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Optimization of Phage Production using Plackett-Burman and Response Surface Methodology

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Striped catfish is one of the most important farmed fish species in Vietnam but often susceptible to hemorrhagic septicemia caused by *Aeromonas hydrophila.* Phage therapy is a promising solution alternative to antibiotic usage due to the bacterial antibiotic resistance. In this study, five individual factors including molasses concentration, KH_2PO_4 concentration, yeast extract concentration, bacterial culture time and phage collection time were investigated for their effects on the producion of phage TG25P. Plackett-Burman design revealed that the bacterial culture time, phage collection time and molasses concentration were the most significant factors affecting the phage production. Application of response surface methodology for optimization of phage production showed that optimal values for these variables were 1.76 h, 2.22 h and 2.03 g/L, respectively. In the optimized medium, the phage production of $(1.44 \pm 0.2) \times 10^9$ PFU/mL was obtained, which agreed well with the predicted value of 1.37×10^9 PFU/mL. The findings really help us produce phage TG25P under the optimal condition at a low cost.

1. Introduction

Striped catfish (*Pangasianodon hypophthalmus*) is one of the most important farmed fish species found in many South East Asian countries. In Vietnam, these catfish are mainly farmed in the Mekong Delta. They contribute the most to the country's annual export turnover, making Vietnam to be the largest producer of striped catfish in the world. The fish are often susceptible to hemorrhagic septicemia, a bacterial disease caused by *Aeromonas hydrophila* (Tran et al., 2021). In order to prevent and treat this disease, many different classes of antibiotics have been applied to farming of these fish (Andreu et al., 2013). Unfortunately, *A. hydrophila* has been found to be highly resistant to many different antibiotics used (Dang et al., 2021), which has been leading to crisis in usage of antibiotics in farming the striped catfish, since this has showed an inadequate control and has led to alarming results of output reduction, export loss, community health and environmental degradation. Due to these adverse impacts, there is an urgent need to come up with more effective solutions that are not only effective but also low in cost and environmentally friendly as a replacement of the antibiotics (Dong et al., 2020). Researches have been turned into alternatives, among which bacteriophage (or phage) therapy is the most promising way. Phage therapy is the therapeutic use of phages to treat pathogenic bacterial infections (Hoang and Pham, 2021). For the purpose of prevention and treatment of hemorrhagic septicemia in striped catfish farmed in Vietnam, we have successfully conducted the phage therapy at the laboratory scale, which showed that phage therapy could reduce the mortality rate of fishes from approximately 70 % to about 10 % (Dang et. al., 2021). As phages need to be prepared in a large enough quantity prior to phage therapy, they must be produced in optimized conditions at a low cost (Le et al., 2022). Therefore, optimization of factors that have significant effects on the phage production plays an important role in the process of phage production. This study aimed to screen the factors including molasses concentration, KH_2PO_4 concentration, yeast extract concentration, bacterial culture time and phage collection time for those that significantly affected the phage production using the Plackett-Burman (PB) design and then optimize the screened factors using the response surface methodology (RSM).

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2. Materials and Methods

2.1 Preparation of phage stock solution

Phage TG25P (Tran et al., 2018) was used in this study to infect *A. hydrophila*. After being defrosted at 4 °C and serially diluted in SM buffer, 100 µL of diluted phage solution together with 100 µL of overnight *A. hydrophila* culture in tryptic soy broth (TSB) were well mixed and used for preparation of phage stock solution as described previously (Hoang et al., 2019). Phage titer was estimated as described (Hoang et al, 2019). The phage stock solution was stored at 4 °C for further use.

2.2 PB design

The PB design (Plackett and Burman, 1946) was used to screen for factors that significantly affected the production of TG25P. The PB design was implemented using the Design Expert software (Version 9, Stat-Ease, Inc., Minneapolis, USA). Five independent variables including molasses concentration, KH_2PO_4 concentration, yeast extract concentration, bacterial culture time and phage collection time, which were respectively coded as factors X_1, X_2, X_4, X_5 and X_6 (Table 1) in the PB design, were investigated. The bacterial culture time was the time taken to culture the host bacteria until the point at which the bacterial culture was added with the phage stock solution. The phage collection time was the interval between the time at which the phage stock solution was added to the bacterial host culture and the time at which the phages were collected from the phage-host mixture. X_3 and X_7 were dummy variables. Each independent variable was set at two levels, the low level (-) and the high level (+). Actual values for these independent variables are also shown in Table 1. The actual values were determined prior to the PB design by investigating the effect of each single variable on the phage production independently. The effect of each single variable on the phage production was studied by changing it while fixing all other variables.

