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OPTIMIZATION OF BIOMASS PRODUCTION FROM *Bacillus licheniformis* B1 USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The production of biomass from Bacillus licheniformsis B1 was investigated in submerged fermentation. Optimization of culture medium was carried out by using response surface methodology (RSM). The B1 biomass production was also significantly affected by dextrose, soya, $CaCl_2$. In addition, while the optimum cultivation parameters were (g/L): 44.026 of dextrose, 34.025 of soya and 3.491 of $CaCl_2$, the prediction of biomass production of B1 was 6.05 x 10^9 CFU ml^{-1}.

Keywords: *Bacillus licheniformis* (B1), Biomass production, Response surface methodology.

1. Introduction

Vibrio parahaemolyticus is one of the pathogenic bacteria in aquatic animals associated with vibriosis, which was identified as a causative agent of acute hepatopancreatic necrosis disease (AHPND). The bacterial strain *Bacillus licheniformis* (B1) was isolated from the intestine of *Mugil Cephalus* in nature showed that antagonistic properties of the strain B1 (at concentration of 10^5 CFU/mL) against pathogenic strain of *V. parahaemolytics*, which a pathogen of AHPND (Vo et al., 2018).

Probiotics are containing *Bacillus spp*. produces a high and diverse amount of friendly antibiotics and bacteriolytic enzymes (Ferrari et al., 1993). Several studies have reported that *Bacillus* produces polypeptide antibiotics, which are directly effective against a wide range of Gram-negative bacteria and reduced mortality and improving crop yields on shrimp farms (Ali Farzanfar, 2006). *Bacillus* is widely used in animal feeds, human dietary supplements, and even in medicines (Cutting, 2006) as a common source of probiotic supplements. In the commercialization of *Bacillus*-based on bio-products, industrial exploitation focuses on high spore yield in bioreaction with less production cost (Khardziani et al., 2017). In addition, the yield per unit biomass or spore was influenced by several factors which including producing strain, media composition such as carbohydrate and nitrogen source, and fermentation condition such as pH, temperature and agitation

(Myers et al., 1995). In early reports, the optimization of culture media and culture conditions was largely studied for higher spore yields for particular *Bacillus* strains, since each strain differed from different nutrient requirements and culture conditions (Khardziani et al., 2017).

The Response Surface Methodology (RSM) can be used to collect and evaluate the relative significance of several factors in the presence of complex interaction, which are useful for developing, improving and optimizing processes (Myers et al., 1995). RSM is a powerful technique for testing multiple - process variables because fewer experimental trials are needed as compared with the study of one variable at a time (Bai et al., 2014). The most popular approach is based on full factorial central composite design (CCD), which is a usefully experimental design for estimating the coefficients of second order (quadratic) model.

In order to apply B1 production in shrimp farms to protect AHPND, we use RSM to evaluate the effect of various carbon, complex nitrogen, mineral sources as well as fermented condition (temperature and shaking) to enhance biomass production of *Bacillus licheniformis* B1.

2. Material and method

2.1. Source of bacterial strains and culture condition

The strain of *Bacillus licheniformis* (B1), which was isolated from fish gut sample, was evaluated inhibitable property *V. parahaemolyticus* in shrimp. Inoculum culture of B1 was enriched in Nutrient broth plus 1.5g NaCl (NB^+) for 18h-24h at 30^0C to obtain the biomass concentration around 10^8 CFU ml^{-1}. Then the 2% culture broth was transferred to 40 mL experimental medium in a 100-milliliter tube. The standard curve is:

Biomass (B1) = ($5.08xOD_{550nm}$ -2.02) x 10^8 CFU ml^{-1} (data not shown).

2.2. Screening the factors affect the biomass

2.2.1. Screening for essential media components by Plackett-Burman Design

Plackett-Burman design (PBD) was employed for screening the significant medium components for growth *Bacillus licheniformis* (B1). Design Expert 11 version was used for experimental designs and subsequent analysis of the experimental data. table 1 shows media components, symbol code and actual low (-1), central (0) and high level (+1) of the variables used in PBD experiments. The experiments were carried out in triplicates. Biomass production (B1) determined for the growth of the *Bacillus licheniformis* (B1) during 48h and were noted as the experimental response (OD_{550nm}). The variables with a confidence level above 95% were considered to have a significant effect on biomass production and thus consider for further optimization (Table 2).

