OPTIMIZING FERMENTATION MEDIUM TO PRODUCE CYCLOSPORIN A USING RESPONSE SURFACE METHODOLOGY

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Abstract

Response surface methodology (RSM) is a three factorial model which illustrates the relationship between one or more independent variables. RSM can be used to optimize the fermentation medium for the production of Cyclosporin A from the isolate *Tolypocladium inflatum.* The optimal point of the response surface area is predicted by using a second-degree polynomial model and applying the statistic model obtained from the central composite design (CCD). The results of optimizing the fermentation medium for Cyclopsorin using the three independent variables of glucose, casein, and KH_2PO_4 show that all three of the independent variables affect the production of Cyclosporin A. There is a positive interaction between the independent variables of glucose and casein, however, there is no visible interaction between glucose with KH_2PO_4 and casein with KH_2PO_4. By using the mathematical model the total optimum result obtained is 1230.5 mg L^{-1}, glucose concentrate 28.5 g L^{-1}, KH_2PO_4 concentrate 0.74 gL^{-1}, and casein concentrate 9.8 g L^{-1}. Laboratory validation shows that Cyclosporin A productivity is 1197.285 mg L^{-1}. There is a value difference of 2.7% between the expected productivity of Cyclosporin A using the mathematical model and the actual production in laboratory tests.

Abstrak

Optimasi Medium Fermentasi untuk Produksi Cyclosporin A Menggunakan *Response Surface Methodology*. *Response surface methodology* (RSM) merupakan model tiga faktorial yang dapat menjelaskan hubungan antar variabel independen satu sama lainnya. RSM dapat digunakan untuk optimasi medium fermentasi produksi Cyclosporin A menggunakan isolat *Tolypocladium inflatum.* Daerah permukaan respon yang merupakan titik optimum dapat diduga dengan menggunakan model polinomial orde kedua dengan menerapkan model statistik *central composite design* (CCD). Hasil optimasi medium fermentasi produksi Cyclopsorin A dengan menggunakan variabel bebas glukosa, kasein, dan KH_2PO_4 menunjukkan bahwa ketiga variabel bebas tersebut memiliki pengaruh nyata terhadap produktivitas Cyclosporin A. Ada interaksi positif diantara variabel bebas glukosa dengan kasein, namun demikian tidak terdapat interaksi nyata diantara glukosa dengan KH_2PO_4 dan kasein dengan KH_2PO_4. Dengan menggunakan model matematik diperoleh data titik optimum sebesar 1230.5 mg L^{-1} pada konsentrasi glukosa 28.5 g L^{-1}, konsentasi KH_2PO_4 0.74 g L^{-1}, dan konsentrasi kasein 9.8 g L^{-1}. Hasil validasi data yang dilakukan dilaboratorium diperoleh produktivitas Cyclosporin A sebesar 1197.28 mg L^{-1}. Dengan demikian terdapat perbedaan 2.7% antara produktivitas Cyclosporin A yang dihasilkan dari nilai dugaan menggunakan model matematik dengan nilai sebenarnya yang diperoleh dari hasil percobaan di laboratorium.

Keywords: Cyclosporin A, optimization, response surface methodology

1. Introduction

Cyclosporin A is a member of a group of cyclic undecapeptides with antiinflammatory, immuno-suppressive, antifungal and antiparasitic properties [1]. Aside from that, Cyclosporin has the characteristic of an *immunosuppressive* that is relatively non-toxic to bone marrow [2-5]. Cyclosporin is known to be produced from *Tolypocladium inflatum* by using the liquid fermentation method [6]. Apart from being produced from *T. inflatum*, Cyclosporin can also be derived from *Rhizopus arrhizus* NRC Fr 113, *Fusarium solani* NRC F.F.13, and *Fusarium oxysporum* NRC FF105. To increase the production of Cyclosporin several modifications to the fermentation medium are carried out [7-8]. Adding L-valine to the solid fermentation is known to increase Cyclosporin A productivity [9-10]. Glucose carbon is known to be the best source of carbon

in the process of Cyclosporin A production [11]. The influence of environmental factors like pH, aeration, and the inocculum density factor also affect Cyclosporin A productivity [12]. However, there is a scarcity of published data on optimizing the fermentation media by using the response surface methodology method (RSM). RSM can be used to evaluate relationships between several explanatory variables and one or more response variables. The aim of this research is to determine the optimum concentration of several variables of the fermentation medium to obtain the highest productivity of Cyclosporin A using RSM.