Table 1: Coded and actual values at the low and high levels in the PB design

Variables	Code	Values of coded levels	
		(-)	(+)
Molasses concentration (g/L)	X_1	0.25	5.25
KH_2PO_4 concentration (g/L)	X_2	0.5	2.5
Yeast extract concentration (g/L)	X_4	0.25	9.25
Bacterial culture time (h)	X_5	0	4
Phage collection time (h)	X_6	0.5	2.5

2.3 RSM design

The factors that were determined to significantly affect the production of phage including the bacterial culture time (A, measured in h), the phage collection time (B, measured in h) and the molasses concentration (C, measured in g/L) were further investigated to find out the optimal settings to maximize the phage production. The RSM on the basis of central composite design (Box and Wilson, 1951) was used to build a quadratic model for the phage production response, which proposed the Eq(1).

$$P = \beta_o+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC \quad (1)$$

where P was the predicted response; β_o was the intercept; β_1, β_2 and β_3 were the linear coefficients; β_{11}, β_{22} and β_{33} were the squared coefficients; and β_{12}, β_{13}, and β_{23} were the interaction coefficients. Optimal values of the variables A, B and C were determined at five distinct levels encoded as $-\alpha$, -1, 0, +1 and $+\alpha$, where 0 was the central level; -1 and +1 were the low and high levels, respectively; $-\alpha$ and $+\alpha$ were the extreme levels corresponding to -1 and +1 levels, respectively, with $\alpha = 2^{3/4}$. Values at the $-\alpha$ and $+\alpha$ levels were determined as M_a (with a was of $-\alpha$ or $+\alpha$) using the Eq(2).

$$M_a = a(H-L)/2 + [(H+L)/2] \quad (2)$$

where H and L were values at +1 and -1 levels, respectively. The coded and actual values at these levels are shown in Table 2. Experimental design, model regression analyses, 3D surface plots and contour graphs were generated by the Design Expert software (Version 9, Stat-Ease, Inc., Minneapolis, USA).

Based on the built quadratic model, numerical optimization option in the Design Expert software was used to find the optimal values of the bacterial culture time, phage collection time and molasses concentration for the optimal phage concentration.

Table 2: Coded and actual values at the central, low, high and extreme levels in the RSM design

Factor	Level				
	-α	-1	0	1	+α
A (h)	0.318	1	2	3	3.68
B (h)	0.318	1	2	3	3.68
C (g/L)	0.318	1	2	3	3.68

2.4 Phage production

Medium used for the phage production contained molasses, KH_2PO_4 and yeast extract. Final pH of the medium was adjusted to 7. 30 mL of the medium contained in each 250 mL flask was inoculated with 1 % (v/v) of overnight *A. hydrophila* culture in TSB.

In the PB design experiment, eight flasks were prepared to contain liquid molasses-based media which varied in compositional concentrations as designed (Tables 1 and 3). After inoculation with *A. hydrophila*, the flasks were shaken at 30 °C, 150 rpm. 100 µL of sample were immediately taken from each of those flasks running at the low level of the bacterial culture time (X_5 = 0 h, i.e., right after the bacterial inoculation), and from each of those flasks running at the high level of the bacterial culture time (X_5 = 4 h, i.e., after 4 h of shaking). These bacterial samples were used for estimating the bacterial population in the flasks, using the spread plating method on Luria-Bertani agar containing plates. The phage concentration of the stock solution was determined as mentioned above before the phage stock solution was added to the flasks so as to achieve multiplicity of infection (MOI, the ratio of phage particles to the bacterial cells) of around 0.001. An appropriate volume of phage stock solution was then added to each of the flasks designed at the low level of the bacterial culture time (i.e., right after the bacterial inoculation) and then to each of those designed at the high level of the bacterial culture time (i.e., after 4 h of shaking). The mixtures of phages and bacteria were continued to be shaken at 30 °C, 150 rpm. At the phage collection time designed at the low level (X_6 = 0.5 h, i.e., after 30 min of shaking since the phage stock solution was added), 100 µL of sample were taken from each of the flasks designed at the low level of the phage collection time; Similarly, at the phage collection time designed at the high level (X_6 = 2.5 h, i.e., after 2.5 h of shaking since the phage stock solution was added), 100 µL of sample were taken from each of the flasks designed at the high level of the phage collection time. These phage containing samples were used to estimate the value of the response variable, the phage concentration, using the phage plaque assay (Hoang et al., 2019). The PB design experiment was carried out in duplicates.

