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Opitimization of PHB production from *Escherichia coli* DH5α harboring phbCAB operon using Plackett-Burman matrix and response surface method-central composite design

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ABSTRACT

Fermentation process of Escherichia coli DH5α harboring phbCAB operon was optimized to achieve highest PHB productivity. Neutral pH (7.0) is optimal for PHB production of E. coli that can be applied at industrial scale as neutral and basic environment (pH ≥ 7) permit bacteria growth. In this study, Plackett – Burman experiment design was used to evaluate the effects of various physical-chemical factors on PHB yield – in which Molasses amount, fermentation time and pH were proved to affect the most. This study also utilized response surface method (RSM) and central composite design (CCD) to achieve optimal value of molasses amount (120g/l), fermentation time (48h) and pH (7.0), which yieled 3.36g/l cell dried weight (CDW) and 0.87g/l PHB products. The design model was applied in 150ml aerated fermentor and 1.5l automatic fermentor, achieved 3,3g/l CDW with 0,86g/l (PHB) and 3,4g/l CDW with 0,9g/l PHB, respectively.

Key worlds: PHB: poly–β–hydroxybutyrate, fermenter, Eschirichia coli DH5α, phbCAB operon, Plackett – Burman, RSM – CCD

INTRODUCTION

Polyhydroxybutyrate (PHB) is a biopolymer which belongs to polyhydroxyalkanoate (PHA) family. It was first discovered in *Bacillus megaterium* in 1926 by French biologist Maurice Lemoigne. This polyester existed as lipid inclusion bodies accumulated inside the cell, makes up to 97 – 98% of the bodies themselves. The other components are protein (2%) and lipid (0.5%) [1]. Industrial scaled manufacture of this polymer has been implemented since the late 1950s; Baptist and Werber of W.R. Grace (U.S.) were considered as pioneers of PHB commercial production [2-3]. Current researches focus on minimization manufacture cost by utilizing cheap materials, efficient PHB-accumulating bacteria, implementation of more effective fermentation and recovery processes. PHB degraded into (D)-3-hydroxybutyric acid, an intermediate of animal metabolic pathway; therefore it is possible to implant this polymer into living bodies without transplant rejection. Such excellent biocompatible enable PHB's wide application in medicine and healthcare, for example, biomedical materials, pharmaceuticals, pharmaceutical capsules, degradable sutures, joints in bone fracture treatment, suturing blood vessels,… without any noxious effects. Moreover, due to similar properties, PHB can be used as replacement for synthetic plastics in wrapping, packages, covering materials... to mitigate environmental pollution. PHB's biodegradability by terrestrial microorganisms enables its application in pesticides, herbicides, fertilizers, seed packages; plant nursery bags... since there is no need of waste treatment after harvesting [4]. Various researches about PHB synthesizing microorganisms has been carried out and resulted in considerable achievements. Much of the works focused on *Ralstonia eutropha*, also known as *Alcaligenes eutrophus* [5-9].

In Vietnam, several papers reported how to obtain PHB from native or mutated bacterial [10-12]. In our previous publication, a 4985 bp DNA fragment including the whole *phb*CAB operon from *Alcaligenes eutrophus* H16 was cloned into *Escherichia coli* DH5α and the PHB powder was harvested for evaluation its chemistry and physical

characters[13-14]. However, none efforts has been made in Vietnam to optimize PHB yields from recombinant *E.coli* using Plackett – Burman matrix and response surface method (RSM) – (central composite design (CCD). Furthermore, it should be noted that inexpensive substrates for PHB production by recombinant *E.coli* still attracts much attention from researchers.

Optimization of fermentation for high yield and increasing manufacture scale is critical in industrial application of theoretical researches. Plackett and Burman (1946) already proposed an optimization multifactor experiment design which is effective, low-cost and able to estimate the optimal value for each factor, and their design has been widely used in screening environmental components in shaking fermentation, followed by experiments using RSM – CCD for optimization of chosen factors[15,16]. This study optimized physical-chemical factors of the fermentation following Plackett – Burman design and RSM – CCD to estimate the maximal cell dried weight (CDW) and PHB amount from recombinant *Escherichia coli* DH5α harboring phbCAB operon.

