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OPTIMIZATION OF CULTURE CONDITIONS TO ENHANCE MYCOPHENOLIC ACID BIOSYNTHEIS BY *Penicillium brevicompactum* BB263 ISOLATED IN VIETNAM

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ABSTRACT

Mycophenolic acid (MPA) is an immunosuppressant utilized in organ transplantation to prevent rejection and in the treatment of autoimmune diseases such as Crohn's disease and systemic lupus erythematosus. Previous studies have reported that MPA is predominantly produced by fungal strains of the genus *Penicillium*, exhibiting immunosuppressive, antiviral, and antitumor activities, making it valuable in medical applications. In this study, we present the optimization of nutritional components in the fermentation medium for the fungal strain *Penicillium brevicompactum* BB263 to enhance MPA biosynthesis. Experiments were designed using the Box-Behnken methodology to assess the effects of three factors–sucrose, yeast extract, and methionine–on MPA production. The optimized medium for *P. brevicompactum* BB263, designated as YES-F1, comprises (per liter): sucrose 38.14 g, yeast extract 18.69 g, methionine 1.29 g, glycine 4 g, and trace elements 1 mL. Fermentation conditions include an initial pH of 6.5, a temperature of 25°C, an agitation speed of 200 rpm, and a harvest time of 114 hours post-inoculation. Following optimization, MPA production increased tenfold compared to the initial YES medium, reaching 969.79 µg/mL, corresponding to a yield exceeding 70% relative to the biomass of *P. brevicompactum* BB263. These findings provide a foundation for the industrial-scale production of MPA from *Penicillium* strains in Vietnam.

Keywords: Box-Behnken design (BBD), conditions of medium, mycophenolic acid, MPA, *Penicillium*, *Penicillium brevicompactum*, submerged fermentation

INTRODUCTION

Mycophenolic acid (MPA) is an antibiotic primarily biosynthesized by the fungus *Penicillium*, serving as an immunosuppressant used to prevent organ transplant rejection and treat autoimmune conditions like Crohn's disease and lupus (Zhang *et al*., 2019). MPA and its derivatives are known for their biological activities, including anti-cancer, anti-inflammatory, anti-psoriasis, antifungal, and

antiviral properties (Ferreira *et al*., 2020). In practice, mycophenolate (a derivative of MPA) is employed to inhibit immune cell proliferation, specifically B and T lymphocytes, through the suppression of the enzyme IMP dehydrogenase (IMPDH) (Bentley, 2000). Unlike typical cells, B and T cells rely solely on IMPDH for purine biosynthesis, making MPA and its derivatives highly effective for immunosuppression. Currently, commercial drugs containing mycophenolate include CellCept (mycophenolate mofetil; Roche) and Myfortic (mycophenolate sodium; Novartis) (Ferreira *et al*., 2020).

On the other hand, numerous studies have reported that MPA is a secondary metabolite of the *Penicillium* genus, particularly species such as *P. brevicompactum, P. stoloniferum*… (Anand & Srivastava, 2020; Regueira *et al*., 2011). *Penicillium* fungi are widely distributed in nature, found in various environments such as forest soils, coastal areas, greenhouses, and agricultural lands worldwide. Furthermore, these species can be found in moldy foods, fruits, and dairy products (Ismaiel *et al*., 2014).

Studies have demonstrated that the administration of MPA in treatment protocols significantly reduces graft rejection rates in organ transplant recipients (Bentley, 2000). This efficacy is attributed to MPA's ability to inhibit the synthesis of DNA and RNA in immune cells (Ramos-Ponce *et al*., 2012). Typically, MPA is produced through aerobic fermentation of various fungal species, including those from the genera *Penicillium, Trichoderma, Monascus, Geotrichum, Candidum, and Byssochlamys* (Zhang *et al*., 2019). In this study, the fungal strain *P. brevicompactum* BB263, isolated in Vietnam, was investigated for optimal culture conditions using a Box-Behnken design (BBD), aiming to maximize MPA biosynthesis. This approach employs response surface methodology (RSM) to optimize environmental parameters, enhancing the efficiency of MPA production.