In the RSM design experiment, twenty flasks were prepared following the experimental design (Tables 2 and 4). The experiment was run in a similar manner as in the PB design experiment, however, in this RSM design, there were only three variables instead of five in the PB design to be investigated for their optimal values, each of which was set at five different levels (Table 2) instead of two levels in the PB design (Table 1).

2.5 Model validation

To validate the optimization model, the predicted values obtained under the optimal condition were experimentally run. The phage concentration obtained from the run was compared with the value predicted by the model.

3. Results and Discussion

3.1 Screening for significant effects of independent variables

The two-level PB design of eight runs was used to analyse the effect of 5 individual variables including the molasses concentration, KH_2PO_4 concentration, yeast extract concentration, bacterial culture time and phage collection time on the production of TG25P. The phage production was estimated as the number of plaque forming units (PFU) in 1 mL of the reaction mixture in each run. Each experimental trial in the PB design was carried out in duplicates. The averages of the responses from each trial are shown in Table 3.

The highest phage concentration was observed in trial number 1, which was 210.5 × 10^6 PFU/mL, while the lowest concentration (1,700 PFU/mL) was observed in trial number 8. The effect of each variable E_{Xi} (i = 1, 2, 3, 4, 5, 6, 7 and 8) was determined using the Eq(3).

$$E_{Xi} = 2[\sum P_i(+) - \sum P_i(-)]/N \quad (3)$$

where $P_i(+)$ and $P_i(-)$ terms were the responses when a given factor was at its high level and low level, respectively, and N was the total number of runs. As N = 8, effects of the variables were assumed to follow a t-distribution (Stowe and Mayer, 1966). The results of variance analysis are shown in Table 4.

The significance of each effect was determined by using the t-test (Stowe and Mayer, 1966). The two dummy variables gave two degrees of freedom for entering the tabulated value of t of 2.92 at p = 0.05, consequently

giving the minimal significance factor effect of 5.42×10^6, which was very much smaller than the absolute values of the effects of the molasses concentration (103.92×10^6), the bacterial culture time (103.92×10^6), and the phage collection time (103.95×10^6), demonstrating that these three variables significantly affected the phage production. Nitrogen in yeast extract was suspected to necessarily be used for phage construction, however, it was surprised that yeast extract did not significantly influence the phage production.

Table 3: The Placket-Burman design variables with phage production as response

Run	Factor							
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Phage concentration (PFU/mL)
1	+	-	-	+	-	+	+	210,500,000
2	+	+	-	-	+	-	+	10,700
3	+	+	+	-	-	+	-	205,250,000
4	-	+	+	+	-	-	+	13,600
5	+	-	+	+	+	-	-	13,850
6	-	+	-	+	+	+	-	71,500
7	-	-	+	-	+	+	+	6,250
8	-	-	-	-	-	-	-	1,700

Table 4: Statistical analysis of the PB design for phage production

Variable	Code	Effect ($\times 10^6$)	Sum of squares ($\times 10^{12}$)
Molasses concentration (g/L)	X_1	103.92	21599
KH_2PO_4 concentration (g/L)	X_2	-1.29	3
Dummy 1	X_3	-1.33	4
Yeast extract concentration (g/L)	X_4	1.33	4
Bacterial culture time (h)	X_5	-103.92	21597
Phage collection time (h)	X_6	103.95	21610
Dummy 2	X_7	1.30	3

3.2 Identifying optimal values for variables having significant effects on the phage production

The objective of the RSM design was to determine the optimal values for the bacterial culture time (A), the phage collection time (B) and the molasses concentration (C) for the phage production. 20 trials were conducted at five distinct levels (Table 2) for these variables. The observed and predicted phage concentrations at these levels are shown in Table 5.

Among the twenty trials, six of them were run at the central levels, i.e., at the bacterial culture time of 2 h, the phage collection time of 2 h and the molasses concentration of 2 g/L (Table 2). At the central levels, the phage concentration was predicted to be 1.343×10^9 PFU/mL. The experimental phage concentrations in these six trials ranged from 1.26×10^9 to 1.48×10^9 PFU/mL, which were also the highest concentrations found among the trials. At the actual levels where the model predicted negative responses, the experimental phage concentrations were actually not high. These low positive values resulted from the experiments were probably due to the experimental errors, especially the plate count method used for estimating the phage concentration. However, multiple regression analyses on th e data (Table 6) showed that the coefficient of determination (R^2) was of 0.9711, indicating the experimental results were fitted to the model. The predicted R^2 of 0.8102 was in reasonable agreement with the adjusted R^2 of 0.945, indicating a good regression model fitness.