EXPERIMENTAL SECTION

Inoculum and fermentation medium

Recombinant *E.coli* DH5α *phb*CAB operon in previously study was used in this work [13]. Cells were maintained in LB 2% glucose agar slant at 35^0C and 150rpm of shaking. Fermentation medium contained (per liter) 120g of molasses, 4g of $(NH_4)_2SO_4$ (for assessment of carbon and nitrogen's effect), 2g of KH_2PO_4, 4g of K_2HPO_4 (for stabilization) with 5% micronutrients content and neutral pH (7.0). Inoculum size was 5% and fermentation time was 48h.

Quantification of cell dried weight and PHB

Cell dried weight (CDW) was quantified by direct measurement, while quantification of PHB was performed via measurement of optical density (OD) at 235mm and construction of a linear correlation directrix to estimate PHB content.

Table 1. Correlation between standard PHB content and OD_{235} value

PHB content (µg/mL)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
OD_{235}	0.017	0.018	0.035	0.051	0.068	0.088	0.094	0.109	0.125	0.150	0.152	0.192

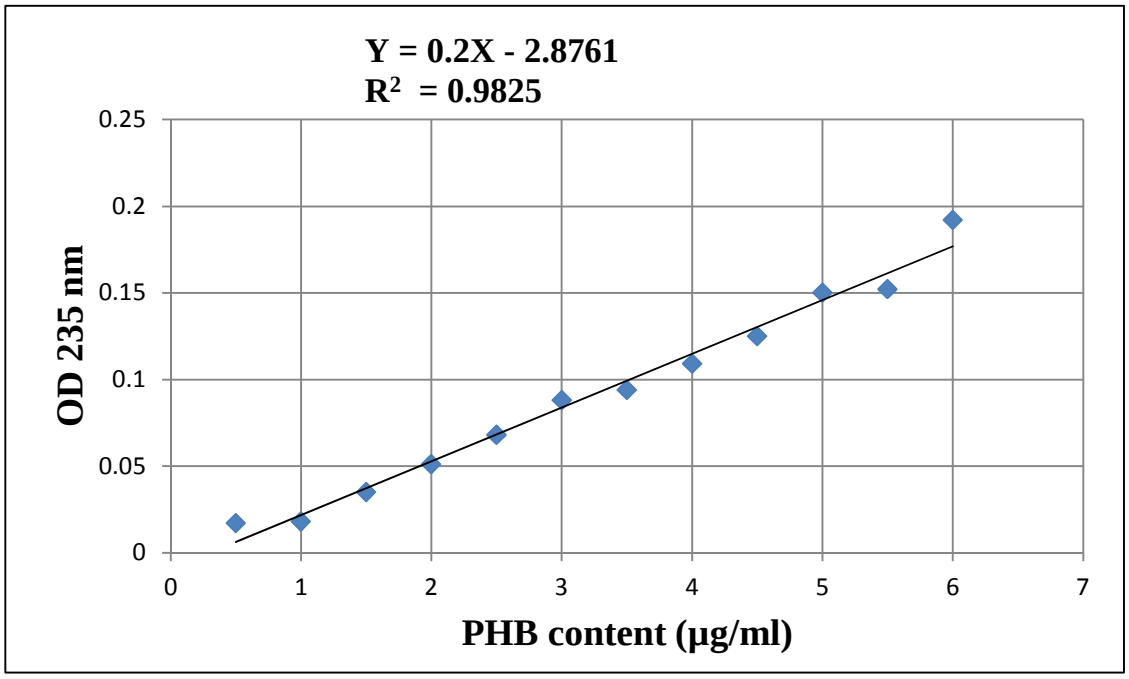

Figure 1: Linear correlation directrix of PHB content

Plackett – Burman design, response surface method (RSM) and central composite design (CCD)

