

## Medium Optimization for 5'-Phosphodiesterase Production from *Penicillium citrinum* Using Response Surface Methodology

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### Summary

Medium optimization for 5'-phosphodiesterase production from *Penicillium citrinum* was studied by applying one-factor-at-a-time method, orthogonal array method and response surface methodology in this paper. The one-factor-at-a-time method was used to study the effects of carbon, nitrogen, phosphorus and metal ion sources on 5'-phosphodiesterase production. Among various carbon and nitrogen sources used, glucose and peptone were the most suitable substances for 5'-phosphodiesterase production, respectively. Subsequently, the concentrations of glucose, peptone, groundnut meal, Zn<sup>2+</sup> and KH<sub>2</sub>PO<sub>4</sub> were optimized using the orthogonal array method. Response surface methodology was also applied for medium optimization. Glucose concentration ( $X_1$ ), peptone concentration ( $X_2$ ) and groundnut meal ( $X_3$ ) were selected as the independent variables. Results showed that the regression models adequately explained the data variation and represented the actual relationships between the parameters and responses. The optimum conditions were glucose at a fraction of 6.5 %, peptone at a fraction of 0.45 % and groundnut meal at a fraction of 1.0 %. Maximum enzyme activity was 353 U/mL under the optimum conditions. Maximum 5'-phosphodiesterase activity in media optimized by orthogonal method and response surface methodology was about 1.286 and 1.456 times, respectively, greater than in the medium optimized by one-factor-at-a-time method.

*Key words:* medium optimization, 5'-phosphodiesterase, response surface methodology

### Introduction

An extracellular enzyme, 5'-phosphodiesterase (EC 3.1.30.1), was first identified by Kuninaka *et al.* (1). This enzyme cleaves 5'-nucleotides (*e.g.* 5'-AMP, 5'-GMP, 5'-CMP and 5'-UMP) successively from 3'-hydroxy termini of 3'-hydroxy-terminated oligonucleotides originating from RNA (2). 5'-nucleotides have been widely used in pharmaceutical and food industries (3–5). They can

be used to synthesize the antiviral and anticancer drugs as the acridine. The nucleotide derivatives have important uses in the treatment of the illness of human central nervous system and circulatory system. The interest in 5'-phosphodiesterase has grown over the last few years due to the application of 5'-nucleotides in antiviral and anticancer treatment.

5'-nucleotides can be produced directly by fermentation using microorganisms, by a chemical method, or

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enzymatic method (3,5). The enzymatic method has some advantages, such as low complexity and high yields, because of which it is the most widely used in the industry. It is important to find a good enzyme source to produce 5'-nucleotides. Enzymes can be obtained from several sources, such as microbial (2,6,7), plant (8–10) or animal source (bovine intestine, kidney, and snake venom). But taking into account economic constraints and possibility of large-scale production, *Penicillium citrinum* is among the most frequently used 5'-phosphodiesterase producers.

Therefore, it is important to optimize the culture medium for 5'-phosphodiesterase production. Rapid fermentation and high 5'-phosphodiesterase activity are desirable to minimize capital costs and energy. To develop a process for the maximum production of 5'-phosphodiesterase, standardization of the medium is crucial. In the conventional one-factor-at-a-time method for optimizing fermentation medium conditions (11–13), (*i.e.* nutrients, temperature, pH, *etc.*), one independent variable is changed while all others are kept at definite levels. This one-dimensional evaluation is tedious and time-consuming, especially for a large number of variables, and usually does not lead to the determination of optimal conditions, mainly due to the ignored interactions. As a solution, fractional factorial experimental designs, including Plackett-Burman design, orthogonal array and response surface methodology (RSM) designs have been introduced, reducing the number of the tests while giving reliable results. These methods have been successfully applied to optimize media components for enzyme production (14–16), spore production (17,18), alcoholic fermentation and other fermentation media (19–22), *etc.* However, to the best of our knowledge, optimization of 5'-phosphodiesterase production by RSM has not yet been reported.