2. Methods

Micro-organisms. Isolates used in this research are *T. inflatum* cultures taken from the Microbiology Laboratory of Biotechnology Research BPPT. The laboratory culture is maintained and kept in an agar medium containing 10 g L^{-1}malt extract, 5 g L^{-1} yeast extract (MYA) and then stored in a refrigerator at a temperature of 4 ºC.

Preparation of inoculum and vegetative culture. The isolate *T. inflatum* kept at a temperature of 4 ºC is refreshed in a new batch of MYA media, positioned at a slanted angle and incubated at a temperature of 25 ºC for 7 days. After spores have grown on the slanted surface, 0.1% of Tween 20 solution is added and then shaken up. As much as 1 mL of the solution containing approximately 10^8 spores is inoculated into 50 mL of the vegetative medium in a 250 L flask. The composition of the vegetative medium is as follows; glycerin 25 g L^{-1}, KH_2PO_4 5 g L^{-1}, peptone, 10 g L^{-1}, NaCl 5 g L^{-1}, demineralised water 50 mL, and the pH is set at 6.1. The vegetative culture is incubated at a temperature of 25 ºC and agitated at 240 rpm for 48 hours.

Fermentation stage. The fermentation medium used in this research is as follows; glucose x g L- (as the optimizing concentration used in RSM), casein y g L^{-1} (as the optimizing concentration used in RSM), KH_2PO_4 z g L^{-1} (as the optimizing concentration used in RSM), L-valine 4 g L^{-1}, (NH_4) $2HPO_4$ 10 g L^{-1}, and the micro compounds (trace elements) Fe $SO_4.7H_20$ 0.05 g L^{-1}, $ZnSO_47H_2O$ 0.0044 g L^{-1}, $CuSO_4$ 5 H_20 0.008 g L^{-1}, $MnCl_2$ $4.H_2O$ 0.0018 g L^{-1}, (NH_4) $6Mo_7O_{24}4H_2O$ 0.0002 g L^{-1} and the medium pH set at 6.1. A total of 5 mL of the vegetative culture is innoculated into 50 mL of the fermentation medium and incubated at a temperature of 25 ºC where it is agitated at 240 rpm for 14 days.

Table 1. Range and Levels of Variables Tested to Optimise the Fermentation Medium to Produce Cyclosporin A

Variabel yang diuji	Kisaran dan taraf				
	-1.68	-1	0	1	1.68
Konsentrasi glukosa (g L^{-1})	16.6	20	25	30	33.4
Konsentrasi KH_2PO_4	0.33	0.5	0.75	1	1.17
Konsentrasi kasein (g L^{-1})	4.64	6	8	10	11.36

Response surface methodology. This research uses the central composite design (CCD) model with 3 independent variables to obtain a response to produce Cyclosporin A, which in this case is in the form of Cyclosporin A concentrate (mg L^{-1}). The trial model uses an 8 point fractional factorial 2^{3-1} design, 6 star points, and 6 central points, making a total of 20 experiments repeated twice. The central values used are glucose concentrate 25 g L^{-1}, casein concentrate 8 g L^{-1}, and KH_2PO_4 concentrate 0.75 g L^{-1}. The range and levels of the variables tested in the optimization tests are presented in Table 1. Defining the optimum points and variable relations were determined by using Design Expert 7.1. program.

Using the three variables a mathematical model is such:

$$Y = bo + b_1X_{1i} + b_2 X_{2i} + b_3 X_{3i} + b_{11}X_{1i}^2 + b_{22}X_{2i}^2 + b_{33}X_{3i}^2 + b_{12}X_{1i}X_{2i} + b_{12}X_{1i}X_{2i} + b_{13}X_{1i}X_{3i} + b_{23}X_{2i}X_{3i} \quad (1)$$

Y = Cyclosporin A concentrate (mg L^{-1})
X_1 = glucose concentrate (g L^{-1})
X_2 = KH_2PO_4 concentrate (g L^{-1})
X_3 = kasein concentrate (g L^{-1})

3. Results and Discussion

The influence of the combination of 3 independent variables of the Cyclosporin A fermentation medium consisting of A: glucose, B: KH_2PO_4; and C: Casein are tested by using the RSM and CCD model. The tests and responses of Cyclosporin A productivity experiments are presented in Table 2.

Results of the diversity analysis from the ANOVA analysis and the estimated coefficient model of successive optimal results are presented in Table 3 and Table 4.