Eight factors were chosen to evaluate the effects on PHB synthesis of recombinant *E.coli* DH5α harboring *phb*CAB operon: molasses, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, pH, inoculum size, fermentation time, micronutrient contents. Experiments were design following Plackett – Burman matrix[15,16] with 8 factors in 12 experiments (Table 2) to screen for the most vital factors affect PHB yield (g/l). Low (-1) and high levels (+1) of the factors were listed in

Table 2. Three main RSM – CCD factors were optimized and evaluated at five levels (-α, -1, 0, +1, +α) (Table 3) in the CCD of 20 experiments (Table 4) [18].

CDW (g/l) and PHB (g/l) were selected response function. The model was expressed by a quadratic equation:

$$Y = bo + b1x1 + b2x2 + b3x3 + b11x12 + b22x22 + b33x32 + b12x1x2 + b23x2x3 + b13x1x3$$

in which b1, b2, b3 were linear coefficients; b11, b22, b33 were quadratic coefficients; and b12, b23, b13 were interactive coefficients of each factors couple; x1, x2, x3, x11, x22, x33, x12, x23, x13 were independent variables. Statistics were analyzed by Design expert 9.0.0® software of Stat-Ease Inc. USA. Optimal value of each factors were inferred from the analyzing results.

Table 2. Variables of Plackett – Burman matrix and their effects

Symbol	Variables	Coded level		Effect level	Reliability
		Low (-1)	High (+1)		
X_1	**Molasses (g)**	**100**	**140**	**0.51[a]**	**0.0556**
X_2	**pH**	**5**	**9**	**0.38[b]**	**0.0785**
X_3	$(NH_4)_2SO_4$ (g)	3	7	0.08[b]	0.3070
X_4	KH_2HPO_4 (g)	1	4	0.008[b]	0.7370
X_5	K_2HPO_4 (g)	2	8	0.005[b]	0.7874
X_6	Inoculum size (%)	4	8	0.14[a]	0.2087
X_7	Micronutrients (%)	0.02	0.1	0.007[b]	0.7537
X_8	**Fermentation time (h)**	**24**	**72**	**6.90[a]**	**0.0015**

[a]Significant at α = 0.1 reliability; [b]Insignificant at α = 0.1 reliability

Table 3. Plackett – Burman experiment matrix

Experiment	Variables								Observed criteria (48h)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	CDW (g/l)	PHB (g/l)
1	1	1	1	-1	1	1	-1	-1	2.94	0.5
2	-1	1	1	-1	-1	1	1	-1	2.86	0.5
3	1	-1	-1	1	1	1	1	1	2.76	0.4
4	-1	1	1	-1	1	-1	1	1	1.07	0.04
5	-1	1	-1	1	-1	1	-1	1	2.19	0.3
6	-1	-1	-1	-1	1	1	1	-1	2.07	0.3
7	1	1	-1	-1	-1	-1	1	1	1.47	0.04
8	1	-1	1	-1	-1	1	-1	1	2.37	0.3
9	1	-1	1	1	1	-1	1	-1	1.16	0.02
10	-1	-1	1	1	1	-1	-1	1	0.74	0.01
11	1	1	-1	1	1	-1	-1	-1	1.18	0.03
12	-1	-1	-1	-1	-1	-1	-1	-1	0.57	0.03

Table 4. Value of studied factors in RSM – CCD

Symbol	Variables	Range of interest	Coded level				
			-α	-1	0	1	+α
X_1	Molasses (g)	86.36-153.63	86.36	100	120	140	153.63
X_2	pH	3.63-10.36	3.63	5	7	9	10.36
X_3	Time (h)	7.63-88.36	7.63	24	48	72	88.36