The purpose of this study was to optimize fermentation medium nutrients to improve 5'-phosphodiesterase production using one-factor-at-a-time design, orthogonal array design and fractional factorial design. At first, the effects of carbon, nitrogen, phosphorous, and metal ion sources on 5'-phosphodiesterase production were investigated by one-factor-at-a-time. Then, the concentration of medium components was optimized using orthogonal method and fractional factorial design.

## Materials and Methods

### Materials

Groundnut meal was a gift from Hangzhou Zhongmeihuadong Pharmaceutical Co., Ltd. (Hangzhou, PR China). Glucose kit was purchased from Ningbo Cicheng Reagent Factory (Ningbo, PR China). All other reagents were of analytical grade.

### Microorganism

The 5'-phosphodiesterase-producing strain of *Penicillium citrinum* used throughout the study was kindly provided by Hangzhou Meiya Biotechnical Co. Ltd., PR China.

### Culture conditions for 5'-phosphodiesterase production

*Penicillium citrinum* was grown at 30 °C in a medium (pH=7.0) consisting of (by mass per volume): sucrose 3 %, potassium dihydrogen phosphate 0.1 %, ferrous sulphate 0.01 %, sodium nitrate 0.3 %, magnesium sulphate 0.05 %, potassium chloride 0.05 %, and potato extract 20 %. This 24-hour grown mother culture (10 mL) was used to inoculate 50 mL of the production medium containing (by mass per volume): glucose 6.5 %, peptone 0.45 %, groundnut meal 1 %, zinc sulphate 0.15 %, calcium carbonate 0.02 %, and potassium dihydrogen phosphate 0.2 %. The initial pH of the medium was adjusted to 5.4 with HCl. Erlenmeyer flasks (500 mL) containing 50 mL of the medium were incubated at 28 °C in an orbital shaker at 240 rpm for 49 h. The 5'-phosphodiesterase solution was harvested by centrifugation at 4000 rpm at 4 °C for 10 min, and the obtained supernatant was used as the crude enzyme preparation.

### 5'-phosphodiesterase assay

5'-phosphodiesterase activity was measured using the methods of Fujishima *et al.* (23) and Fujimoto *et al.* (24). It was measured in terms of the amount of acid-soluble nucleotides produced by the RNA hydrolysis catalyzed by its activity. Enzyme solution (0.10 mL) was incubated with 1.9 mL of substrate solution (1 % RNA (by mass per volume), 0.125 M acetate buffer, pH=5.4 and 3 mM Zn<sup>2+</sup>) at 69 °C for 15 min. The reaction was stopped by adding 2 mL of ice-cold nucleic acid precipitator (0.25 % of ammonium molybdate dissolved in 2.5 % of perchloric acid, by mass per volume). The mixture was settled in ice-bath for more than 10 min. The precipitated RNA was removed by centrifugation at 4000 rpm and 4 °C for 10 min. The supernatant fluid was diluted 50-fold with distilled water. The absorbance at 260 nm of the diluted solution was read against a blank solution without enzymes. 5'-phosphodiesterase activity was calculated according to the following formula:

$$\text{Enzyme activity}/(\text{U}/\text{mL}) = \frac{A_{260} \times 4.0 \times 50 / 0.1 \times 15}{= A_{260} \times a \times 133.3} \quad /1/$$

where *a* was a dilution factor of the enzyme before the enzyme activity assay. In this work the dilution factor was always 2.0.

One unit of enzyme activity was defined as the amount of 5'-nucleotides that increased the absorbance for 1.0 within 1 min at 260 nm.

### Determination of glucose concentration

Glucose was determined with an enzymatic glucose kit, based on the following principles (25): glucose was oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacted with *o*-dianisidine in the presence of peroxidase to form a coloured product. Oxidized *o*-dianisidine reacted with sulphuric acid to form a more stable coloured product. The absorbance of the pink colour measured at 540 nm was proportional to the original glucose concentration.