MATERIALS AND METHODS

Fungal strain/organism and culture

The fungal strain *Penicillium brevicompactum* BB263 was isolated from the northern coastal region of Vietnam by the Fermentation Technology Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology. Cultivation conditions for this strain were as follows: temperature at 25°C, initial pH of 6.5, fermentation in shake flasks at 200 rpm, and incubation period ranging from 4 to 7 days. Spores were harvested on potato dextrose agar (PDA) medium and diluted with distilled water to a concentration of 10^7 CFU/mL, following the methodology described by Patel *et al*. (2016).

The cultivation medium is an improved YES medium (yeast extract sucrose agar) (Fisvad, 1981) comprising (per liter): 20 g yeast extract, 50 g sucrose, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 g glycine, 1 g methionine, 1 mL trace element solution; distilled water 1000 mL; pH 6.5 ± 0.2. The trace element solution consisted of (g/L): $FeSO_4 \cdot 7H_2O$ 2.2; $CuSO_4 \cdot 5H_2O$ 0.3; $ZnSO_4 \cdot 7H_2O$ 2.4; $MnSO_4 \cdot 4H_2O$ 0.16 and $KMoO_4$ 0.2. All experiments were repeated three times.

Optimize the medium for MPA production using the Box-Behnken design

The modified YES medium is used for optimization according to the Box-Behnken design. The changing factors include a carbon source (sucrose), a nitrogen source

(yeast extract) and a precursor source (methionine) (Table 1).

Tables 1. The concentrations of the factors used in the experimental design matrix are as follows

Factors	Symbol code	Units	Coded values		
			-1	0	+1
Sucrose	X_1	g/L	20	35	50
Yeast extract	X_2	g/L	10	17.5	25
Methionine	X_3	g/L	0.5	1	1.5

To determine the optimal values of three key factors in the fermentation process, a Box-Behnken design was employed. Each factor was evaluated at three levels (-1, 0, and +1), resulting in a matrix comprising 15 experiments, including three center points. The experimental design was constructed using Design-Expert 11 software. Factors exhibiting high significance ($P < 0.05$) were incorporated into the optimization model utilizing RSM with the BBD. The response variable selected for this study was the concentration of MPA produced (µg/mL). The relationship between the response and the independent variables was modeled using a second-order polynomial equation:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{11}X_{12} + B_{22}X_{22} + B_{33}X_{32}$$

Where: Y is the response variable (MPA concentration). B_0 is the intercept term. B_1, B_2, B_3 are the linear coefficients. B_{11}, B_{22}, and B_{33} are the quadratic coefficients. B_{12}, B_{13}, and B_{23} are the interaction coefficients between pairs of factors. X_1, X_2, and X_3, are the independent variables. Data analysis was performed using Design-Expert® 11.0 software (Stat-Ease, Inc., USA). This analysis facilitated the determination of optimal levels for the factors under investigation to achieve maximum MPA biosynthesis.

Mycophenolic acid analysis

The fungal fermentation broth obtained after 4 days of incubation at 25°C was diluted with methanol at a 1:25 ratio and subsequently filtered through a 0.45 µm Millex-HV filter (Merck Millipore) prior to analysis using high-performance liquid chromatography (HPLC) (LaChrom Elite - Hitachi). A C18 column (Symmetry® C18, 5 µm, 4.6 x 250 mm) was employed at 40°C. The mobile phase consisted of double-distilled water and acetonitrile (50:50 v/v), adjusted to pH 3.0, with a flow rate of 0.5 mL/min. The injection volume was 10 µL. Detection was performed using a photodiode array detector at 220 nm (Elbarbry & Shoker, 2007).