Table 5. Experiment outline for optimization of CDW follow RSM – CCD

Experiment	Factors			Observed criteria	Predicted from model
	Molasses	pH	Time (h)	Y_1 (CDW) g/l	
	X_1	X_2	X_3		
1	-1	-1	-1	1.49	1.52
2	1	-1	-1	1.46	1.48
3	-1	1	-1	1.34	1.42
4	1	1	-1	1.24	1.35
5	-1	-1	1	2.87	2.98
6	1	-1	1	2.81	3.02
7	-1	1	1	2.48	2.56
8	1	1	1	2.69	2.98
9	-α	0	0	1.64	1.92
10	+α	0	0	2.97	3.12
11	0	-α	0	0.67	0.79
12	0	+α	0	0.93	1.01
13	0	0	-α	1.12	1.18
14	0	0	+α	2.68	2.86
15	0	0	0	2.98	3.04
16	0	0	0	2.92	2.99
17	0	0	0	3.02	3.22
18	0	0	0	3.06	3.36
19	0	0	0	3.30	3.42
20	0	0	0	3.05	3.14

Table 6. Experiment outline for optimization of PHB production follow RSM – CCD

Experiment	Factors			Observed criteria		Predicted from model
	Molasses	pH	Time (h)	Y_1 (CDW) g/l		
	X_1	X_2	X_3			
1	-1	-1	-1	0.05	2.68%	0.06
2	1	-1	-1	0.04	3.42%	0.06
3	-1	1	-1	0.04	2.99%	0.05
4	1	1	-1	0.03	2.42%	0.05
5	-1	-1	1	0.5	17.42%	0.6
6	1	-1	1	0.4	16.13%	0.5
7	-1	1	1	0.4	14.23%	0.5
8	1	1	1	0.4	14.87%	0.6
9	-α	0	0	0.3	18.29%	0.4
10	+α	0	0	0.7	23.57%	0.9
11	0	-α	0	0.1	14.93%	0.2
12	0	+α	0	0.2	21.51%	0.3
13	0	0	-α	0.02	1.79%	0.02
14	0	0	+α	0.6	22.39%	0.7
15	0	0	0	0.7	23.49%	0.8
16	0	0	0	0.7	23.97%	0.8
17	0	0	0	0.7	23.18%	0.7
18	0	0	0	0.8	26.14%	0.9
19	0	0	0	0.8	24.24%	1.0
20	0	0	0	0.7	22.95%	0.8

Evaluation of experiment model

Experiments of theoretical optimal fermentation model were performed in 150ml aerated fermentor and 1.5l Bioflo 110 Bioreactor (New Brunswick Scientific, USA). The 1.5l bioreactor was self-controlled at pH 7.0, 30^0C, 25% dissolved oxygen (DO) and automatically relgulated aeration rate.

RESULTS AND DISCUSSION

Recombinant *E.coli* DH5α harboring phbCAB operon could grow in a broad rage of pH (5 to 9), that consistent with previous researches about the bacterium[20-21]. Optimal pH value for PHB synthesis was 7.0. Hence, recombinant *E.coli* DH5α harboring phbCAB operon may be able to function in basic environment (pH > 7) and can be used in industrial production. Highest PHB productivity was achieved after 48h and strated to decrease after 72h.

Various growth conditions proved to highly affect the CDW of recombinant *E. coli*. The first to be mentioned was fermentation time. Molasses was another important factor for observed criteria due to its role both as carbon source and material for cell components. The next one should be pH since bacteria growth is only favourable in basic or neutral condition. K_2HPO_4 and KH_2PO_4 toghether at certain ratios could stabilize the pH and considerably increase

the observed criteria. $(NH_4)_2SO_4$ as a nitrogen source also made its own contribution. Micronutrients such as Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}… functioned as ion supply and co-factor of various essential enzymes for microorganisms' growth [19, 21-25].

Screening for vital factors of CDW and PHB accumulation of recombinant *E.coli* DH5α harboring phbCAB operon

Plackett – Burman matrix resulted in 0.57 – 2.94 g/l of CDW and 0.03 – 0.5g/l of PHB from ferment extracts (Table 3). Effect of each fator on PHB and CDW yield was calculated by Design expert® 9.0.0 software (Table 2). Positive and high effect value meant great effects on the PHB yield. Amongst the evaluated factors, fermentation time, molasses concentration and pH exerts highest effect on PHB yield with significance of α= 0.05, therefore they were chosen for the next RSM – CCD experiments.

