American Journal of Analytical Chemistry, 2013, 4, 594-599
http://dx.doi.org/10.4236/ajac.2013.410070 Published Online October 2013 (http://www.scirp.org/journal/ajac)

Optimization of Fermentation Medium for Producing α-Hydroxyphenylacetic Acid by Using Plackett-Burman Design and Response Surface Methodology*

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Received May 9, 2013; revised June 9, 2013; accepted July 10, 2013

ABSTRACT

Plackett-Burman design and response surface methodology were applied in order to optimize the fermentation medium of (R)-α-hydroxyphenylacetic acid ((R)-HPA) producing *Bacillus* sp. HZG-19. The factors playing important roles in the production of (R)-HPA were selected based on Plackett-Burman design. The path of steepest ascent was undertaken to optimize said fermentation medium. Finally, the optimal levels of the factors with the greatest change in regard to product yield were further optimized using Box-Behnken and response surface analysis. The optimal conditions were found to be as follows: casein peptone 30.49 ($g \times L^{-1}$), glycerol 14.09 ($g \times L^{-1}$), KH_2PO_4 0.1345 ($g \times L^{-1}$), K_2HPO_4 0.01 ($g \times L^{-1}$), $CaCl_2$ 0.1 ($g \times L^{-1}$), $MnSO_4$ 0.01 ($g \times L^{-1}$). Under the optimal conditions described above, the yield of (R)-HPA reached 63.30%, which indicated an increase of 14.9%, as compared to the yield obtained before optimization.

Keywords: α-Hydroxyphenylacetic Acid; Fermentation Medium; Biotransformation; Response Surface Analysis

1. Introduction

α-Hydroxyphenylacetic acid (mandelic acid) (HPA) and its derivatives are key intermediates for the production of various pharmaceuticals, such as semi-synthetic penicillins and cephalosporins [1-3]. It is also used as a chiral resolving agent and chiral synthon for the synthesis of anti-tumor and anti-obesity agents [4]. Many methods have been reported for the preparation of enantiomerically pure (S)-or (R)-α-Hydroxyphenylacetic acid [5-9]. For example, the synthesis of enantiopure α-Hydroxyphenylacetic acid has been investigated through diastereomeric crystallization [10]. It has also been prepared by the chemo-enzymatic routes [11], from methyl mandelate by lipase-catalyzed hydrolysis [12], or from kinetic resolution [13]. However, these approaches are limited in their industrial application due to the expensive catalysts, limited efficiency and low yields.

Recently we have reported a new bacterial strain, *Bacillus* sp. HZG-19, which is capable of degrading phenylglyoxylic acid (PGA) and affording (R)-HPA with high optical purity. Numerous variables will have an effect on the production of HPA, hence, factors playing important roles in the production of pure enantimomeric (R)-HPA are crucial for large scale production. Plackett-Burman, Box-Behnken design and response surface methodology are inexpensive and accurate methods for further optimization of the fermentation medium.

2. Materials and Methods

2.1. Strain and Chemicals

The strain (*Bacillus* sp. HZG-19) was preserved in the lab of Chemical Engineering Department, Fuzhou University. (R)-HPA and (S)-HPA were purchased from Sigma (St. Louis, MO USA). Phenylglyoxylic acid (PGA, >98%) was supplied by Pharmaceutical & Chemical Co., Ltd of Taizhou, China. Methyl alcohol of HPLC grade was purchased from Merck, Germany. Hydroxypropyl-

*The work was supported by the Doctoral Fund of Ministry of Education (No. 20103514110002) and the Major Plan Project of Science and Technology of Fujian Province (No. 2009N0046).
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β-cyclodextrin (HP-β-CD) was supplied by Fluka (Neu Ulm, Germany). All other chemicals were obtained from local suppliers and of reagent grade.

2.2. Medium

Seed medium (g × L^{-1}): casein peptone 10, beef extract 5, maltose 10, $NaCl_2$, pH 7.2.

Fermentation medium (FM) (g × L^{-1}): casein peptone 20, glycerol 10, K_2HPO_4 0.01, KH_2PO_4 0.1, $CaCl_2$ 0.1, $MnSO_4$ 0.01, pH 7.2.

Medium for slant culture (SM): same as seed medium with 2% (w/v) of agar.

