a new strategy to overcome on HIV in human body [5, 6]. In addition, MPA and some of its derivatives such as mycophenolate mofetil (MMF) and sodium mycophenolate have immunosuppressive properties [7, 8]. The food and drug administration (FDA) approved them as immunosuppressive drugs for decreasing the incidence of graft rejection after organ transplantation [9-12]. MPA has an inhibitory effect on inosine monophosphate dehydrogenase to block conversion of inosine monophosphate to guanosine monophosphate [13, 14]. In other word, MPA prevents the biosynthesis of DNA and RNA, especially in B and T

lymphocytes cells, which consequently stops cell proliferation completely [15-17]. Several species of *Penicillium* especially *P. brevicompactum* [18-26], *Penicillium stoloniferum* [27] and *Penicillium roqueforti* [28] can produce MPA as a secondary metabolite by submerged and solid-state fermentation processes. MPA production processes have been done in batch [18-28], repeated batch with immobilized cells [20] and continuous [18] modes. Process optimization for different cell products was done in some previous researches [29-30]. Investigation of the effects of methionine and acetate concentrations on MPA production by *P. brevicompactum* MUCL 19011 in submerged batch culture [19], introducing of an optimized medium for MPA production by *P. brevicompactum* ATCC 16024 using the orthogonal methodology [20] as well as optimization of MPA production in solid state fermentation using response surface methodology [23] are some of these efforts.

The objective of this study is optimization of *Penicillium brevicompactum* ATCC 16024 culture composition to reach the maximum MPA production in batch submerged culture.

MATERIALS AND METHODS

Microorganism and Inoculum: *Penicillium brevicompactum* that produces mycophenolic acid was employed in present study. A stock culture of *P. brevicompactum* ATCC 16024 was prepared as slants containing potato dextrose agar (PDA) at pH 6.5, sterilized at 120 °C for 15 min and incubated at 27 °C for one week, then, stored at 4 °C [18].

Spore suspension was used as inoculum. For inoculum preparation, spores were transferred to PDA petri plates and incubated at 27 °C for 3 days. Then, spores were shaved and extracted with a sterile loop under aseptic conditions and dispersed in distilled water. The number of spores in inoculum were counted with a Thoma lam and adjusted to 10⁷-10⁸ spores per mL [18].

Qualitek 4 Software for Experiment Design: Qualitek 4 software for automatic design and analysis of Taguchi experiments was used to identification of the individual influence of each factor, determination of the optimum conditions and also estimation of performance at the optimum condition.

In Taguchi method, orthogonal arrays are used to describe a large number of experimental situations mainly to reduce experimental errors and to enhance the efficiency and reproducibility of laboratory experiments [29]. The symbolic design of these arrays represents the numbers of the experimentation, e.g. L16 has 16 trials. In this work, four factors, each one in four different levels were studied (Table 1).

The layout of the L16 orthogonal arrays used in the present study is presented in Table 2.

Medium Preparation: Using Taguchi approach, 16 shake flasks with defined culture composition were prepared to evaluate the optimization procedures. All of those were contained 5 g.L⁻¹ of KH₂PO₄ and 1 g.L⁻¹ of MgSO₄•7H₂O.

Table 1: Factors and their levels assigned to different columns

Serial number	Factor	Level 1	Level 2	Level 3	Level 4
1	Glucose Concentration (g.L ⁻¹)	60	80	100	120
2	Enzymatically Hydrolyzed Casein (g.L ⁻¹)	10	15	20	25
3	Methionine Concentration (g.L ⁻¹)	0.1	0.4	0.8	1.2
4	Glycine Concentration (g.L ⁻¹)	5	10	15	20

Table 2: The layout of the L16 orthogonal arrays designed using Taguchi method

Run Number	Factor Level			
	Glucose Concentration	Enzymatically Hydrolyzed casein	Methionine Concentration	Glycine Concentration
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	1	4	4	4
5	2	1	2	3
6	2	2	1	4
7	2	3	4	1
8	2	4	3	2
9	3	1	3	4
10	3	2	4	3
11	3	3	1	2
12	3	4	2	1
13	4	1	4	2
14	4	2	3	1
15	4	3	2	4
16	4	4	1	3

Also, 1 mL.L⁻¹ of trace elements mixture, which included (g.L⁻¹): FeSO₄•7H₂O, 2.2; CuSO₄•5H₂O, 0.3; ZnSO₄•7H₂O, 2.4; MnSO₄•4H₂O, 0.16 and KMoO₄, 0.2 was added to each shake flask [20]. The glucose (GLU), enzymatically hydrolyzed casein (CHE), methionine (MET) and glycine (GLY) concentrations were different in each shake flask based on experiment design proposed by Taguchi orthogonal array.