Optimization for maximum CDW and PHB yield

After screening, the chosen factors were optimized using RSM – CCD and Design expert® 9.0.0 software. Observed and predicted values of response function were presented in Table 5 and 6. After performing ANOVA, CDW and PHB yield were estimated via regression equation in the following model:

$$Y = 0{,}33 - 0{,}031x_1 - 0{,}027x_2 - 0{,}16x_3 + 3{,}89.10^{-3}x_1x_2 - 8{,}24.10^{-3}x_1x_3 - 0{,}014x_2x_3 - 4{,}28.10^{-3}x^2_1 + 0{,}26x^2_2 + 0{,}048x^2_3$$

In which Y was CDW yield (g/l), X_1, X2, X_3 were molasses concentration (g/l), pH and fermentation time (h), respectively. Regression coefficient (R^2) was identified as 0.981, which means 98.1 % of experiment data was compatible with the value predicted via the model. $R^2 > 0.75$ means the model was also compatible with experiments. Predicted R^2 value (0.7714) was on accord with modified R^2 (0.981) with deviation of 0.1556 < 0.2. Signal: noise ratio was 19,301 > 4, which means the signals were sufficient. Response surface diagram (Figure 2) presented the interaction between each couple of factors and from the diagram it was possible to identify the optimal of each ones for the maximum value of response function. The model predict the optimal molasses concentration (120g/l), fermentation time (48h) and pH (7.0) to achieve maximal CDW and PHB yields (3.4g/l and 0.9g/l, respectively). Increase of CDW and PHB yields couple with the rise of molasses content, however molasses and pH only increased to a certain extend and decreased afterward.

Model experiment in 150ml and 1.5ml fermentors

The model was experimented in aerated 150 ml fermentor and 1.5 l automated bioreactor. Experiment in 150ml fermentor was repeated 3 times while the other one was done once. Results were presented in Table 7. The CDW reached 3.36 ± 0.5g/l in 150ml fermentor, similar to predicted value (3.42g/l). Automated bioreactor (at 30^0 C ± 3; pH 7.0 ± 0,2; airflow 1 vvm; aerated rate 300 rpm) managed to yielded 3.4g/l CDW and 0.9g/l PHB.