2.3. Culture Conditions

The strain, *Bacillus* sp. HZG-19, was inoculated into 20 ml seed medium in 50 ml Erlenmeyer flasks and cultured aerobically at 32˚C on a shaker (180 rpm). When the cells reached logarithmic phase, cultures were then inoculated (1%, v/v) into 50 ml flasks containing 20 ml of FM medium at the same fermentation conditions. PGA solution, with a final concentration of 15 mM, was added directly into the fermentation broth under aseptic conditions. After 24 h of incubation, the supernatant that was obtained after centrifugation from the medium and was subjected to HPLC analysis and HPCE analysis to determine the concentration and purity of the HPA generated.

2.4. Analytical Methods

The concentrations of substrate and product were determined by HPLC using a reverse phase column (Agilent HC-C 18, Ø 4.6 mm × 250 mm, 5 μm). The mobile phase is composed of methanol and phosphate buffer (25 mmol/L) (15:85, v/v) containing 6 mmol/L of tetrabutylammonium bromide (pH 6.8) at the rate of 1.0 ml/min. A UV detector (at 220 nm) was employed for quantification.

The yield of HPA is expressed as:

$$\text{Yield}(\%) = \text{CHPA}/\text{CPGA} \times 100\%,$$

where CHPA and CPGA represent the concentration of the HPA generated and initial concentration of PGA, respectively. The concentration of (R)-HPA and (S)-HPA were determined by HPCE. Detection was made at 214 nm using a buffer solution of Tris-phosphoric acid (100 mmol/L, pH 7.6) containing 150 g/L of hydroxypropyl-β-cyclodextrin. A voltage of 20 KV was applied at a temperature of 20˚C.

$$\text{Enantiomeric excess}(\text{e.e.}\%) = ([\text{R}]-[\text{S}])/([\text{R}]+[\text{S}]) \times 100\%,$$

where [R] and [S] are the concentration of (R)-HPA and (S)-HPA, respectively.

3. Results and Discussion

3.1. Plackett-Burman Design

The Plackett-Burman (PB) design is extremely useful in screening and selecting for the most vital factors within a large candidate pool [14]. The experiments were carried out according to the design matrix shown in **Table 1**, with each row representing one trial while each column represents a single variable. The six factors listed are: casein peptone, glycerol, KH_2PO_4, K_2HPO_4, $CaCl_2$, $MnSO_4$, respectively. Three variables (X_4, X_6, X_8) are dummy variables employed to evaluate the standard errors of the experiment. Elements (1) and (−1) are representative of the relative amounts each variable factor, either high or low, within each experimental trial. Yield of HPA is considered as response value.

Levels selection and significance evaluation of experimental variables are summarized in **Table 2**. Generally, effects with a confidence level of greater than 95% are considered to be actual effects, whereas a confidence level of less than 95% indicates that the effect may have resulted due to chance. As shown in **Table 2**, the confidence level of factors casein peptone (X_1), glycerol (X_2) and KH_2PO_4 (X_3) are shown to be above 95% and considered to be significant. The rest of the factors, K_2HPO_4 (X_5), $CaCl_2$ (X_7) and $MnSO_4$ (X_9) had a confidence of below 95% in HPA production and hence, were considered insignificant. The high level of X_5, X_7 and the low level of X_9 were selected during further optimization studies.

Table 1. Design matrix and experimental results of Plackett-Burman design.

Run	X_1	X_2	X_3	(X_4)	X_5	(X_6)	X_7	(X_8)	X_9	Yield/%
1	1	−1	1	−1	−1	−1	1	1	1	59.52
2	1	1	−1	1	−1	−1	−1	1	1	60.71
3	−1	1	1	−1	1	−1	−1	−1	1	52.83
4	1	−1	1	1	−1	1	−1	−1	−1	61.72
5	1	1	−1	1	1	−1	1	−1	−1	62.34
6	1	1	1	−1	1	1	−1	1	−1	61.31
7	−1	1	1	1	−1	1	1	−1	1	56.63
8	−1	−1	1	1	1	−1	1	1	−1	54.02
9	−1	−1	−1	1	1	1	−1	1	1	42.12
10	1	−1	−1	−1	1	1	1	−1	1	51.32
11	−1	1	−1	−1	−1	1	1	1	−1	51.90
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	45.05

Each experiment was performed in triplicates, the same below.

Table 2. Levels selection and significance evaluation of experimental variables.

Code	Levels		T-test	Pr > |t|	Significancy
	Low (−1)	High (1)			
X_1	20	30	13.1833	0.0057	1*
X_2	10	15	7.7648	0.0162	3*
X_3	0.1	0.15	7.9100	0.0156	2*
X_5	0.01	0.015	−2.8302	0.1054	6
X_7	0.1	0.15	2.9269	0.0996	5
X_9	0.01	0.015	−3.2172	0.0845	4

*Indicate that the significancy is of more than 95% confidence.