All medium components were autoclaved separately at 121 °C for 15 min. Glycine and methionine solutions as well as the trace elements mixture were sterilized by a 0.2 µm filter (Millipore, USA). For all shake flasks, the medium pH was adjusted to 6.0 before autoclaving using a 2N HCl or NaOH solution. Then, to prepare final medium, all sterile components were transferred to a proper sterile vessel and mixed together, under aseptic conditions.

Fermentation Process: A 50 mL liquid medium was used in 250 mL shake flasks. A rotary shaker incubator (JAHL-JSH 20LUR, Iran) was used for this reason. After inoculation with 0.5 mL of the spore suspension (~5×10⁷ cell per mL) under aseptic conditions, shake flasks were incubated at 27 °C for 300 h [18].

Analytical Procedures: Mycophenolic acid concentration was assayed by high performance liquid chromatography (HPLC) method as mentioned in the previous work [20]. HPLC grade MPA (Biochemica, Germany) was used as standard for analysis. The stock solution of MPA and working standard solutions were prepared as mentioned before [20]. MPA measurement experiments were repeated twice.

RESULTS AND DISCUSSIONS

MPA Production in Batch Fermentation: MPA production results are presented in Table. 3. MPA concentration was varied from the lowest amount 1.22 g. L⁻¹ for trial number 14 to the highest value 1.893 g. L⁻¹ at trial number 7. At trial number 7, the concentrations of glucose, enzymatically hydrolyzed casein, methionine and glycine were in levels 2, 3, 4 and 1, respectively. In the other word the concentrations were 80, 20, 1.2 and 5 g. L⁻¹ for glucose, enzymatically hydrolyzed casein, methionine and glycine, respectively.

Table 3: The obtained results of MPA production in batch fermentation by *Penicillium brevicompactum*

Run Number	MPA Concentration (g/L)	
	Repeat 1	Repeat 2
1	1.390	1.388
2	1.421	1.420
3	1.647	1.645
4	1.879	1.871
5	1.439	1.430
6	1.669	1.671
7	1.890	1.893
8	1.853	1.846
9	1.267	1.259
10	1.329	1.327
11	1.410	1.410
12	1.390	1.387
13	1.329	1.326
14	1.224	1.220
15	1.289	1.288
16	1.297	1.299

Table 4: The main effects of each factor in optimization process of MPA production by *Penicillium brevicompactum*

Serial Number	Factor	Level 1	Level 2	Level 3	Level 4	L2-L1
1	Glucose Concentration (g.L ⁻¹)	3.922	4.616	2.581	2.114	0.693
2	Enzymatically Hydrolyzed Casein (g.L ⁻¹)	2.619	2.927	3.709	3.979	0.307
3	Methionine Concentration (g.L ⁻¹)	3.139	2.755	3.359	3.980	-0.384
4	Glycine Concentration (g.L ⁻¹)	3.245	3.458	3.047	3.482	0.213

Table 5: The interactions of different factors in optimization process of MPA production by *Penicillium brevicompactum*

Serial Number	Factors	Columns	SI (%)	Col	Opt
1	CHE*GLY	2*4	22.4	6	[3,1]
2	CHE*MET	2*3	22.2	1	[3,4]
3	GLU*MET	1*3	19.96	2	[2,4]
4	GLU*CHE	1*2	14.83	3	[2,3]
5	GLU*GLY	1*4	5.13	5	[2,1]
6	MET*GLY	3*4	0.89	7	[4,1]

^a Columns—represent the column locations to which the interacting factors are assigned.

^b SI—interaction severity index (100% for 90° angle between the lines, 0% for parallel lines).

^c Col—shows column that should be reserved if this interaction effect were to be studied (2-L factors only).

^d Opt—indicates the factor levels desirable for the optimum conditions (based strictly on the first two levels). If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without considerations of any interaction effects.