The model F-value of 37.27 with $p < 0.0001$ implies that the model was significant. There was only a 0.01 % chance that an F-value this large could occur due to noise. The “Lack of Fit” F-value of 4.99 means that the Lack of Fit was insignificant, which is a good indication of the model. The TG25P production using *A. hydrophila* as host bacteria was expressed by the Eq(4).

$$\text{Phage production (PFU/mL)} = (134.3 - 15.24A + 13.76B + 2.63C - 40.16A^2 - 41.22B^2 - 37.42C^2 - 18.8AB + 0.71AC - 0.29BC) \times 10^7 \quad (4)$$

It can be seen among the three variables that only the bacterial culture time (A) and the phage collection time (B) exerted linear effects on the phage production, with p-values of 0.0024 and 0.0045, respectively. The quadratic effects of all the three variables on the phage production were obvious (p-values < 0.0001), the response surfaces thus contain curvatures.

Table 5: The RSM design variables with phage production as response

Run	A	B	C	Phage concentration (PFU/mL, ×10^3)	
				Observed	Predicted
1	2	0.318	2	71	-54,520
2	2	2	2	1,340,000	1,343,000
3	3	1	3	17	89,070
4	2	2	3.68	340,000	328,600
5	0.318	2	2	280,000	463,200
6	3	1	1	13	16,410
7	2	2	2	1,480,000	1,343,000
8	2	2	2	1,260,000	1,343,000
9	1	3	1	800,000	616,400
10	3	3	3	2,410	-17,540
11	1	1	3	19,500	3,806
12	2	2	2	1,300,000	1,343,000
13	3	3	1	380	-78,460
14	2	2	2	1,400,000	1,343,000
15	2	2	2	1,300,000	1,343,000
16	1	3	3	760,000	649,100
17	3.68	2	2	146	-49,390
18	2	2	0.318	95,000	240,100
19	2	3.68	2	220,000	408,300
20	1	1	1	34,000	-40,590

Table 6: Regression analysis of the TG25P production

Source	Coefficient Estimate (×10^7)	Sum of Squares (× 10^{16})	df	Mean Square (× 10^{16})	F-value	p-value	
Model		653.80	9	653.80	37.27	< 0.0001	significant
Intercept	134.3		1				
A-Bacterial culture time	-15.24	31.72	1	31.72	16.28	0.0024	
B-Phage collection time	13.76	25.86	1	25.86	13.27	0.0045	
C-Molasses	2.63	0.95	1	0.95	0.4858	0.5017	
AB	-18.8	28.27	1	28.27	14.5	0.0034	
AC	0.71	0.04	1	0.04	0.0205	0.889	
BC	-0.29	0.01	1	0.01	0.0035	0.9538	
A²	-40.16	232.40	1	232.40	119.27	< 0.0001	
B²	-41.22	244.90	1	244.90	125.66	< 0.0001	
C²	-37.42	201.80	1	201.80	103.56	< 0.0001	
Residual		19.49	10	19.49			
Lack of Fit		16.24	5	16.24	4.99	0.0512	not
Pure Error		3.25	5	3.25			
Cor Total		673.30	19				

R^2 = 0.9711; R^2_{adj} = 0.945; R^2_{pred} = 0.8102

These effects were all negative on the phage production response. The interaction effect that was significant in this model was that between the bacterial culture time and the phage collection time (p-value = 0.0034). This means the bacterial culture time and the phage collection time were interdependent on each other in exerting effect on the phage production. To gain a better understanding of the interaction effect of these two variables, 3D response surface plot and contour graph for the phage production were generated, which are visually shown in Figure 1. Numerical optimization using the Design Expert software revealed that the optimal phage concentration was of 1.37 $\times$ 10^9 PFU/mL, at the optimal values of the bacterial culture time, phage collection time and molasses concentration of 1.76 h, 2.22 h and 2.03 g/L, respectively. The verification experiment was performed at these predicted optimal values in three 250 mL flasks each of which contained 30 mL of medium with KH_2PO_4 (2 g/L), yeast extract (10 g/L), pH = 7. The bacterial inoculation was of 1 % (v/v) overnight *A. hydrophila* culture in TSB. The average phage concentration obtained was of (1.44 $\pm$ 0.2) $\times$ 10^9 PFU/mL. This value was within the predicted range at the 95 % prediction interval, between 1.16 $\times$ 10^9 and 1.60 $\times$ 10^9 PFU/mL.