A standard solution was prepared using pure MPA (HPLC grade) as a control. The stock solution of MPA (1 mg/mL) was prepared in methanol and stored at -20°C. When required, the stock solution was serially diluted with methanol to obtain MPA concentrations ranging from 2.5 to 125 µg/mL. All MPA measurements were conducted in triplicate. The formula for calculating MPA concentration:

$$X = \frac{Y - 5245911.81}{1186680.641}$$

Where:

X: MPA concentration (µg/mL)

Y: Absorbance area at the wavelengths (nm)

Statistical analysis

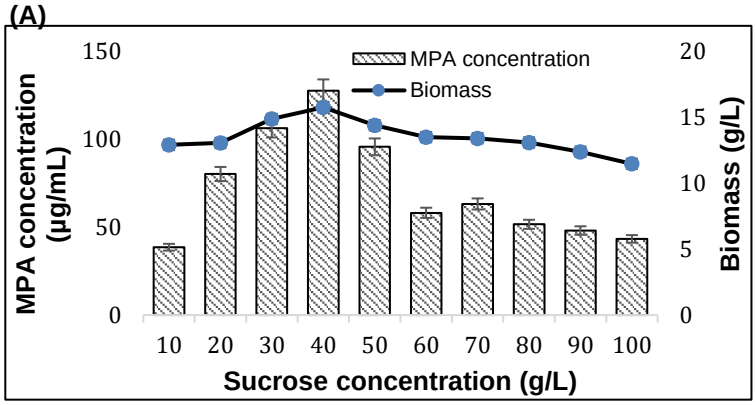
The statistical error analysis method used is ANOVA, which can be employed to analyze the variability between samples in an experiment.

RESULTS AND DISCUSSION

Screening experiments by the single-factor test for MPA production

The Box-Behnken design is based on the results of single-factor experiments with sucrose, yeast extract, and methionine regarding their effects on the synthesized MPA content of the *P. brevicompactum* BB263 strain (Figure 1). The experiment selected three factors as follows: sucrose (20-50 g/L), yeast extract (10-25 g/L), and methionine (0.5-1.5 g/L) (Table 1), with the MPA content reaching 80.18-127.57 µg/mL, 144.52-263.92 µg/mL, and 96.27-236.76 µg/mL, respectively. The MPA content obtained in the initially improved YES medium was 95.62 µg/mL, which will be used for comparison with the experimental samples after optimization. The results from 15 experiments showed that 3 experiments at the center yielded the highest MPA content, reaching 646.714-666.116 µg/mL, with only minor discrepancies from the design. The experiments showed no significant difference with the predictions.

Based on the study of the three single-factors (Figure 1), the significant ranges have been established for the objective function, which is MPA, to be incorporated into the BBD (Table 1).



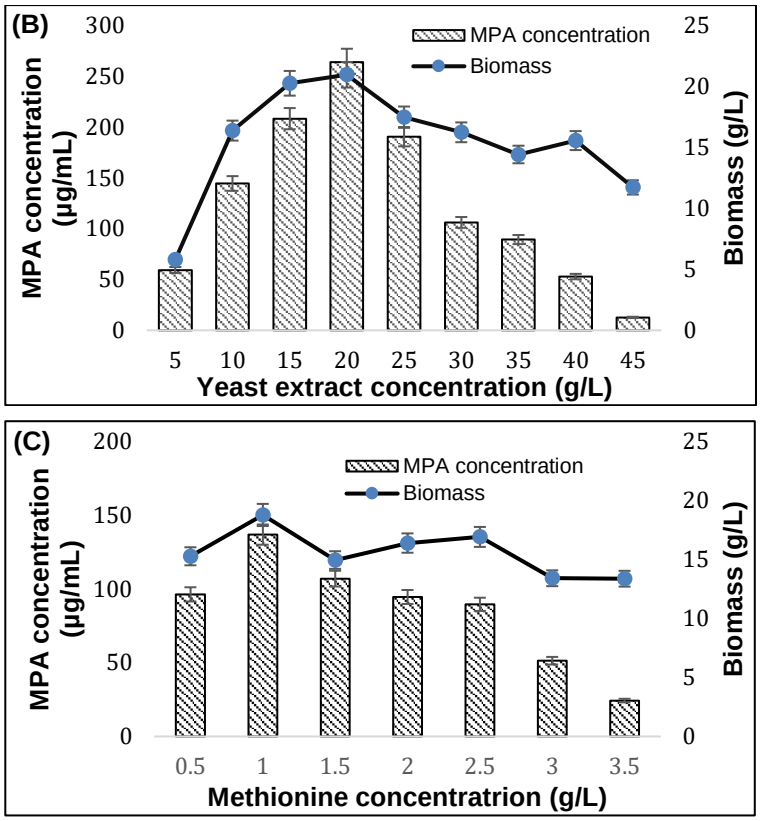


Figure 1. Effects of the single-factor test on the production of MPA and biomass. (A) Different concentration of sucrose (10-100 g/L); (B) Different concentration of yeast extract (5-35 g/L) and (C) Different concentration of methionine (0.5-3.5 g/L).