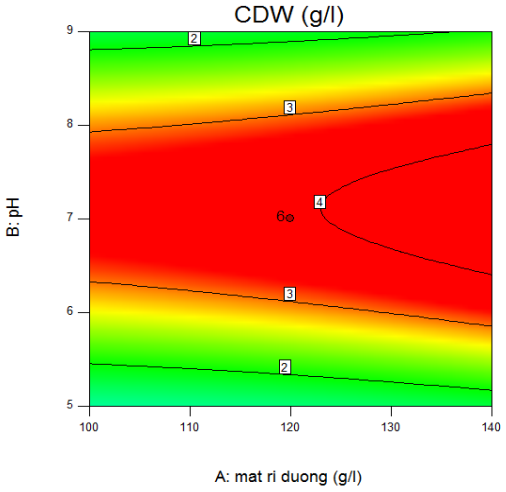

Figure 2 CDW response surface by pH and molasses

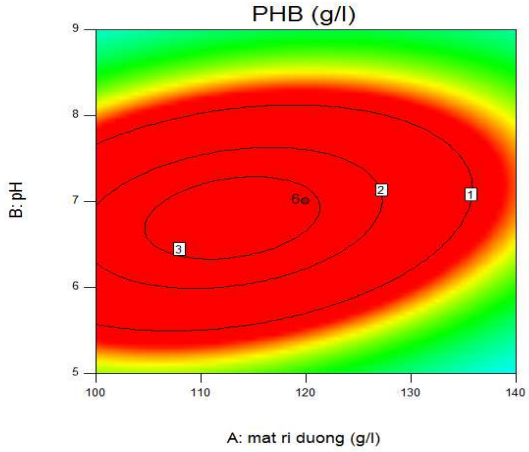

Figure 3 PHB response surface by pH and molasses

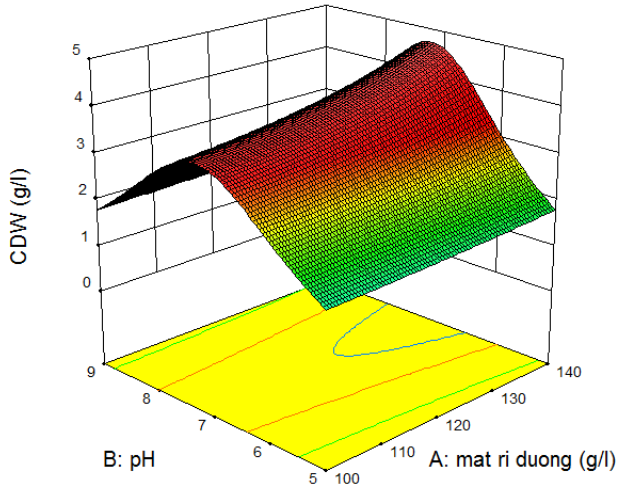

Figure 4 Three-dimensional CDW response surface by pH and molasses

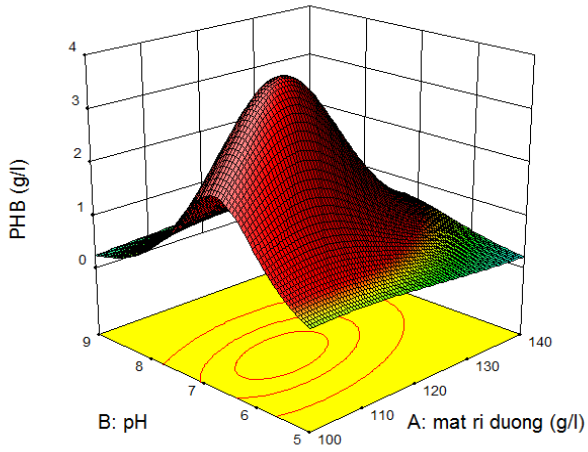

Figure 5 Three-dimensional PHB response surface by pH and molasses

Table 7. PHB and CDW yields from recombinant *E.coli* DH5α harboring *phb*CAB operon

Capacity	Fermentation mode	CDW (g/l)	PHB (g/l)
150ml	Aerated	3.36	0.86
1.5l	Automated fermentor	3.4	0.9

Plackett – Burman optimization multifactor experiment design and response surface method – central composite design can be considered as powerful instruments for screening and optimization of factor values to achieve maximum function response. Using these instruments together with Design expert® software will reduce time consumption and number of experiments; the user is also able to select one amongst the solutions proposed by the software.

Molasses is cheap and yields considerable PHB amount, nonetheless it is still inferior to some other carbon sources. That is an obstacle in up-scaling PHB production. Unfavorable working conditions rendered non-molasses substrates inaccessible to this study. Due to high specificity of recombinant *E.coli* DH5α harboring *phb*CAB operon, it is necessary to conduct further researches on other different carbon sources.

CONCLUSION

This study managed to identify a number of optimal nutrient and environmental conditions for PHB production by recombinant *E.Coli* in which 120g/L of molasses, 48 hours of fermentation, neutral pH, 4g/L of $(NH_4)_2SO_4$ as nitrogen source, 2g/l of KH_2PO_4, 4g/L of K_2HPO_4, 0.05% of micronutrient contents and inoculum size 5%. Via fermentation experiments using pre-determined optimal medium and system, this study managed to achieve similar results to other studies in 150ml fermentor, in which the CDW amounted to 3.3 ± 0.05g/L and PHB accumulation of 26.2 ± 0.05% (w/w). Although the result was relatively modest, it can be used as basis for further studies to improve the PHB synthesis efficiency.

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