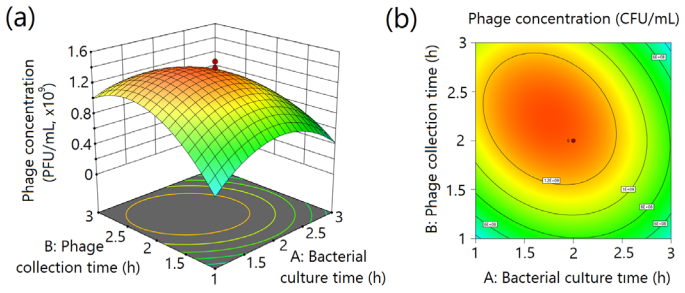

Figure 1: 3D response surface plot (a) and contour graph (b) showing the interaction between the bacterial culture time and the phage collection time

4. Conclusions

This study shows that the time taken to culture the *A. hydrophila* prior to TG25P infection and the time left for the phages in the mixture with the bacterial hosts, and the molasses concentration influenced the phage production significantly. All these three factors exerted quadratic effects while only the bacterial culture time and the phage collection time exerted linear effects on the phage production. Interestingly, the phage production was found to be strongly affected by the interdependence on each other of the bacterial culture time and the phage collection time. At the bacterial culture time of 1.76 h, the phage collection time of 2.22 h and the molasses concentration of 2.03 g/L, the optimal TG25P concentration of $(1.44 \pm 0.2) \times 10^9$ PFU/mL was indeed obtained. The findings have real economic implications for our TG25P phage production. Other factors such as bacterial culture temperature and rotation shaking rate will be investigated for their effects on the TG25P production before this phage is produced at a larger scale.

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References

Andreu R., Tran M.P., Kriengkrai S., Jiang M., Shahabuddin A.M., Patrik J.G.H., Francis J.M., David C.L., Anders D., Paul J.V.B., 2013, Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia, Aquaculture, 412-413, 231-243.

Box G.E.P., Wilson K.B., 1951, On the experimental attainment of optimum conditions, Journal of the Royal Statistical Society: Series B (Methodological), 13, 1-38.

Hoang, H.A., Pham, D.M., 2021, Phage cocktails to inactivate *Edwardsiella ictaluri*, an infectious agent in striped catfish *Pangasianodon hypophthalmus*. Chemical Engineering Transactions, 89, 535-540.

Dang, T.H.O., Xuan, T.T.T., Duyen, L.T.M., Le, N.P., Hoang, H.A., 2021, Protective efficacy of phage PVN02 against haemorrhagic septicaemia in striped catfish *Pangasianodon hypophthalmus* via oral administration. Journal of Fish Diseases, 44, 1255-1263.

Dong Z., Sha S., Li C., Hashim H., Gao Y., Ong P., Lee C.T., Zhang Z., Wu W.M., 2020, Potential risk of antibiotics pollution in aquaponic system and control approaches. Chemical Engineering Transactions, 78, 265-270.

Hoang A.H., Tran T.T.X., Le P.N., Dang T.H.O., 2019, Selection of phages to control *Aeromonas hydrophila* – an infectious agent in striped catfish, Biocontrol Science, 24, 23-28.

Le T.M.D., To H.N., Tran X.T.T., Hoang A.H., Le P.N., 2022, Influencing Factors on Up-stream Process of Edwardsiella ictaluri Bacteriophage Preparation, Chemical Engineering Transactions, 97, 313-318.

Plackett R.L., Burman J.P., 1946, The design of optimum multifactorial experiments, Biometrika, 33, 305-325.

Stowe R.A., Mayer R.P., 1966, Efficient screening of process variables, Industrial and Engineering Chemistry, 58, 36-40.

Tran T.T.H., Annette S.B., Tran T.M.D., Dang T.M.T., Nguyen T.T.H., Roger W.H., Nguyen T.P., 2021, Nursing stages of striped catfish (*Pangasianodon hypophthalmus*) in Vietnam: Pathogens, diseases and husbandry practices, Aquaculture, 533, 736114.

Tran T.T.X., Le D.T., Hoang A.H., 2018, Stability and activity of TG25P phage in control of *Aeromonas hydrophila* in striped catfish pond water, Science & Technology Development Journal, 21, 64-70.