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Medium Optimization for The Production of Mycophenolic Acid by *Penicillium Brevicompactum* through Statistical Assessment At Shake Flask Level

Science

KEYWORDS : Mycophenolic acid, Penicillium, Plackett Burman, Factorial design, Screening of Medium components

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ABSTRACT

Penicillium brevicompactum is an fungal isolate used for the production of mycophenolic acid (MPA). MPA is an immunosupressive agent also having antineoplastic, antifungal, antiinflammatory properties. One of the efforts carried out to meet the increasing demand of MPA and its cost effective production, is media optimization through statistical approach. To determine the best carbon and nitrogen source and also the type of casein enzyme hydrolysate to increase the MPA production, a categorical factorial experiment was conducted. Thereafter nine components were screened using Plackett Burman design which were then optimized using full factorial design and further by one variable at a time. Along with the conventional method like Analysis of Variance, Half Normal, Normal plot and Pareto chart were employed to screen the significant factors. Casein enzyme hydrolysate, and polypropylene glycol were found to be the most significant factors that increase the production by 50%. The optimum concentration of 25 g/l of casein enzyme hydrolysate and 1 g/l of PPG gave the maximum yield of 8.85 mg/gm.

Introduction

Mycophenolic acid (MPA) and its derivatives such as mycophenolate mofetil (MMF), have diverse biological properties such as an tineoplastic,immunosuppressive, antifungal anti-inflammatory, antiviral, and antipsoriasis activity (Sadhukhan, et al,1991).MPA has inhibitory effect on inosine monophosphate dehydrogenase enzyme "IMPDH". This is the rate-limiting enzyme in denovo biosynthetic pathway of purine nucleotides. MPA stops the biosynthesis of DNA and RNA and cell reproductivity (Ardestani, Fatemi and Yakchali, 2011). MPA is a secondary metabolite which produced by several species of Penicillium like Penicillium brevicompactum and Penicillium roqueforti by submerged fermentation (Sadhukhan, et al,1991).

Growth media contributes to thirty to forty percent of the manufacturing cost of metabolite production industrially.The production of mycophenolic acid depends on the composition of the fermentation medium (Pranaw,et al, 2014 and Rajendran, Thirugnanam and Thangavelu, 2007). For bulk production, designing an appropriate fermentation medium is very crucial in optimizing the product yield. One-variable-at-a-time is the most frequently used conventional experimental approach, but this strategy not only is time consuming and laborious but also requires a large number of experiments to be conducted since the effect of each factor on metabolite production needs to be investigated individually, and their interaction effect on production process cannot be quantified exactly (Pranaw,et al, 2014). Plackett burman and full factorial design (Category and Numerical) allows reliable short listing of medium components in fermentation for further optimization and allows one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations. This statistical approach for the optimization of media effectively deals with the problem, which involves specific design of experiments thus minimizing the error in determining the effect of each variable (Rajendran, Thirugnanam and Thangavelu, 2007).

The current study involves the designing of the medium using the above mentioned statistical tools. Categorical full factorial design, numerical full factorial design, Plackett Burman design were employed to screen the significant components of the medium.All the above statistical analysis were done using the Design expert software (Stat-Ease Inc., Version 8.0.7.1).

Material and Methods

Microorganism

The fungus Penicillium brevicompactum was used for the mycophenolic acid fermentation. It is an ascomycetous fungus belonging to the genus Penicillium. The organism was grown on Potato dextrose agar slants. The slants were incubated at 25ºC for 15 days. The spores were harvested with normal saline and were used as inoculum.