### Orthogonal arrays for medium optimization

To examine the interactions among nutritional components of the medium and optimize their concentra-

tions for 5'-phosphodiesterase production,  $L_{16}$  orthogonal arrays were used (Table 1). The orthogonal arrays, data analysis and ANOVA were obtained using Qualitek-4 (2000) software based on the Taguchi method.

Table 1. Orthogonal experimental design combinations of factors and levels used for the optimization of 5'-phosphodiesterase

Factor <i>w</i> /%	Level			
	1	2	3	4
Zn <sup>2+</sup>	0.005	0.010	0.015	0.020
KH <sub>2</sub> PO <sub>4</sub>	0.06	0.10	0.20	0.30
Glucose	5.50	6.00	6.50	7.00
Groundnut meal	0.50	0.80	1.20	1.50
Peptone	0.20	0.30	0.40	0.50

### Fractional factorial design for medium optimization

A 3×3 fractional factorial design was employed to optimize medium components. The factors and levels used are shown in Table 2.

Table 2. Values of coded levels used for fractional factorial design ( $a=1.682$ )

Factor <i>w</i> /%	Symbol	Coded level		
		-1	0	1
Glucose	X <sub>1</sub>	5.0	6.5	8.0
Peptone	X <sub>2</sub>	0.20	0.45	0.70
Groundnut meal	X <sub>3</sub>	0.5	1.0	1.5

The responses were analyzed using Minitab 14.0 software. In developing the regression equation the test factors were coded according to the equation:

$$x_i = (X_i - X_0) / \Delta X_i \quad /2/$$

where  $x_i$  was the coded value of the independent variable,  $X_i$  was the actual value of the independent variable,  $X_0$  was the actual value of the independent variable at the central point and  $\Delta X_i$  was the steep change value.

A quadratic polynomial regression model was assumed to predict both  $Y$  responses. The model response of  $Y$  was:

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 b_{ij} X_i X_j \quad /3/$$

where  $b_0$  was an intercept;  $b_i$  first-order model coefficient,  $b_{ii}$  quadratic coefficient for the variable,  $b_{ij}$  interaction coefficient for the interaction of variables  $i$  and  $j$ , and  $X_i$  and  $X_j$  were the independent variables. The goodness of fit model was evaluated by the coefficient of determination ( $R^2$ ) and the analysis of variance (ANOVA).

Contour plots were developed using the fitted quadratic polynomial equations obtained by keeping one of the independent variables at a constant value and changing the levels of the other two variables. All data errors in this paper were  $\pm 2.00$ .

## Results and Discussion

### One-factor-at-a-time method

#### Effect of carbon source

To choose a suitable carbon source for 5'-phosphodiesterase production, seven carbon sources including glucose, glycerol, mannitol, dextrine, maltose, sucrose and starch were investigated at fractions of 3 and 6 % in the basal medium. As shown in Table 3, among the carbon sources evaluated, the highest enzyme activity was 213 and 221 U/mL at glucose and sucrose fractions of 6 %, respectively.

Table 3. Effect of carbon sources on 5'-phosphodiesterase activity

Carbon source	<i>w</i> /%	Enzyme activity/(U/mL)
Mannitol	3	32
	6	35
Glycerol	3	56
	6	69
Glucose	3	123
	6	213
Maltose	3	51
	6	131
Dextrine	3	53
	6	64
Sucrose	3	127
	6	221
Starch	3	48
	6	48

Subsequently, the effect of glucose and sucrose fractions (0–9 %) in basal medium was studied on 5'-phosphodiesterase production. The highest enzyme activity was about 213 and 221 U/mL at glucose and sucrose fractions of 6 %, respectively (Fig. 1). Sucrose was more expensive than glucose, so glucose was selected as the carbon source.