Table 3 shows that the model used has a value F (F-test) of 22.7 and p-value (Prob > F) below 0.0001. The higher the F value or the lower the p-value is (Prob > F) the more significant is the relationship with the model used [13]. The model developed has the coefficient determination R2 value of 95% that shows the height of correlation between the values of observation and the values of assumption and the value adj R2 of 0.91 signifies that this model has high significance with the

Table 2. Central Composite Design Consists of 20 Designed Experiments and the Cyclosporin A Productivity Response

Glukosa ($g\ L^{-1}$)	KH_2PO_4 ($g\ L^{-1}$)	Kasein ($g\ L^{-1}$)	Notasi			Respon Cyclosporin A ($mg\ L^{-1}$)
X_1	X_2	X_3	X_1	X_2	X_3	
20	0.5	6	-1	-1	-1	885.1
30	0.5	6	1	-1	-1	892.1
20	1	6	-1	1	-1	895
30	1	6	1	1	-1	891.1
20	0.5	10	-1	-1	1	1090
30	0.5	10	1	-1	1	1158
20	1	10	-1	1	1	922
30	1	10	1	1	1	1218.7
16.6	0.75	8	-1.68	0	0	1009.6
33.4	0.75	8	1.68	0	0	1165.6
25	0.33	8	0	-1.68	0	1042.8
25	1.17	8	0	1.68	0	1066.6
25	0.75	4.64	0	0	-1.68	822.5
25	0.75	11.36	0	0	1.68	1014.4
25	0.75	8	0	0	0	1194.5
25	0.75	8	0	0	0	1194
25	0.75	8	0	0	0	1194.9
25	0.75	8	0	0	0	1195
25	0.75	8	0	0	0	1194
25	0.75	8	0	0	0	1193

Table 3. Diversity Analysis (ANOVA) Optimizing the Fermentation Medium for Cyclosporin A Production

Source	Sum of Squares	df	Mean Square	Value	p-value Prob > F
Model	333343.48	9	37038.16	22.76	<0.0001
A-glukosa	29132.46	1	29132.46	17.90	0.0017
B-KH_2PO_4	254.66	1	254.66	0.16	0.7
C-Kasein	96423.3	1	96423.3	59.25	<0.0001
AB	5896.98	1	5896.98	3.62	0.0861
AC	16290.13	1	16290.13	10.01	0.01
BC	1670.42	1	1670.42	1.03	0.3349
A^2	24221.85	1	24221.85	14.88	0.0032
B^2	39916.44	1	39916.44	24.53	0.0006
C^2	146429.59	1	146429.6	89.97	<0.0001
Residual	16274.5	10	1627.45		
Lack of Fit	16271.77	5	3254.35	5953.09	<0.0001

Information: C.V: 3.8%, R-Square: 0.95, Adj R-Squared: 0.91

independent variables of glucose, KH_2PO_4, and casein that strongly influence the response produced. This model also has a CV value of 3.8% that indicates a quite high level of accuracy (precision) in the tests, for the lower the CV value is the higher the level of accuracy is in the experiment [13].

Results of the diversity analysis and the coefficiency model estimate in Tables 3 and 4 show that glucose gives positive linear influence and a positive quadratic towards Cyclosporin A productivity. KH_2PO_4 does not appear to have linear influence on Cyclosporin A productivity which is evident from its p-value (Prob > F) of 0.7, however KH_2PO_4 does have a negative quadratic influence towards Cyclosporin A productivity. Casein shows a positive linear influence and a negative quadratic influence towards Cyclosporin A productivity.

Observing the interaction between the variables, the interaction between glucose and casein is seen to have a real p-value (Prob > F) of <0.01. This interaction has a positive influence on increasing Cyclosporin A concentrate. Therefore, changes in each variable concentrate will in turn affect the Cyclosporin A concentrate it produces. Still, there appeared to be no interaction between glucose and KH_2PO_4 or between casein and KH_2PO_4. Results of the Normality Test show that the normal model has distributed normally and freely with relatively homogeneous diversity (Figure 1). Following that, the results of the test used the coefficient model estimation of output data processing Design Expert 7.1 Program which showed that the mathematical formula developed from test results is such;

$$Y = 1194.72 + 46.19X_1 + 84.02X_3 + 45.13X_1X_3 - 41X_1^2 - 52.63X_2^2 - 100.8X_3^2 \quad (2)$$

Table 4. Estimated Coefficient Model of an Optimal Fermentation Medium for Cyclosporin A Production