Optimization using BBD method

The model was designed for the *P. brevicompactum* strain BB263, including 3 factors with 15 experiments, where there are 3 trials at the center, numbered 13, 14, and 15 (Table 2), with an MPA concentration reaching 647.529 µg/mL. The overall model has statistical significance (p-value = 0.0048). In Table 3, the analysis of variance for the regression model shows that the R^2 value is close to 1, indicating that the model explains 96.19% of the variability in the data, demonstrating the model's adequacy. The adjusted R^2 value is in the range of 0.7-0.95 (adjusted R^2 = 0.8933), and the predicted R^2 is in the range of 0.5-0.7 (predicted R^2 = 0.5971), ensuring the generalizability of the experiment. Variables X_1 and X_3 have p-values less than 0.05, indicating statistical significance and a significant effect on the dependent variable, while variable X_2 has a p-value greater than 0.05, suggesting it does not significantly affect the dependent variable, though it is still close to the significance level.

Regarding the interaction values of each pair of factors, the pair X_1X_2 is statistically significant (p-value < 0.05) and shows a significant interaction between the two variables. The other pairs, X_1X_3 and X_2X_3, do not have statistically significant interaction effects, as their p-values are greater than 0.05, indicating that these pairs do not significantly affect the dependent

variable. The effects of the squared values of the factors, such as X_1^2 and X_2^2, are statistically significant with p-values < 0.01, indicating that these values have a substantial and nonlinear impact on the dependent variable. As for the factor X_3^2, its p-value is close to the significance level (p-value > 0.05) but is not sufficient to assert a clear impact in the model.

Table 2. Box-Behken design with experimental and predicted values of MPA concentration

				MPA concentration (µg/ml)		
Run	**X_1**	**X_2**	**X_3**	**Predicted**	**Experimental**	**Difference**
1	-1	-1	0	375.799	327.946	47.853
2	1	-1	0	255.204	249.916	5.288
3	-1	1	0	238.706	243.994	-5.288
4	1	1	0	625.463	585.188	40.275
5	-1	0	-1	320.582	326.951	-6.369
6	1	0	-1	434.626	398.431	36.195
7	-1	0	1	500.436	536.631	-36.195
8	1	0	1	564.427	558.058	6.369
9	0	-1	-1	294.411	335.895	-41.484
10	0	1	-1	432.661	421.004	11.657
11	0	-1	1	514.970	527.627	-12.657
12	0	1	1	521.758	480.274	41.484
13	0	0	0	647.529	648.559	-1.03
14	0	0	0	647.529	646.714	0.815
15	0	0	0	647.529	662.116	-14.587

Table 3. Analysis of variance for response surface model of MPA

Source	**Sum of squares**	**Degree of freedom**	**Mean square**	**F-value**	***p*-value**
Model	282600	9	31397.49	14.03	0.0048
X_1	15848.3	1	15848.3	7.08	0.0448
X_2	10517.99	1	10517.99	4.7	0.0824
X_3	47942.95	1	47942.95	21.42	0.0057
X_1 X_2	43937.18	1	43937.18	19.63	0.0068
X_1 X_3	626.33	1	626.33	0.2798	0.6195
X_2 X_3	4320.58	1	4320.58	1.93	0.2234
X_1^2	75839.32	1	75839.32	33.88	0.0021
X_2^2	91458.41	1	91458.41	40.86	0.0014

X_3^2	10817.92	1	10817.92	4.83	0.0793
Residual	11192.05	5	2238.41	-	-
Lack of Fit	11050.57	3	3683.52	5.07	0.0689
Pure Error	141.47	2	70.74	-	-
Cor Total	293800	14	-	-	-
R^2	-	-	0.9619	-	-