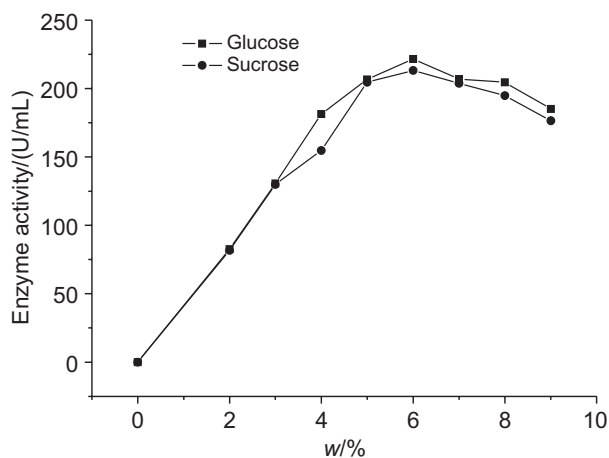


Fig. 1. Effect of glucose and sucrose fractions on 5'-phosphodiesterase activity

### Effect of nitrogen source

To investigate the effect of nitrogen sources on 5'-phosphodiesterase production, conidia were inoculated into the medium containing 6 % glucose and various nitrogen sources including sodium nitrate, sodium nitrite, ammonium sulphate, corn steep powder, urea, peptone and yeast extract, where each nitrogen source was added to the basal medium at fractions of 0.2 and 0.5 %. As shown in Table 4, amongst 8 nitrogen sources examined, peptone led to the highest 5'-phosphodiesterase activity of 227 U/mL.

Subsequently, the effect of peptone and corn steep powder fractions (0–1.5 %) on 5'-phosphodiesterase production was studied. The highest enzyme activity was about 227 and 216 U/mL at peptone fraction of 0.2 % and corn steep powder fraction of 0.5 %, respectively (Fig. 2).

Table 4. Effect of nitrogen sources on 5'-phosphodiesterase activity

Nitrogen source	w/%	Enzyme activity/(U/mL)
Peptone	0.2	227
	0.5	213
Yeast extract	0.2	133
	0.5	147
Peptone+yeast extract	0.2	171
	0.5	160
Urea	0.2	53
	0.5	27
Ammonium sulphate	0.2	0
	0.5	0
Sodium nitrate	0.2	155
	0.5	204
Sodium nitrite	0.2	147
	0.5	205
Corn steep powder	0.2	171
	0.5	216

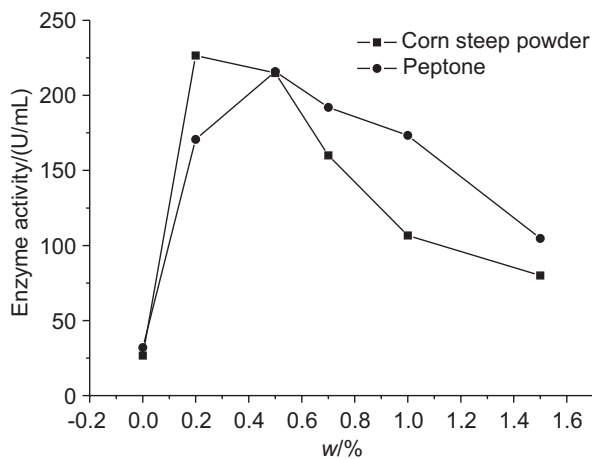


Fig. 2. Effect of peptone and corn steep powder fractions on 5'-phosphodiesterase production

### Effect of phosphorus source

To choose a suitable phosphorus source for 5'-phosphodiesterase production, three phosphorus sources, namely  $K_2HPO_4$ ,  $KH_2PO_4$ , and  $KH_2PO_4+K_2HPO_4$  were investigated at different concentrations. Amongst the phosphorous sources, the highest enzyme activity value was 232 U/mL in  $K_2HPO_4$  medium at fraction of 0.1 % (Table 5).