Growth media

Seed media comprises of dextrose 50g//l, soya peptone 15g/l, yeast extract 10 g/l, malt extract 10 g/l, magnesium sulphate 1 g/l, potassium dihydrogen phosphate 1 g/l and sodium nitrate 2.5 g/l, pH 5.8. 1 ml of the harvested spores suspension was inoculated in 35ml seed in 250 ml erlenmeyer flasks. Seed flasks were incubated at 26ºC at 240 rpm on shaking incubator for 40 hrs. 8% of the seed was transferred to 250 ml flasks containing 35ml of production media. Basal production media was used for the optimization. Basal media composition: sucrose 40 g/l, yeast extract 10 g/l, soya peptone 5 g/l, magnesium sulphate 1g/l, potassium dihydrogen phosphate 2.5 g/l and ammonium sulphate 2 g/l. Flasks were incubated at 26ºC and 240 rpm. The yield was assessed through HPLC

Quantification of MPA by HPLC

Mycophenolic acid produced in the culture broth was determined by HPLC. The culture broth of 2.5 gm was taken in 20 ml volumetric flask with 20 ml methanol and sonicated for 20 minutes and the volume was made up with methanol. The resulting extracted solution was injected into the HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (250 mm X 4.6 mm) for the estimation of mycophenolic acid. Concentration of MPA was calculated by comparison of peak areas with those standard mycophenolic acid and subsequently MPA activity was calculated.

Categorical full factorial design

Categorical designs allows the selection of the best carbon and nitrogen sources. The cell growth and the accumulation of the MPA in the cells depends greatly on the carbon sources and nitrogen sources (Rajendran, Thirugnanam and Thangavelu, 2007). In the study carbon sources chosen were sucrose with dextrose . The nitrogen sources were cotton seed meal and corn steep liquor and casein enzyme hydrolysate (Type 1 with Type 2). Type 1 casein comprised of 60% concentration and type 2 comprised of 70% composition from Enzochem, India. The code sheet for the categorical factorial design is shown in table 1.

Table 1: Code sheet for Categorical Factorial Design

Component	Code	Low level (-1)	High Level (+1)
Carbon source	A	Dextrose (60g/l)	Sucrose (60g/l)
Nitrogen Source	B	Corn steep liquor (10g/l)	Cotton seed meal (10g/l)
Casein Type	C	60% (15g/l)	70% (15g/l)

Plackett Burman Design

The Plackett-Burman design is based on the first order model (Plackett and Burman, 1946). It is used for screening and evaluating the important medium components that influence the production of secondary metabolites. All the experiments were carried in triplicate according to designed matrix using the equation 1:

Y= β0 + Σ βiXi (i = 1, …. k) (Equation 1) (Deshmukh and Puranik, 2010)

In the study 9 factors were studied by using 11 variable, 12 run design. The code sheet for Plackett burman design is shown in table 2

Table 2: Code sheet for Plackett Burman design

Component	Code	Low Level (-1)	High Level (+1)
Sucrose	A	50	80
Soyaflour	B	15	25
Cotton seed meal	C	5	10
Glycerol	D	10	20
Casein enzyme hydrolysate	E	5	15
PPG	F	2	4
Magnesium sulphate	G	0.5	2
Potassium dihydrogen phosphate	H	1.5	3
Ammonium sulphate	J	2	4
D1	K	-1	1
D2	L	-1	1

Note: D1, D2: Dummy variables

Numerical Full Factorial Design

The two level factorial design is considered to be a multivariable sequential search technique in which the effects of two or more factors are studied simultaneously and the responses are analyzed statistically to arrive at a decision (Dutta, Franca and Lopes, 2006 and Anbu et al, 2006). A two level three factorial design was carried out on the basis of the results obtained from the Plackett Burman design. The components analyzed were casein enzyme hydrolysate, polypropylene glycol (PPG) and ammonium sulphate. The code sheet for the design is represented in table 3.

Table 3: Code sheet for numerical full factorial design

Component	Code	Low Level (-1)	High Level (+1)
Casein enzyme hydrolysate	A	15	20
PPG	B	1	3
Ammonium sulphate	C	1.5	3

One variable at a time

The components screened from full factorial design of experiment were further optimized using one variable at a time method (OVAT). This method involves the variation of the concentration of one component at a time while keeping the others constant. This is done to determine the final concentration of the significant components giving maximum yield. Casein enzyme hydrolysate and PPG were tested using this method.