The lack of fit factor has a p-value of 0.0689, indicating that the model is statistically significant but may require adjustments or the addition of other variables to improve the results. The pure error value shows variability that is not caused by the model, and this error is acceptable within the experimental data. Thus, it can be observed that among the three factors, sucrose and methionine have a significant impact on MPA formation, while the third factor, yeast extract, does not have a significant effect. However, the correlation between the pairs of factors, sucrose-yeast extract, has a direct influence on MPA values, while the other two pairs, yeast extract-methionine and sucrose-methionine, do not significantly affect the MPA content produced.

Based on the linear regression equation, the intercept of 652.46 represents the baseline value of the dependent variable when all three independent variables are equal to zero. The coefficients in the equation indicate that an increase in the coefficients of the independent variables will result in an increase in the MPA produced. Specifically, a combination of increased sucrose and yeast extract will have a positive interaction that boosts MPA levels. However, the other two pairs of factors will lead to a decrease in the MPA content produced. This regression model is quite complex, as it not only includes the independent variables but also their interactions and nonlinear effects. When the independent variables are increased, the nonlinear coefficients (squared terms) for all three factors—sucrose, yeast extract, and methionine—will reduce the amount of MPA produced. This interaction indicates that a change in just one variable will also affect the MPA yield. The results of ANOVA analysis have yielded the following linear regression equation:

$$Y = 652.46 + 44.51X_1 + 36.26X_2 + 77.41X_3 + 104.81X_1X_2 - 12.51X_1X_3 - 32.87X_2X_3 - 143.32X_{12} - 157.38X_{22} - 54.13X_{32}$$

In the formula, Y is the MPA content (µg/mL); X_1 represents sucrose, X_2 represents yeast extract and X_3 represents methionine. The results of the analysis of variance for the regression model show that the F value of the model is greater than 9. And the coeficient of the model (R^2) was 0.9619 with adjusted R^2 (0.8933) and prediction R^2 (0.5971), as well as the not significantly lack of fit (0.0689) (Table 3). To determine the optimal level of each variable for the optimal environment for MPA production by strain BB263, a three-dimensional interaction surface plot was constructed with the Z-axis representing MPA content and any two independent variables, while the remaining variable was maintained at its optimal level.