Table 5. Effect of phosphorus sources on 5'-phosphodiesterase activity

Phosphorus source	w/%	Enzyme activity/(U/mL)
$KH_2PO_4$	0.1	214
$KH_2PO_4$	0.2	219
$K_2HPO_4$	0.1	232
$K_2HPO_4$	0.2	179
$KH_2PO_4+K_2HPO_4$	0.05+0.05	195
$KH_2PO_4+K_2HPO_4$	0.1+0.1	181

### Effect of groundnut meal concentration

When groundnut meal was added into the production medium, 5'-phosphodiesterase activity was increased, so it was necessary to find a suitable concentration to get the highest 5'-phosphodiesterase activity. As seen in Fig. 3, the most suitable groundnut meal fraction was 0.7 %. 5'-phosphodiesterase activity changed greatly with the groundnut meal fraction.

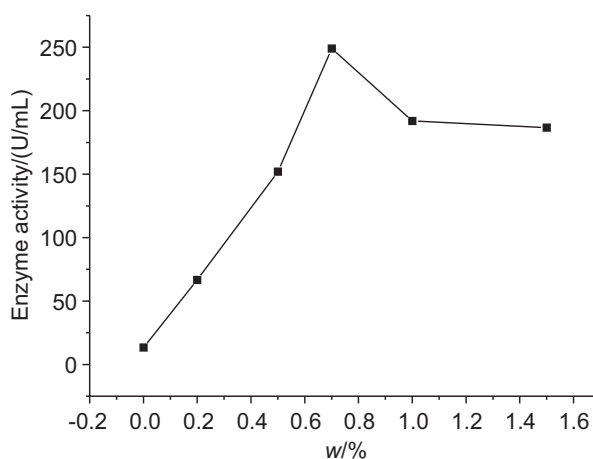


Fig. 3. Effect of groundnut meal fractions on 5'-phosphodiesterase activity

### Effect of metal ion source

Some literature reports that  $Zn^{2+}$  is necessary for 5'-phosphodiesterase activity, so  $Zn^{2+}$  should be added into the medium (25). To investigate the effect of other metal ion sources on 5'-phosphodiesterase activity, conidia were inoculated into the medium containing glucose at a fraction of 6 % and various metal ion sources including  $Ca^{2+}$  ( $CaCO_3$ ,  $CaCl_2$ ),  $NaCl$ ,  $FeSO_4$ ,  $KCl$ ,  $Cu^{2+}$  ( $CuSO_4$ ,  $CuCl_2$ ),  $BaCl_2$ ,  $Li_2SO_4$ ,  $MnCl_2$ ,  $SnCl_2$ ,  $Pb(NO_3)_2$  and  $Al(NO_3)_3$ , where each metal ion source was added to the basal medium at a fraction of 0.05 %. As shown in Table 6, amongst 13 metal ion sources examined,  $CaCO_3$  led to the highest 5'-phosphodiesterase activity (229 U/mL).

Table 6. Effect of metal ion sources on 5'-phosphodiesterase activity

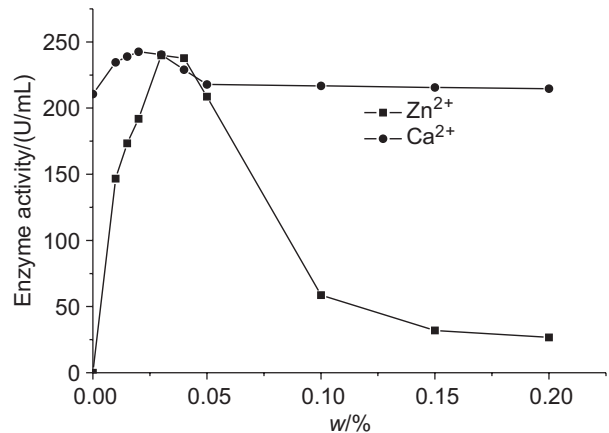
Metal ion source	Enzyme activity/(U/mL)
CaCO <sub>3</sub>	229
CaCl <sub>2</sub>	203
NaCl	123
FeSO <sub>4</sub>	117
KCl	208
CuSO <sub>4</sub>	0
CuCl <sub>2</sub>	0
BaCl <sub>2</sub>	149
Li <sub>2</sub> SO <sub>4</sub>	139
MnCl <sub>2</sub>	187
SnCl <sub>2</sub>	141
Pb(NO <sub>3</sub> ) <sub>2</sub>	160
Al(NO <sub>3</sub> ) <sub>3</sub>	179
None	155

