Iranica Journal of Energy & Environment 4 (4): 330-335, 2013
ISSN 2079-2115
IJEE an Official Peer Reviewed Journal of Babol Noshirvani University of Technology
DOI: 10.5829/idosi.ijee.2013.04.04.03

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

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(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, enzymaticaly hydrolyzed casein, methionine and glycine concentrations were determined. Optimum glucose, enzymaticaly 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.

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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 10^7-10^8 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 KH_2PO_4 and 1 $g.L^{-1}$ of $MgSO_4 \cdot 7H_2O$.

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	Enzymaticaly 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

	Factor Level			
Run Number	Glucose Concentration	Enzymaticaly 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}): $FeSO_4$•$7H_2O$, 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 was added to each shake flask [20]. The glucose (GLU), enzymaticaly 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 (~5×10^7 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, enzymaticaly 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, enzymaticaly hydrolyzed casein, methionine and glycine, respectively.

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

	MPA Concentration (g/L)	
Run Number	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	Enzymaticaly 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	Enzymaticaly 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	Enzymaticaly 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 enzymaticaly hydrolyzed casein concentration as 22.4 and 22.2%, respectively. This parameter for methionine and enzymaticaly 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 (enzymaticaly 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 (enzymaticaly 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 Isalmic Azad University, Qaemshahr Branch.

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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.