2.2.2. Fractional Factorial two level design

The factorial design was employed for screening the most significant medium component from Plackett-Burman for growth *Bacillus licheniformis* (B1) after eliminated insignificant factors. Design Expert 11 version was used for experimental designs and subsequent analysis of the experimental data. The coded value of the independent factor and design layout is shown in Table 3.

2.3. Experimental design of response surface methodology

The culture medium was incubated in various treatments combination on aerobic condition for 48 hours at 30^0C. The three variables including dextrose, soya, and calcium chloride were selected for application in the RSM. Each variable was evaluated at five coded levels ($-\alpha$, -1, 0, +1, $+\alpha$). A Central Composite Design for three factors which set of 20 experiments included 8 running factors, 6 axial runs and 6 replicates at the central point were carried out for optimization. For evaluating the maximum point, a second-order polynomial function was fitted to the experimental results. For three factors this equation is:

$$Y\ (X_i) = ß_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i\ X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \varepsilon$$

Where Y (X_i) represents the response variable (OD_{550nm}), X_i is the independent variable, β_0 is the interception coefficient, β_i the coefficient of the linear effect, β_{ii} is the coefficient for the quadratic effect, β_{ij} corresponds to the coefficient for the interaction effect and ε is the random error.

Table 1. *Screen of treatment combinations with maximum biomass as the response*

Run	Dextrose [X_1[a]] (g/L)	Soya [X_2[a]] (g/L)	$CaCl_2$ [X_3[a]] (g/L)	Response [b] OD_{550nm} 48h
1	43.68 [0[c]]	37.00 [0]	3.45 [0]	12.435
2	43.68 [0]	37.00 [0]	4.22 [1.628]	11.100
3	43.68 [0]	37.00 [0]	3.45 [0]	12.320
4	40.00 [-1]	30.00 [-1]	3.91 [1]	11.620
5	47.36 [1]	44.00 [1]	3.91 [1]	10.503
6	40.00 [-1]	44.00 [1]	3.91 [1]	10.680
7	43.68 [0]	37.00 [0]	3.45 [0]	12.381
8	47.36 [1]	30.00 [-1]	3.00 [-1]	11.520
9	40.00 [-1]	30.00 [-1]	3.00 [-1]	9.9200
10	43.68 [0]	37.00 [0]	3.45 [0]	12.223
11	43.68 [0]	37.00 [0]	2.68 [-1.682]	11.380
12	43.68 [0]	25.22 [-1.682]	3.45 [0]	11.950
13	43.68 [0]	37.00 [0]	3.45 [0]	12.497

14	43.68 [0]	48.77 [1.682]	3.45 [0]	11.442
15	40.00 [-1]	44.00 [1]	3.00 [-1]	9.648
16	47.36 [1]	30.00 [-1]	3.91 [1]	10.900
17	43.68 [0]	37.00 [0]	3.45 [0]	11.970
18	47.36 [1]	44.00 [1]	3.00 [-1]	11.060
19	49.86 [1.682]	37.00 [0]	3.45 [0]	10.290
20	37.49 [-1.682]	37.00 [0]	3.45 [0]	9.800

[a] X_1, X_2, X_3 are Dextrose, Soya and Calcium chloride monohydrate, respectively, express in g/L; [b] Maximum biomass achieved at 48 hours, expressed in OD_{550nm}; [c] -1.682 (-α), -1, 0, +1, and 1.682 (+α) are coded levels.

The experiment was independently repeated three times. Results were analyzed using Design Expert 11. In the regression model, which permitted evaluation of the effects of the linear, quadratic, and interactive terms of the coded independent variables of dextrose, soya and calcium chloride monohydrate. Three – dimensional surface plot was drawn to illustrate the main and interactive effects of the independent variables on the number of viable cells, the response variable is $\log_{10}$ CFU per milliliter culture. The optimum value of the selected variables was obtained by both solving the regression equation and analyzing the response surface contour plots. The significant differences of every term in selected will be set as 5%.

2.4. Validation of the optimum medium and evaluation of modified medium

The maximum biomass of B1 was performed in the modified medium, brain heart infusion broth and nutrient broth, in order to measure the adequacy of the model and to verify the validity of the optimum medium.