Result and Discussion

Categorical Full Factorial Design

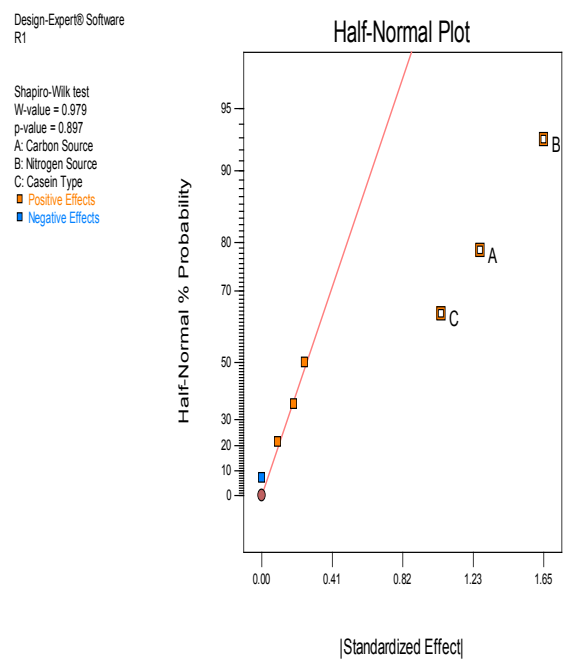
To screen the best carbon, nitrogen and casein enzyme hydrolysate type which support the growth of Penicillium brevicompactum and also enhances the yield of mycophenolic acid (MPA), the 2^3 categorical design was employed. The design matrix along with the corresponding response is depicted in table 4.

Table 4: Categorical full factorial design along with the response

Run	A:Carbon Source	B:Nitrogen Source	C:Casein Type	Response (mg/g)
1	-1	-1	1	4.508
2	1	-1	-1	4.892
3	-1	1	-1	5.012
4	-1	-1	-1	3.523
5	1	1	1	7.678
6	-1	1	1	6.123
7	1	-1	1	5.502
8	1	1	-1	6.194

The half-normal probability plot is a graphical tool that uses the ordered estimated effects to help assess which factors are important and which are not important. According to the half normal plot in figure 1 the three factors A, B and C are seen to be away from the noise line (red line). The more the distance from the noise line, the greater is the effect of the factor on the yield of MPA. The high level for the three factors is significant It thus implies that the best carbon source is sucrose and the best nitrogen source is cotton seed meal. The casein enzyme hydrolysate with 70% concentration is better than the 60% one. Thus these three factors were further optimized along with the other components through Plackett Burman design.

Figure 1: Half normal plot for categorical design



Plackett Burman Design

The carbon, nitrogen and casein type along with salts and surfactant (PPG) were screened through 11 variable 12 run design of Plackett Burman (PB).The design along with the response is shown in table 5

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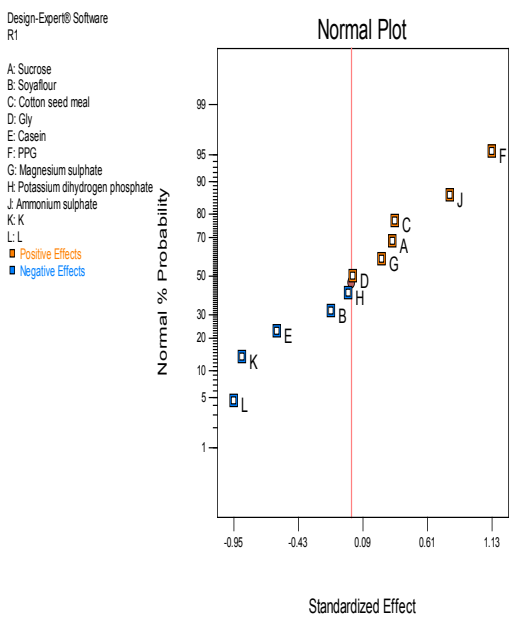
Table 5: Plackett Burman Design along with the response

Run	A	B	C	D	E	F	G	H	J	K	L	Response (mg/g)
1	1	1	-1	1	1	1	-1	-1	-1	1	-1	5.643
2	-1	-1	1	-1	1	1	-1	1	1	1	-1	6.985
3	1	-1	-1	-1	1	-1	1	1	-1	1	1	3.932
4	1	1	-1	-1	-1	1	-1	1	1	-1	1	6.954
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	7.859
6	-1	1	1	-1	1	1	1	-1	-1	-1	1	5.028
7	1	1	1	-1	-1	-1	1	-1	1	1	-1	6.488
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	5.753
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	5.012
10	-1	1	-1	1	1	-1	1	1	1	-1	-1	6.074
11	-1	1	1	1	-1	-1	-1	1	-1	1	1	4.357
12	-1	-1	-1	1	-1	1	1	-1	1	1	1	6.367

The response was analyzed by normal plot (figure 2).