Table 6: Proposed optimum conditions to achieve the maximum MPA production

Serial Number	Factor	Level Description	Level	Contribution
1	Glucose Concentration (g.L ⁻¹)	80	2	1.307
2	Enzymatically Hydrolyzed Casein (g.L ⁻¹)	25	4	0.670
3	Methionine Concentration (g.L ⁻¹)	1.2	4	0.671
4	Glycine Concentration (g.L ⁻¹)	20	4	0.174

Table 7: ANOVA table in optimization process of MPA production by *Penicillium brevicompactum*

Serial Number	Factor	DOF (f)	Sums of Squares (S)	Variance (V)	F-Ratio (F)	Pure Sum (S')	PercentP(%)
1	Glucose Concentration (g.L ⁻¹)	3	15.67	5.223	62.57	15.419	62.854
2	Enzymatically Hydrolyzed Casein (g.L ⁻¹)	3	5.105	1.701	20.384	4.854	19.778
3	Methionine Concentration (g.L ⁻¹)	3	2.925	0.975	11.679	2.674	10.902
4	Glycine Concentration (g.L ⁻¹)	3	0.581	0.193	2.322	0.331	1.349
	Other/Error	3	0.25	0.083			5.107
	Total	15	24.532				100%

Analysis of the Results: Table 4 presented the average affects of each factor and interactions at the designed levels on MPA production. The difference between the average value of each factor at level 2 and 1 indexed the relative influence of the affect. The larger difference was indicated the higher influence. The sign of the difference value indicates whether the change from level 1 to level 2 or 3 increased (+) or decreased (-) the result (Table 4).

Thus, it can be seen that the glucose showed the highest influence to that of other factors and the least contribution was noticed with methionine concentration with the assigned levels. The interactions of different factors were presented in Table 5. The highest severity index percentage was showed for glycine as well as methionine concentration (the most little important factors) versus enzymatically hydrolyzed casein concentration as 22.4 and 22.2%, respectively. This parameter for methionine and enzymatically hydrolyzed casein concentration versus glucose concentration (the most impact factor) was evaluated 19.96 and 14.83%, respectively. The severity index percentage for glycine concentration (the least influence factor) versus glucose concentration (the highest influence factor) was only 5.13%. Also, the least severity index percentage was obtained for glycine concentration versus methionine concentration (two the less important factors) as 0.89%. These results suggest that in optimizing MPA production process parameters, the influence of one factor on MPA production was dependent on the condition of the other factors. Proposed optimum conditions to achieve the highest MPA production are given in Table 6. Based on the results, optimum conditions for the glucose concentration was its level 2 (80 g. L⁻¹). Other three factors (enzymatically hydrolyzed casein, methionine and glycine concentrations) must adjust at their level 4 (25, 1.2 and 20 g. L⁻¹, respectively) to achieve the optimum

condition for MPA production. The contribution of each factor to reaching this product yield is presented in the mentioned table too. The results showed glucose concentration had a significant contribution and glycine concentration played the least role in MPA production in the investigated process.

The percentage contribution of each factor is shown in the last column of ANOVA table (Table. 7). As can see in the table, glucose concentration was the most influence factor in MPA production with 62.854% confidence level and a considerable higher level in comparison with other factors. While, sum of the contribution of other three factors (enzymatically hydrolyzed casein, methionine and glycine concentrations) was only about 32%. Glycine concentration showed negligible influence as 1.349%. The analysis showed that the expected result for MPA concentration under optimal conditions was 2.025 g. L⁻¹. At the next step, MPA production under the proposed optimum conditions was performed experimentally in a batch submerged culture of *Penicillium brevicompactum*. An acceptable consistency of 98.5% was observed between the theoretical suggested MPA concentration and its actually obtained amount in optimum conditions.

CONCLUSION

Four effective factors each one in four different levels were identified in the MPA production by *Penicillium brevicompactum* ATCC 16024 to achieve its maximum product yield. Based on the analysis, glucose concentration was found to be the most significant factor on MPA production in the investigated process. Glycine concentration was the less important factor in MPA production. The optimum culture composition to achieve maximum MPA yield was determined. Using Taguchi methodology for optimizing the yield of MPA production was found to be an efficient strategy.

ACKNOWLEDGMENTS

The present work was accomplished and financially supported by Islamic Azad University, Qaemshahr Branch.