The effect of Zn<sup>2+</sup> and Ca<sup>2+</sup> fractions (0–2 %) on 5'-phosphodiesterase activity was studied. The highest enzyme activity was about 240 and 243 U/mL at Zn<sup>2+</sup> fraction of 0.03 % and Ca<sup>2+</sup> fraction of 0.02 %, respectively (Fig. 4).

#### Orthogonal array designs

Table 7 summarizes 5'-phosphodiesterase activity detected under different treatment conditions that ranged from 109 to 312 U/mL.

Using the orthogonal design L<sub>16</sub> approach, the relationships between medium component variables and their concentrations could be calculated. The factors could be ranked according to importance (magnitude in parentheses), namely peptone concentration (contribution percentage, CP=99.00) > groundnut meal concentra-

Fig. 4. Effect of Zn<sup>2+</sup> and Ca<sup>2+</sup> fractions on 5'-phosphodiesterase activity

tion (CP=98.67) > Zn<sup>2+</sup> concentration (CP=37.67) > glucose concentration (CP=37.66) > KH<sub>2</sub>PO<sub>4</sub> concentration (CP=26.33). This shows that peptone concentration and groundnut meal concentration were the most important factors in producing 5'-phosphodiesterase, whereas KH<sub>2</sub>PO<sub>4</sub> concentration was the least important factor.

Table 7 presents the optimal combination and fractions of substrates required to achieve the highest 5'-phosphodiesterase activity. The expected optimum for 5'-phosphodiesterase was 312 U/mL, a value that was about 1.286 times greater than the highest activity (243 U/mL) achieved in one-factor-at-a-time experiment.

Table 8 shows the analysis of variance (ANOVA) of the results after 49 h of fermentation. This indicates that 5'-phosphodiesterase could be enhanced using a combination of solutes at different fractions in the fermentation medium. Groundnut meal and peptone fractions were the most significant factors in the production of 5'-phosphodiesterase, followed by glucose fraction.

Table 7. L<sub>16</sub> orthogonal array applied for 5'-phosphodiesterase activity

Runs	w(Zn <sup>2+</sup> )/%	w(KH <sub>2</sub> PO <sub>4</sub> )/%	w(glucose)/%	w(groundnut meal)/%	w(peptone)/%	Enzyme activity/(U/mL)
1	1	1	1	1	1	253
2	1	2	2	2	2	304
3	1	3	3	3	3	227
4	1	4	4	3	4	131
5	2	1	2	3	4	109
6	2	2	1	4	3	180
7	2	3	4	1	2	277
8	2	4	3	2	1	293
9	3	1	3	4	2	200
10	3	2	4	3	1	291
11	3	3	1	2	4	208
12	3	4	2	1	3	312
13	4	1	4	2	3	293
14	4	2	3	1	4	187
15	4	3	2	4	1	193
16	4	4	1	3	2	200

Table 8. Results of ANOVA and optimal factor levels by the Taguchi method

Factors <i>w</i> /%	DF	SS	MS	F	CP	OL	Significance
Zn <sup>2+</sup>	3	3478.45	1159.48	2.26	37.67	3	
KH <sub>2</sub> PO <sub>4</sub>	3	1540.38	513.46	1.00	26.33	3	
Glucose	3	2858.96	952.99	1.86	37.66	2	
Groundnut meal	3	24782.63	8260.88	16.09	98.67	2	*
Peptone	3	26444.32	8814.77	17.17	99.00	1	*
Error	0.07						

DF degree of freedom, SS sum of squares, MS mean square, CP contribution percentage, OL optimum level

\*Significance<0.05

### Fractional factorial design

#### Model fitting

Table 9 shows the responses of enzyme activity. The regression coefficients of the second order polynomial equations, and the results of the linear, quadratic and interaction terms and ANOVA are presented in Tables 10 and 11, respectively.