Factor	Coefficient Estimate	p-value
Intercept	1194.73	<0.0001
A-glukosa	46.19	0.0017
B-KH_2PO4	-4.32	0.7007
C-Kasein	84.02	<0.0001
AB	27.15	0.0861
AC	45.13	0.01
BC	-14.45	0.3349
A^2	-41	0.0032
B^2	-52.63	0.0006
C^2	-100.8	<0.0001

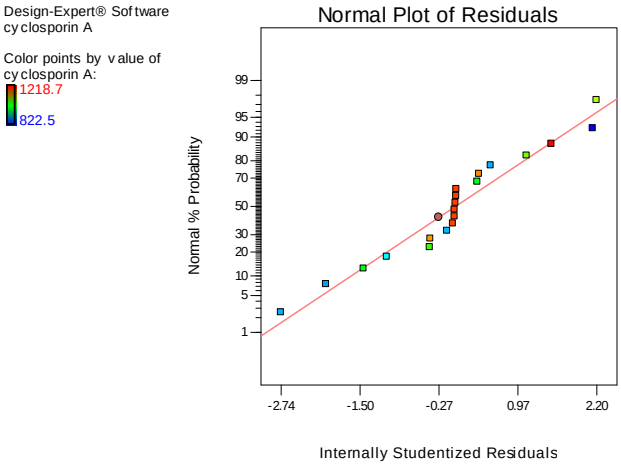

Figure 1. The Normal Probability Plot of Cyclosporin A Productivity Produced by Isolate *Toplypocladium inflatum*

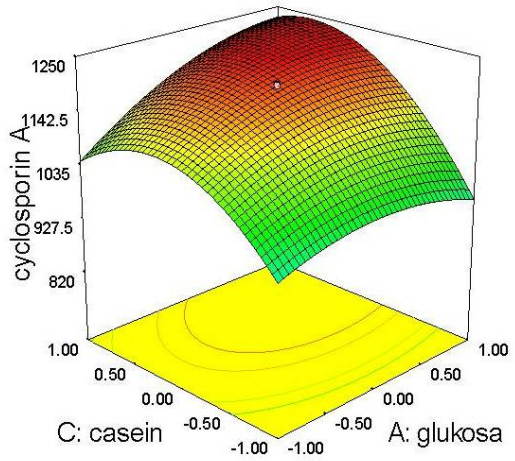

Figure 2. The Response Surface of Cyclosporin A Production Influenced by Glucose and Casein

Relationships between the variables of glucose and casein can be depicted by using the response surface as Figure 2.

Figure 2 shows that an increase in glucose and casein concentrates have an actual effect on the production of Cyclosporin A. Glucose is reported to be the best source of carbon in the process of Cyclosporin A production [11,14]. An increase of casein concentrate appears to have more influence on Cyclosporin A production than an increase of glucose concentrate. Relationships between the two variables show that there is interaction between one another.

Figure 3 showed that an increase in glucose and KH_2PO_4 concentrates have an actual influence on Cyclosporin A productivity. Mineral salts, especially those containing divalent ion strongly influence Cyclosporin productivity. This is closely linked to the reaction of the enzyme in the metabolism process of Cyclosporin A formation [15]. Furthermore, an increase in the KH_2PO_4 concentrate that exceeds the concentration 0.75 g L-1 level (0) shows a significant decline in Cyclosporin A production. This is due to the toxoid characteristics of the metal ion on the cell network in excessive concentrations.

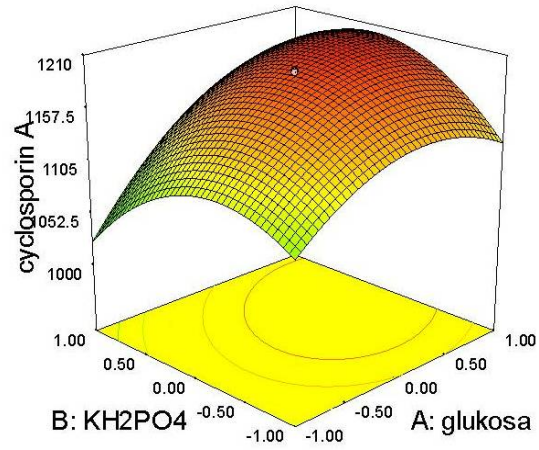

Figure 3. The Response Surface of Cyclosporin A Production Influenced by Glucose and KH_2PO_4