3.2. Steepest Ascent Design to Approach the Optimal Region

The steepest ascent method was applied in order to investigate optimal substrate concentration, by slowly increasing substrates along the path of steepest ascent until no further increase in response is observed [15]. Based on the experimental results of PB, casein peptone, glycerol and KH_2PO_4 should be increased due to their positive effect (positive value for t). **Table 3** represents the design and results of the steepest ascent search experiment. As shown in **Table 3**, optimal fermentation medium should be around run 2, so the level of run 2 is considered as center point in the follow-up response surface experiment.

3.3. Response Surface Analysis (RSM) and Establishment of Optimum Fermentation Medium

RSM is a statistical method by which one can find the best conditions in a multi-factor system [16]. Among RSM, Box-Behnken design (BBD) method and central composite design are more frequently used than the others. When the number of the experimental factors does not exceed 5, Box-Behnken method is more economic. Casein peptone, glycerol and KH_2PO_4 were determined to be the main factors as shown through the PB experiment.

X_1, X_2, X_3 represent casein peptone, glycerol, KH_2PO_4 respectively and the yield of HPA shows the response value. Factor levels were determined by steepest ascent experiment. In order to investigate the optimum levels of those variables and study their interactions, a three-factor three-level BBD was applied.

A total of 15 experiments were performed in triplicates, 12 of which used different factors while 3 trials were used as controls. Controls were used to estimate experimental error. **Table 4** illustrates the coded and non-coded values of the experimental variables, with results of the BBD experiments given in **Table 5**.

Table 3. Design and results of the steepest ascent search experiment.

Run	casein peptone (g/L)	glycerol (g/L)	KH_2PO_4 (g/L)	Yield (%)
1	25	12.5	0.125	54.12
2	28	13.5	0.135	61.92
3	31	14.5	0.145	59.37
4	34	15.5	0.155	54.10
5	37	16.5	0.165	52.35

Table 4. The coded and non-coded values of the experimental variables.

Factors	Code	Coded level		
		−1	0	1
casein peptone (g/L)	X_1	25	28	31
glycerol (g/L)	X_2	12.5	13.5	14.5
KH_2PO_4 (g/L)	X_3	0.125	0.135	0.145

Table 5. Design and results of BBD experiments.

Run	X_1	X_2	X_3	Yield/%
1	−1	−1	0	55.53
2	−1	1	0	57.04
3	1	−1	0	56.3
4	1	1	0	62.43
5	0	−1	−1	54.13
6	0	−1	1	50.39
7	0	1	−1	56.06
8	0	1	1	56.89
9	−1	0	−1	52.78
10	1	0	−1	58.78
11	−1	0	1	52.68
12	1	0	1	56.42
13	0	0	0	61.83
14	0	0	0	62.02
15	0	0	0	61.93

Based on the experimental results of BBD (**Table 5**) and regression analysis, a quadratic polynomial equation was established to identify the relationship between yield and variables. The model of coded units can be expressed as:

$$\begin{aligned} Y_1 = {} & 61.92667 + 1.9875X_1 + 2.00875X_2 \\ & - 0.67125X_3 - 1.652083X_1^2 + 1.155X_1X_2 \\ & - 0.565X_1X_3 - 2.449583X_2^2 + 1.1425X_2X_3 \\ & - 5.109583X_3^2 \end{aligned} \quad (1)$$

where Y_1 is the yield of HPA, X_1, X_2 and X_3 represent casein peptone, glycerol and KH_2PO_4, respectively.

Analysis of variance (ANOVA) is important in determining the adequacy and significance of the quadratic model. ANOVA summary was shown in **Table 6**. The F-value of 63.1957 implies that the model was significant. A p value of <0.0001 indicated that there was only a 0.01% chance that model's large F-value could occur due to noise. Values of "prob > F" less than 0.0500 indicated the significant model terms. The mathematical model was reliable with an R^2 value of 0.9913. It suggested that model was unable to explain only 0.87% of the total variations. A low value of coefficient of the variation (*C. V.*) (1.0298) clearly indicated a very high degree of precision and a good deal of reliability of the experimental values.