REFERENCES

1. Robertson, C.M., L.L. Hermann and K.M. Coombs, 2004. Mycophenolic acid inhibits *avian reovirus* replication. *Antiviral Research*, 64: 55-61.
2. Hermann, L.L. and K.M. Coombs, 2004. Inhibition of *reovirus* by mycophenolic acid is associated with the M1 genome segment. *Virology*, pp: 6171-6179.
3. Diamond, M.S., M. Zachariah and E. Harris, 2002. Mycophenolic acid inhibits *dengue virus* infection by preventing replication of viral RNA. *Virology*, 304: 211-221.
4. Vinokurova, N.G., N.E. Ivanushkina, G.A. Kochkina, M.U. Arinbasarov and S.M. Ozerskaya, 2005. Production of mycophenolic acid by fungi of the genus *Penicillium* link. *Applied Biochemistry and Microbiology*, 41: 83-86.
5. Ichimura, H. and J.A. Levy, 1995. Polymerase substrate depletion: a novel strategy for inhibiting the replication of the human immunodeficiency virus. *Virology*, 211: 554-560.
6. Margolis, D., A. Heredia, J. Gaywee, D. Oldach, G. Drusano and R. Redfield, 1999. Abacavir and mycophenolic acid an inhibitor of inosine monophosphate dehydrogenase, have profound and synergistic anti-HIV activity. *Journal of Acquired Immune Deficiency Syndromes*, 21: 362.
7. Allison, A.C. and E.M. Eugui, 1993. The design and development of an immunosuppressive drug mycophenolate mofetil. *Springer Semin Immunopathol*, 14: 353-380.
8. Platz, K.P., H.W. Sollinger, D.A. Hullett, D.N. Eckhoff, E.M. Eugui and A.C. Allison, 1991. Rs-61443-A new potent immunosuppressive agent. *Journal of Transplantation*, 51: 27-31.
9. Mele, T.S. and P.F. Halloran, 2000. The use of mycophenolate mofetil in transplant recipients. *Immunopharma*, 47: 215-245.
10. Halloran, P.F., 2004. Immunosuppressive drugs for kidney transplantation. *The New England Journal of Medicine*, 351: 2715-29.
11. Geevasinga, N., L. Wallman and C. Katelaris, 2005. Mycophenolate mofetil: a review of indications and use in a large tertiary hospital. *Iranian Journal of Allergy, Asthma and Immunology*, 4(4): 159-166.
12. Allison, A.C., 2000. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharma*, 47: 63-83.
13. Sintchak, M.D. and E. Nimmesgern, 2000. The structure of inosine 5-monophosphate dehydrogenase and the design of novel inhibitors. *Immunopharma*, 47: 163-184.
14. Jonsson, C.A. and H. Carlsten, 2003. Mycophenolic acid inhibits inosine 5-monophosphate dehydrogenase and suppresses immunoglobulin and cytokine production of B cells. *International Immunopharmacology*, 3: 31-37.
15. Allison, A.C. and E.M. Eugui, 2000. Mycophenolate mofetil and its mechanism of action. *Immunopharmacology*, 47: 85-118.
16. Wu, J.C., 1994. Mycophenolate mofetil: Molecular mechanisms of action. *Drug Dis*, 2: 185-204.
17. Allison, A.C. and E.M. Eugui, 1996. Purine metabolism and immunosuppressive effects of mycophenolate mofetil. *Clinical Transplantation*, 10: 77-84.
18. Ardestani, F., S.S.A. Fatemi, B. Yakhchali, S.M. Hosseyni and G. Najafpour, 2010. Evaluation of mycophenolic acid production by *Penicillium brevicompactum* MUCL 19011 in batch and continuous submerged cultures. *Biochemical Engineering*, 50: 99-103.
19. F., S.S.A. Fatemi, B. Yakhchali, S.M. Hosseyni and G. Najafpour, 2009. The effects of methionine and acetate concentrations on mycophenolic acid production by *Penicillium brevicompactum* MUCL 19011 in submerged culture. *International Journal of Natural and Engineering Sciences*, 2(2): 83-86.
20. Xu, Z. and S. Yang, 2007. Production of mycophenolic acid by *Penicillium brevicompactum* immobilized in a rotating fibrous-bed bioreactor. *Enzyme and Microbial Technology*, 40: 623-628.
21. Bartman, C.D., D.L. Doerfler, B.A. Bird, A.T. Remaley, J.N. Peace and I.M. Campbell, 1981. Mycophenolic acid production by *Penicillium brevicompactum* on solid media. *Applied and Environmental Microbiology*, pp: 729-736.
22. Doerfler, D.L., C.D. Bartman and I.M. Campbell, 1979. Mycophenolic acid production by *Penicillium brevicompactum* in two media. *Canadian Journal of Microbiology*, 25: 940-943.