Figure 2: Normal plot for Plackett Burman design



As per normal plot the factor A (sucrose), C (Cotton seed meal), D (Glycerol), F (PPG), G (Magnesium sulphate) and J (Ammonium sulphate) are on positive scale whereas the rest (B, E, H) are on the negative scale. Also it shows that F, E and J are significant as they are away from the noise line (red line).

Evaluation of PB design through ANOVA

Table 6: ANOVA analysis for PB design

Source	Sum of squares	df	Mean square	F value	p value Prob > F
Model	11.86	5	2.37	14.81	0.0025
E-Casein enzyme hydrolysate	1.08	1	1.08	6.75	0.0407
F-PPG	3.85	1	3.85	24.07	0.0027
J-Ammonium sulphate	1.9	1	1.9	11.84	0.0138
K-K	2.33	1	2.33	14.55	0.0088
L-L	2.7	1	2.7	16.85	0.0063
Residual	0.96	6	0.16		
Cor Total	12.82	11			

R-Squared	0.9251
Adj R-Squared	0.8626
Pred R-Squared	0.7003
Adeq Precision	12.594

The Model F-value of 14.81 implies the model is significant. There is only a 0.25% chance that a "Model F-Value" this large could occur due to noise. .The "Pred R-Squared" of 0.7003 is in reasonable agreement with the "Adj R-Squared" of 0.8626."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable.The ratio of 12.594 indicates an adequate signal. This model can be used to navigate the design space.

P value less than 0.0500 indicates that the model terms are significant. In this case E (Casein enzyme hydrolsate), F (PPG), J (Ammonium sulphate) are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The factors E, F and J were further screened by 2 level full factorial design. Factors K and L are dummy variable and hence not considered for further analysis.

Full factorial design

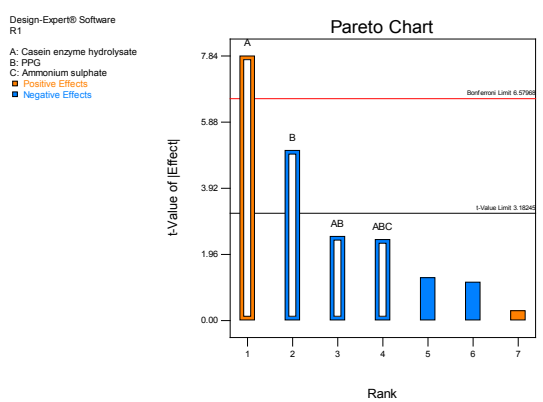
The factors casein enzyme hydrolysate, PPG and ammonium sulphate were further optimized through 2^3 factorial design.The response with the design is represented in table 7.

Table 7 : Full factorial design with response

Run	A	B	C	Response (mg/g)
1	-1	1	-1	4.449
2	-1	-1	1	5.559
3	1	1	-1	6.884
4	1	-1	1	8.654
5	1	-1	-1	7.963
6	1	1	1	5.664
7	-1	-1	-1	5.478
8	-1	1	1	5.212

The obtained response was analyzed with Pareto chart (figure 3). A Pareto Chart is a series of bars whose heights reflect the frequency or impact of variables.