Figures 1-2 show the response and contour curves for casein peptone, glycerol and KH_2PO_4. Contour curves

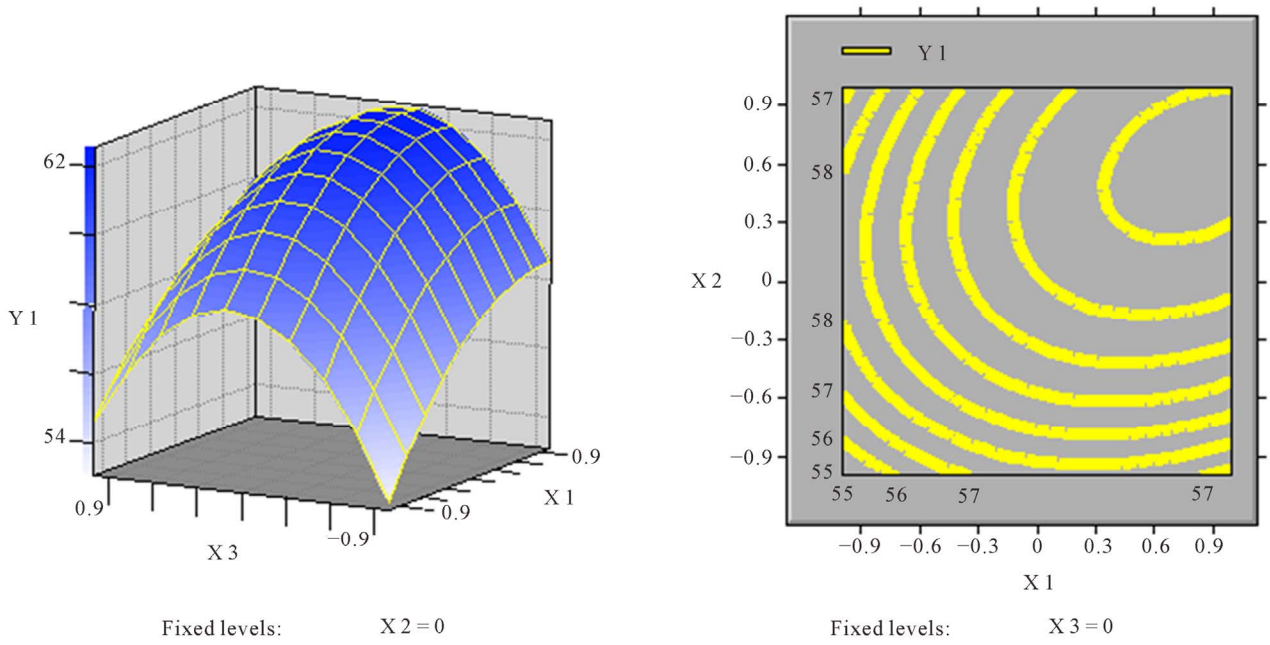

Figure 1. Response surface plot and contour plot for HPA yield (%) as a function of casein peptone and KH_2PO_4 (X_1 and X_3 represent casein peptone and KH_2PO_4, respectively).