3. Results and Discussion.

3.1. Screening the factor affect the biomass B1

3.1.1. Screening the essential media components by Plackett-Burman design

Investigating 11 factors impacting on Bacillus B1 cell density including selected carbon and nitrogen sources were based on single impact surveys (data not show). In this design, the pH was stabilized at about 7.0 ± 0.2 as *Bacillus licheniformis* B1 grows well at pH 5-8 (Vo et al., 2018). Table 2 shows five factors have a positive effected to *Bacillus licheniformis* B1 cell density including shaking rate, $CaCl_2$, soya, yeast extract and dextrose with a level of $\alpha = 0.05$ (p-value <0.05). The biomass production of the *Bacillus* B1 strain with optical has the following equation:

$Y\ (OD_{550nm}) = 5.89 + 0.12X_1 + 0.32X_5 + 0.13X_6 + 0.18X_7 + 2.06X_{11}$.

In which, Y is the target function (OD_{550} optical density), X1, X5, X6, X7, X11 are dextrose elements, yeast extract, soya flour, $CaCl_2$ and agitation.

Table 2. *Standardized effected of Plackett-Burman for evaluated*

Variable	Unit	Actual factor level at the coded factor level of			Effect	
		-1*	0*	+1*	Standardized effect	Prob>F
X_1	g/L	5[b]	17.5	30	0.25[a]	0.026
X_2	g/L	1	3	5	0.17[b]	0.052
X_3	g/L	5	12.5	20	-0.14[b]	0.075
X_4	g/L	5	17.5	30	0.063[b]	0.266
X_5	g/L	5	10	15	0.63[a]	0.004
X_6	g/L	5	17.5	30	0.26[a]	0.023
X_7	g/L	0.5	1.25	2	0.37[a]	0.012
X_8	g/L	0.5	1	1.5	-0.11[b]	0.117
X_9	g/L	0.5	1	1.5	-0.28[a]	0.021
X_{10}	^{0}C	30	33.5	37	-0.014[b]	0.762
X_{11}	**Rev/min**	**50**	**125**	**200**	**4.13[a]**	**<0.0001**

[a] Significant at $\alpha = 0.05$; [b] Not significant at $\alpha = 0.05$

* -1, 0, and +1 are coded levels

3.1.2. Fractional factorial two-level design

The fractional factorial design was designed with the main influencing factors: dextrose, yeast extract, soya, $CaCl_2$ and agitation. Table 3 represents the effect and p-value of each component.

Table 3. *Statistical analysis of the effects of medium components on biomass production*

Variables	Symbol	Unit	Actual factor level at the coded factor level of			Effect	p-value
			-1*	0*	+1*		
Agitation	X_1	Rev/min	150	200	250	-0.35[b]	0.318
Dextrose	X_2	g/L	25	32.5	40	1.43[a]	<0.001
Yeast Extract	X_3	g/L	15	22.5	30	0.73[a]	0.006
Soya	X_4	g/L	5	17.5	30	1.63[a]	<0.001
$CaCl_2$	**X_5**	**g/L**	**1.5**	**2.25**	**3**	**1.75[a]**	**<0.001**

R^2=93.03%, Adj R^2=83.32

* -1, 0, and +1 are coded levels; [a] Significant at $\alpha \leq 0.05$; [b] Not significant at $\alpha \geq 0.05$

According to the regression equation analysis, dextrose, yeast extract, soya and $CaCl_2$ have a p-value <0.05, indicating that the presence of these factors was significant in the regression equation. Although yeast extract significantly affects the cell density of *Bacillus licheniformis* B1 (0.73), the level of effect was lower than the other three factors. Moreover, the survey range of yeast extract (15-35 g/L) was quite high and if reaching the extreme area, it can lead to high cost than soya. In addition, the shaking rate factor (150-

200 rpm) has a value of p = 0.138 (> 0.05), showing that the shaking rate did not significantly affect the density of B1.

3.2. Experimenting of Response surface methodology

The effect of dextrose (X_1), Soya (X_2), and calcium chloride (X_3) were examined using central composite design (CCD). The response on maximum biomass at 48 hours varied from OD_{550}=9.648 to OD_{550nm}=12.497 (Table 1). The analysis of variance for the evaluation of the second-order model is presented in Table 4.