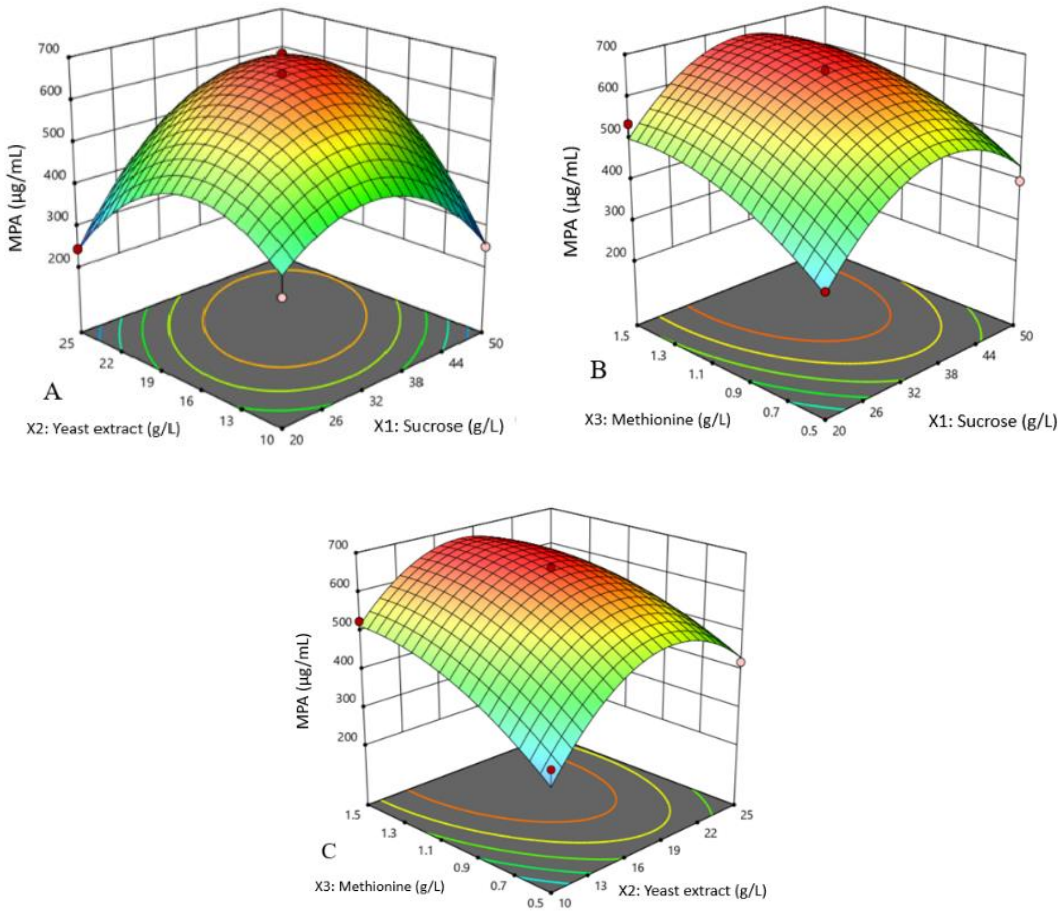


Figure 2. The 3D-plot of response surface represent the interaction between two factors in MPA production (µg/mL) by keeping the other two variables constant: (A) Sucrose (X_1) – Yeast extract (X_2); (B) Sucrose (X_1) – Methionine (X_3); (C) Yeast extract (X_2) – Methionine (X_3).

In the charts A, B, C of Figure 2, the small ellipses within the contour lines indicate the maximum optimal values of the variables in the model (Dong *et al*., 2009, 2015). The diagrams illustrate the effects of the three factors—sucrose, yeast extract, and methionine—on the formation of MPA. The 3D chart describes the optimization of MPA and the interactions between the factor pairs among the three factors: sucrose and yeast extract (Figure 2A) show a direct proportional interaction, while the other two pairs—sucrose and methionine (Figure 2B) and yeast extract and methionine (Figure 2C) exhibit off-center ellipses, indicating a lack of interaction between these factors.

Based on the results from the optimized factors processed by software, 100 optimal formulations were generated, from which three formulations with the highest predicted MPA content were selected for experimental testing. The results showed a significant difference between the predicted MPA content and the experimental values (Table 4). The experiments were repeated three times.

Table 4. Design and experimental results of the Box-Behnken design

Experiment formula	Sucrose concentration (g/L)	Yeast extract concentration (g/L)	Methionine concentration (g/L)	MPA concentration (µg/mL)	
				Predicted	Experimental
F1	38.143	18.686	1.288	682.985	969.79 ± 7.82
F2	36.153	18.331	1.308	682.328	861.19 ± 2.34
F3	39.296	19.0	1.321	681.283	693.36 ± 4.27

The MPA concentrations were found to be higher by 41.99, 26.22, and 0.2% for formulations F1, F2 and F3, respectively. The substantial discrepancy between the predicted values from the model and the experimental results suggests that the model may not fully account for all the factors affecting the MPA levels produced, and variations among these factors could lead to better MPA yields. Therefore, it may be beneficial to consider adding additional factors not included in the model that could influence MPA production. Nevertheless, based on these results, the optimal medium has been identified, with the highest MPA production of this strain content achieved at 969.79 ± 7.82 (µg/mL) in the YES-F1 medium (g/L), which includes 38.14 g of sucrose, 18.69 g of yeast extract, 1.29 g of methionine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 4 g of glycine, and trace salts.

We have successfully optimized the culture medium for the *P. brevicompactum* BB263 strain to produce MPA at a concentration of 969.79 (µg/mL), 29 times higher than the original improved YES medium of 33.62 (µg/mL). This result opens up the direction of producing mycophenolic acid from fungi, creating products with practical application potential to help Vietnamese people have the opportunity to access and use therapeutic products in the treatment of anti-rejection in organ transplants at a lower cost than imported ones.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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