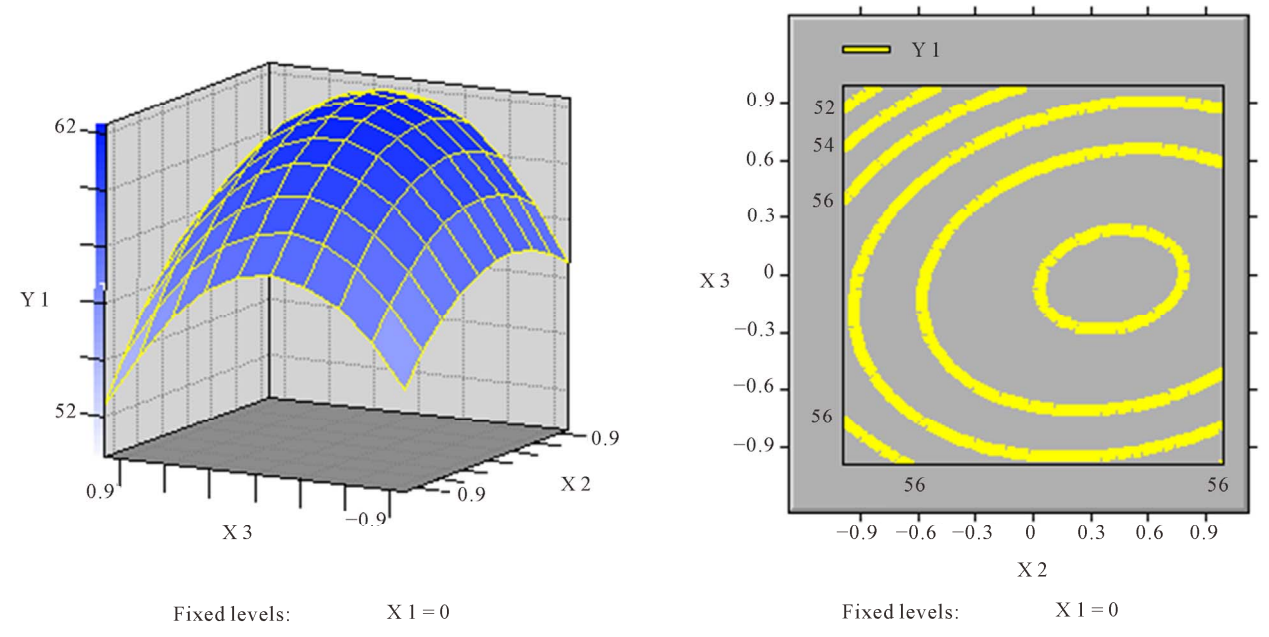

Figure 2. Response surface plot and contour plot for HPA yield (%) as a function of glycerol and KH_2PO_4 (X_2 and X_3 represent glycerol and KH_2PO_4, respectively).

Table 6. Estimated value of regression equation partial regression coefficient and analysis of square deviation.

Term	Parameter estimate	Standard error	T-test	Pr > |t|
X_1	1.9875	0.2076	9.5750	0.0002
X_2	2.0088	0.2076	9.6774	0.0002
X_3	−0.6713	0.2076	−3.2338	0.0231
$X_1 * X_1$	−1.6521	0.3055	−5.4071	0.0029
$X_1 * X_2$	1.155	0.2936	3.9346	0.0110
$X_1 * X_3$	−0.565	0.2936	−1.9247	0.1122
$X_2 * X_2$	−2.4496	0.3055	−8.0173	0.0005
$X_2 * X_3$	1.1425	0.2936	3.8920	0.0115
$X_3 * X_3$	−5.1096	0.3055	−16.7233	0.0001
Source	DF	SS	MS	F-value	Prob > F
Model	9	196.0455	21.7828	63.1957	0.0001
Linear	3	67.4865	22.4955	65.2633	0.0002
Quadratic	3	116.7248	38.9083	112.8796	0.0001
Interaction	3	11.8342	3.9447	11.4444	0.0112
Error	5	1.7234	0.3447		
Total	14	197.769	R^2 = 99.13% CV = 1.0298		

represent the HPA yield as a function of concentrations of two independent variables with another variable being at a fixed level. **Figure 1** shows that HPA yield increases firstly and decreases slowly afterward with the increase of casein peptone, and that moderate KH_2PO_4 results in high HPA yield. **Figure 2** demonstrates that HPA yield increases firstly and then decreases slowly with decreasing glycerol, whereas moderate KH_2PO_4 caused HPA yield increasing. This could be attributed to the fact that casein peptone, KH_2PO_4 and glycerol were directly related with the activity of cell. As seen from **Figures 1** and **2**, there was a maximum response at the optimum level of each variable and exist the interaction among the three variables, so it was not simple linear relationship for the effect of response value.

The ridge analysis indicated that the regression equation has no singularity. Simultaneously it obtained the best response surface conditions and the predictive value of yield from the regression equation. The best theoretical levels in experiment were: X_1 = 0.816, X_2 = 0.592 and X_3 = −0.045, that is, casein peptone 30.49 g·L^{-1}, glycerol 14.09 g·L^{-1} and KH_2PO_4 0.1345 g·L^{-1}. Predicted value of yield given these conditions was 63.35%. Validation under the optimized conditions was performed in a 50 ml Erlenmeyer flask containing 20 ml reaction medium. The experiments were conducted in triplicate. Under optimized conditions, HPA yield achieved in the verification experiment was 63.30%, which was very close to the value predicted by model based on BBD (63.35%).

4. Conclusion

Selective reduction of PGA with *Bacillus* sp. HZG-19 is a very promising technology for production of HPA. Results of this study clearly indicate that RSM is an effective method for the optimization of fermentation medium. Optimum casein peptone, glycerol and KH_2PO_4 were found to be 30.49, 14.09 and 0.1345 g·L^{-1}, respectively.

5. Acknowledgements

This work was financially supported by the Doctoral Fund of Ministry of Education (No. 20103514110002) and the Major Plan Project of Science and Technology of Fujian Province (No. 2009N0046).

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