Table 4. *Analysis of variance for evaluation of the second-order model*

Regression	Degree of Freedom	Sum of Squares	R-Square	F Value	P Value
Linear	3	1.3444	0.0840	6.47	0.010
Quadratic	3	13.5073	0.7495	57.75	0.000
Interaction	3	1.9716	0.1232	9.49	0.003
Total Model	9	15.31	**0.9567**	24.57	**0.000**
Residual	Degree of Freedom	Sum of Squares	Mean Square	F Value	P Value
Lack of Fit	5	0.5138	0.1028	2.87	**0.1356**
Pure Error	5	0.1787	0.0357		
Total Error	10	0.6925	0.0692		

R^2= 0.9567, Adj R^2= 0.9178

The lack of fit and R-square testes showed how well the model fits the data (Bezerra et al., 2008). As showed in Table 4, the data obtained were fitted to a polynomial model with the R^2 value were significant (R^2 = 0.9567) and significance of the model was also supported by statistically insignificant lack of fit, as was evident from the lower calculated F-value (2.87). On the other hand, the term adjusted R^2 has been introduced which arranges the R^2 value for the sample size and for the number of variables in the model. Addition of insignificant model term in the model leads to a decrease in adjusted R^2 value, so the value of R^2 (0.9567) should be as close as that of adjusted R^2 (0.9178). By considering only significant factors, a second-order model actual values were shown in equation:

$$Y\ (OD_{550\,nm}) = 12.31 + 0.2152x_1 - 0.2141x_2 - 0.8374x_1^2 - 0.2537x_2^2 - 0.4149x_3^2 - 0.4886x_1x_3$$

Hence x_1, x_2 and x_3 represent dextrose, soya, calcium chloride and $ß_0$, $ß_1$, $ß_2$, $ß_3$ $ß_{11}$, $ß_{22}$, $ß_{33}$, $ß_{12}$, $ß_{13}$, $ß_{23}$ are constant coefficients.

The relationship between independent and dependent variables was expressed via response surface plots include contour plots and 3-dimensions plots simulated by the adjusted model showed in Figures 1. The plots of second-order predicted model illustrated that, for high dextrose and calcium chloride, the biomass production of viable cells was increasing.

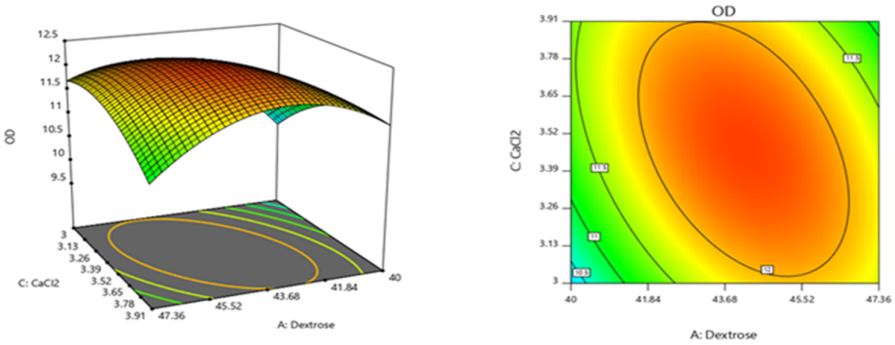

Figure 1. *Response surface plot and the Contour plot for effects of Dextrose and $CaCl_2$*

Through the use of numerical optimization in Design Expert 11, the quadratic model predicted that maximum biomass production was OD_{550nm} = 12.369, when the optimal values of test factors were dextrose = 44.026 g/L, soya = 34.025 g/L and $CaCl_2$ = 3.491 g/L.