23. Sadhukhan, A.K., M.R. Murthy, R.A. Kumar, E. Mohan, G. Vandana, C. Bhar and V.K. Rao, 1999. Optimization of mycophenolic acid production in solid state fermentation using response surface methodology. *Journal of Industrial Microbiology and Biotechnology*, 22: 33-8.
24. Alania, F., J.A. Grove, W.A. Anderson and M.M. Young, 2009. Mycophenolic acid production in solid-state fermentation using a packed-bed bioreactor. *Biochemical Engineering Journal*, 44: 106-110.
25. Sircar, A., Sh. Suryanarayan, A.P. Khedkar, P. Subramaniyam and Sh. P. Tambe, 2005. Manufacture and purification of Mycophenolic acid. United States Patent, PCT No: PCT/INOO/00017.
26. Ozaki, H., M. Ishihara, T. Kida, S. Yamanaka and H. Shibai, 1987. Mycophenolic acid production by drug-resistant and methionine or glutamic-acid requiring mutants of *Penicillium brevicompactum*. *Agricultural and Biological Chemistry*, 51: 2509-2514.
27. Castanhas, B.G., J. Rui, M.C. Vazao, J. Joao, B.F.T. Almedia and M. Monica, 2006. P.C. Barbosa and E.P. Antonio, Process for the production of mycophenolic acid. European Patent, EP1624 070.
28. Lafont, P., J.P. Debeaupuis, M. Gaillardin and J. Payen, 1979. Production of mycophenolic acid by *Penicillium roqueforti* Strains. *Applied and Environmental Microbiology*, 37: 365-368.
29. Azin, M., R. Moravej and D. Zareh, 2007. Production of xylanase by *Trichoderma longibrachiatum* on a mixture of wheat bran and wheat straw: Optimization of culture condition by Taguchi method. *Enzyme and Microbial Technology*, 40: 801-805.
30. Ashish, K., K. Naveen, M.M. Hasan, R. Chaudhary, A. Noor Siddiquee and A.K. Zahid, 2010. Production of biodiesel from thumba oil: optimization of process parameters. *Iranica Journal of Energy and Environment*, 1(4): 352-358.

Persian Abstract

DOI: 10.5829/idosi.ijee.2013.04.04.03

چکیده

مایکوفنولیک اسید به عنوان یک متابولیت ثانویه پنی سیلیوم بروی کامپکتوم با خواص آنتی بیوتیکی و متوقف کننده سیستم ایمنی شناخته شده است. بهینه سازی پارامترهای ترکیبی محیط کشت با استفاده از آرایه ارتوگونال L16 و نرم افزار تاگوچی برای طراحی خودکار آزمایش ها و تجزیه و تحلیل نتایج آزمایشگاهی در فرایند تولید مایکوفنولیک اسید توسط پنی سیلیوم بروی کامپکتوم در محیط کشت غوطه ور ناپیوسته انجام گردید. سطوح بهینه پارامترهای کلیدی شامل غلظت گلوکز، کازئین هیدرولیز شده آنزیمی، متیونین و گلیسین به ترتیب برابر با ۸۰، ۲۵، ۱/۲ و ۲۰ گرم بر لیتر به دست آمد. غلظت مایکوفنولیک اسید تولید شده در شرایط بهینه در آزمایشگاه و همچنین غلظت تولیدی مورد انتظار تئوری آن تحت شرایط بهینه به ترتیب برابر با ۱/۹۹۵ و ۲/۰۲۵ گرم بر لیتر به دست آمد که دارای ۹۸ درصد تطابق با یکدیگر بوده اند. تجزیه و تحلیل نتایج نشان داد که غلظت گلوکز بیشترین سطح تاثیر را در تولید مایکوفنولیک اسید داشته است و غلظت گلیسین نیز دارای کمترین میزان تاثیر بر تولید این ماده بوده است.