Figure 4 shows that an increase of casein concentrate and KH_2PO_4 concentrate has an actual effect on Cyclosporin A productivity. An increase of casein concentrate appears to have a greater effect on Cyclosporin A productivity than an increase of KH_2PO_4 concentrate. From the level of casein (-1) up to level (0) it appears that increasing levels of casein concentrate correlate with higher Cyclosporin A productivity. An increase in KH_2PO_4 concentrate positively effects a rise in Cyclosporin A productivity but only at level (0). Amongst the three variables used in the optimalization

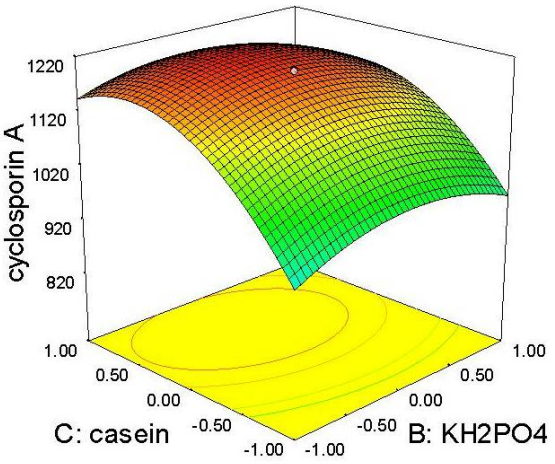

Figure 4. The Response Surface of Cyclosporin A Production Influenced by KH_2PO_4

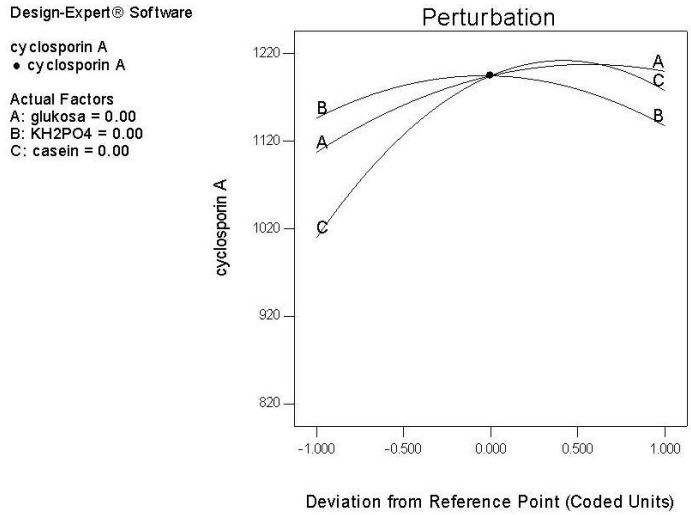

Figure 5. Relationship Changes within the Concentrates of Each Variable Towards the Changes in Cyclosporin A Productivity

process it is seen that the effect of changes in the casein concentrate appears to be greater than glucose and KH_2PO_4. This is seen from the pattern changes in the concentrates from the three variables that are depicted in the pertubation plot (Figure 5). Casein contains several amino acids that can act as a precursor in the metabolism process of Cyclosporin A formation [16].

The Design Expert 7 program mathematically determines the optimal value of the variables of glucose, casein, and KH_2PO_4. Results of the mathematical calculation give variables in encoded units, that is X_1= 0.7 or glucose = 28.5 g L^{-1}; X_2 = -0.05 or KH_2PO_4= 0.74 g L^{-1}; X_3= 0.88 or casein = 9.8 g L^{-1} with the response produced (expected value) of Y = 1230.5 mg L^{-1}. After carrying out validation of the model in the laboratory with a glucose concentrate of 28.5 g L^{-1}, KH_2PO_4 0.74 g L^{-1} and casein 9.8 g L^{-1} it was proven that Cyclosporin A productivity attained 1197.28 mg L^{-1}. The value of Cyclosporin A concentrate produced from the tests is 2.7% less than the estimated response value taken from the mathematical model used. The difference between the estimated value (model response) with the response value produced by laboratory tests of only 2.7% shows that the model used is appropriate and can be used to explain the test data used.

4. Conclusion

This research concludes that the independent variables used in the process of optimizing Cyclosporin A production have a real influence on Cyclosporin A productivity. The variable casein has the most influence on Cyclosporin A productivity compared to the other variables. Casein and glucose have a positive interaction in Cyclosporin productivity, however there appears to be no interaction between glucose and KH_2PO_4, and casein with KH_2PO_4. Results optimizing the fermentation medium produced 1197.28 mg L^{-1a} of Cyclosporin A, or 2.7% less than the expected value of Cyclosporin A productivity which shows that the model used is appropriate and can validate the test data used.

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