3.3. Validation of the optimum medium and evaluation of modified medium

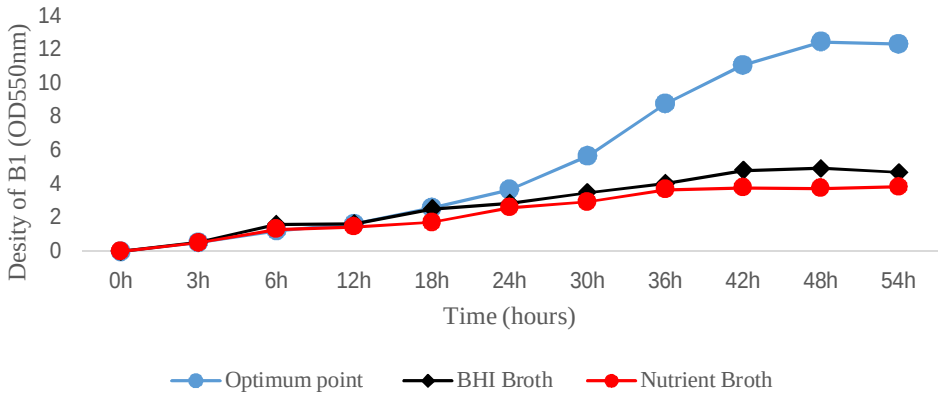

Figure 2. *The growth curve of Bacillus licheniformis B1 with different medium*

The result of the analyzed strain growth on optimal medium confirmed the usefulness of the new medium than the $BHIB^+$ and NB^+ (Figure 2). In the first eighteen hours, there was no significant difference in the density between three mediums. From the twenty-fourth hour, the density of B1 rises more quickly in the optimal medium than in the other. The highest density was reached approximate 6.02 $x10^9$ CFU ml^{-1} (OD_{550nm}=12.443) after forty-eight hours in modified medium. However, density of B1 in BHI broth and NB broth were about 2.27$x10^9$ CFU ml^{-1} and 1.67$x10^9$ CFU ml^{-1}, respectively.

3.4. Discussion

Optimization of the formulation of growth medium is the one of the key factors that need to be considered in the enhancement of any fermentation processes. In the fermentation, carbon and nitrogen sources were required for both growth and product formation. The characteristic features and nature of carbon and nitrogen play a major role in microorganism metabolism (Anderson et al., 2003) in which an appropriate nitrogen supplementation can cause the microflora. As a general rule, each organism has its own requirement for maximum biomass and bacteriocin production (Elibol, 2004). Even the oversupply could lead to the inhibition of bacterial growth. Despite the essential of an environment for biomass optimum, no defined medium has yet been established for mass production used in aquaculture. This requirement necessitated the present study to optimize carbon and nitrogen source for *Bacillus licheniformis* (B1) so that a commercial production process could be involved.

There are few previous reports on the selection of carbon and nitrogen sources for the groups of *Bacillus sp.* The current study makes a new move in the way bio-factors are chosen for a specific bacterial. In this current study, dextrose, soya and $CaCl_2$ were identified as preferential carbon and nitrogen sources for biomass production in *Bacillus licheniformis* (B1). In the medium for biomass production, the linear and quadratic effects of dextrose, soya and $CaCl_2$ were significant. This suggests that factors had directly effects relationship with biomass production, this can be explained as follow: soya was considered to be vital importance by virtue of its stimulatory effect on microbial cell growth because of soya is the rich peptone, amino acid and vitamin (Zendo et al., 2005). Far further, $CaCl_2$ was a precursor for building skeleton, improving metabolism process for start culture and likely played a role in activating enzyme systems necessary for sporulation (Ren et al., 2018). Otherwise, dextrose is a carbon source, which commonly just add into the fermentation media for facilitate growth.

In this study, the response surface plot and three-dimensional plots of biomass indicating a strong correlation between of two factors. The result of present study showed that contour plot was elliptical shape thus indicate significant interaction effect between dextrose and $CaCl_2$ (Figure 1). The optimum biomass production of *Bacillus licheniformis* strains reaches pH value 6.5- 7.0 and temperature 30^0C and 37^0C.

4. Conclusion

The response surface methodology was successfully employed to the growth of *Bacillus lincheniformis* (B1). The second-order polynomial model gave a satisfactory description of the experimental data. Dextrose concentration, soya concentration and $CaCl_2$ in the culture media were the most important factors affecting the growth of the microorganism ($p< 0.05$). Predicted and experimental results showed high similarity, which reflected the accuracy and applicability of RSM process optimization of biomass production. The present study introduced the new production conditions for Bacillus strains that are potentially useful in aquaculture.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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TỐI ƯU SẢN XUẤT TẠO SINH KHỐI CHỦNG *Bacillus licheniformis* B1 BẰNG PHƯƠNG PHÁP ĐÁP ỨNG BỀ MẶT

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TÓM TẮT

Nghiên cứu khả năng tạo sinh khối của Bacillus licheniformsis B1 được thực hiện bằng phương pháp lên men chìm. Thành phần môi trường tối ưu được thực hiện bằng phương pháp đáp ứng bề mặt ứng bề mặt (RSM). Khả năng tạo sinh khối của chủng B1 phụ thuộc vào các yếu tố như dextro, đậu nành, $CaCl_2$. Hơn nữa, khi các yếu tố dextro đạt giá trị tối ưu 44.026 g/L; đậu nành 34.025 g/L và 3.491 g/L $CaCl_2$ thì sinh khối của B1 tạo ra lớn nhất và đạt $6.05x10^9$ CFU ml^{-1}.

Từ khóa: *Bacillus licheniformis* (B1); Sinh khối, phương pháp đáp ứng bề mặt.