Figure 3: Pareto chart for full factorial design



In the Pareto chart, the bars are arranged in descending order of height from left to right. This means the categories represented by the tall bars on the left are relatively more significant than those on the right (Scholtes, 1988). Here the bars for the components A, B and interaction of AB are located towards the extreme left indicating that these components contribute more to the MPA yield. It also shows that component A (Casein enzyme hydrolysate) affects the MPA production on a positive scale whereas component B (PPG) and interaction of PPG and casein enzyme hydrolysate (AB) affects the yield on the negative scale. This implies that the concentration of casein enzyme hydrolysate needs to be increased while that of PPG needs to be decreased to get the maximum production of MPA.

Evaluation of full factorial results by ANOVA

The results were validated using ANOVA. Table 8 represents the analyzed data.

Table 8: ANOVA for full factorial design

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	14.41	4	3.6	24.75	0.0124
A-Casein enzyme hydrolysate	8.96	1	8.96	61.54	0.0043
B-PPG	3.71	1	3.71	25.45	0.015
AB	0.91	1	0.91	6.23	0.0881
ABC	0.84	1	0.84	5.77	0.0957
Residual	0.44	3	0.15		
Cor Total	14.85	7			

R-Squared 0.9706
Adj R-Squared 0.9314
Pred R-Squared 0.7908
Adeq Precision 13.678

The Model F-value of 24.75 implies the model is significant.There is only a 1.24% chance that a "Model F-Value" this large could occur due to noise.

In this case, casein enzyme hydrolysate and PPG are significant model terms as their p value is lesser than 0.05. The other terms are insignificant as their p value is greater than 0.05.

The "Pred R-Squared" of 0.7908 is in reasonable agreement with the "Adj R-Squared" of 0.9314."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable.The ratio of 13.678 indicates an adequate signal.This model can be used to navigate the design space.

One variable at a time

Based on the Pareto chart and ANOVA analysis the components casein enzyme hydrolysate and PPG were significant. Here PPG acts as a surfactant promoting the pelletization. Increased pelletization improves growth and yield. Very high quantities of surfactant could be detrimental to the productivity. Addition of casein enzyme hydrolysate leads to accumulation of the greater amounts of nitrogen culminating in increased MPA production (Ardestani, Fatemi and Yakchali, 2011).Their concentration was further optimized using OVAT. Concentration were determined based on the results obtained from Plackett Burman and full factorial design. Table 9 and 10 represent the concentration and yield of casein enzyme hydrolysate and PPG respectively.

Table 9: OVAT for casein enzyme hydrolysate

Run	Concentration g/l	Yield mg/g
1	15	6.405
2	20	7.182
3	25	8.825
4	30	7.234
5	35	6.582

Table 10: OVAT for PPG

Run	Concentration g/l	Yield mg/g
1	0.5	7.186
2	1	7.854
3	1.5	7.509
4	2	6.892

It can be observed that maximum yield was seen in run comprising of 25 g/l of casein enzyme hydrolysate. Run with 1 g/l of PPG gave maximum yield. OVAT experiments revealed that optimum concentration of casein enzyme hydrolysate is25g/l and PPG is 1 g/l. Thus media was designed using the optimum concentrations of PPG and casein enzyme hydrolysate along with minimal concentrations of the other components. The experiment was performed in triplicates. The average yield obtained was 8.85 g/l which was in agreement with statistically analyzed data.

Conclusion

Categorical factorial design with different carbon sources showed that sucrose is the best carbon source, cotton seed meal is the best nitrogen source. 70% casein enzyme hydrolysate is better than 60%. Plackett Burman design with various carbon, nitrogen and salt sources revealed that casein enzyme hydrolysate, ammonium sulphate and polypropylene glycol were the most significant components and hence have highest impact on yield of MPA. Full factorial design with the chosen components showed that PPG and casein enzyme hydrolysate are the most significant out of the three. ANOVA analysis was applied to validate the Plackett Burman and full factorial design and the results were in agreement with the former. The concentration of the PPG and casein enzyme hydrolysate was optimized further by OVAT. The optimized concentration of casein enzyme hydrolysate was 25 g/l and PPG is 1 g/l in production media. There was an overall increase of almost 50 % increase in the yield. This shows that the model under study is significant, robust and highly effective for optimization of medium at shake flask level to increase the productivity. This work will be useful for mass production of mycophenolic acid from Penicillium brevicompactum.

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