Table 9. Coded level combinations of a three fractional factorial design for medium optimization

Runs	Coded levels			Enzyme activity/(U/mL)
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	
1	1	1	1	14
2	1	1	-1	53
3	1	-1	1	349
4	1	-1	-1	6
5	-1	1	1	16
6	-1	1	-1	67
7	-1	-1	1	376
8	-1	-1	-1	5
9	-1.682	0	0	26
10	1.682	0	0	239
11	0	-1.682	0	6
12	0	1.682	0	17
13	0	0	-1.682	98
14	0	0	1.682	29
15	0	0	0	355

Table 10. Regression coefficients and probability values (p) for three dependent variables

Term	Coeff.	SE Coeff.	T	p
Constant	353.38	36.11	9.769	0.000
X <sub>1</sub>	23.29	23.96	0.972	0.354
X <sub>2</sub>	-41.52	23.96	-1.733	0.114
X <sub>3</sub>	37.80	23.96	-1.548	0.153
X <sub>1</sub> <sup>2</sup>	-66.25	23.32	-2.831	0.018
X <sub>2</sub> <sup>2</sup>	-109.19	23.32	-4.673	0.001
X <sub>3</sub> <sup>2</sup>	-90.71	23.32	-3.881	0.003
X <sub>1</sub> X <sub>2</sub>	1.33	31.30	0.042	0.967
X <sub>1</sub> X <sub>3</sub>	-1.94	31.30	-0.062	0.952
X <sub>2</sub> X <sub>3</sub>	-100.59	31.30	-3.214	0.009

Table 11. Results of the ANOVA test

Source	DF	SS	Adj SS	Adj MS	T	p
Regression	9	428106	428106	47567.4	6.070	0.005
Linear	3	49731	49731	16577.0	2.110	0.162
Square	3	297386	297386	99128.7	12.650	0.010
Interaction	3	80989	80989	26996.3	3.440	0.060
r-error	5	78381	78381	7838.1		
Lack of fit	3	78381	78381	15676.2	9.501	0.00098
Pure error	2	0	0	0		
Total	14	506487				

The responses and variables were fitted to each other by multiple regressions. A good fit was obtained and there were no outliers observed for the regression:

$$Y = 353.38 + 23.29X_1 - 41.52X_2 + 37.8X_3 - 66.25X_1^2 - 109.19X_2^2 - 90.71X_3^2 + 1.33X_1X_2 - 1.94X_1X_3 - 100.59X_2X_3 \quad /4/$$

The  $R^2$  value and standard error (SE) were 0.885 and 0.845, respectively, and the probability (p) values of regression models were less than 0.01 with no significant lack of fit ( $p=0.00098<0.01$ ). These results indicate that there were not other significant factors affecting the enzyme activity except glucose, peptone and groundnut meal concentration. The predicted result, according to the models for enzyme activity, was close to the observed experimental responses. The  $R^2$  and SE values between the experimental and predicted results for enzyme activity were 0.941 and 0.919, respectively, which demonstrates that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

#### Effects of parameters

ANOVA results showed that enzyme activity was affected by the first-order (linear) ( $p=0.162$ ) and second-order ( $p=0.01$ ) of the variables. It was found that the first-order parameters ( $X_1$ ,  $X_3$ ) had a positive effect on enzyme activity, with peptone concentration ( $X_2$ ) being the most significant factor ( $p=0.114$ ). At the same time, the second-order ( $X_1^2$ ,  $X_2^2$ ,  $X_3^2$ ) variables had a negative effect. During three interactions between the variables, only  $X_2X_3$  had a prominent influence on the production of 5'-phosphodiesterase with  $p=0.009$ . These results suggested that the linear, second-order and interaction effects were the primary factors to determine enzyme activity.

### Optimization of the medium

Eq. 4 showed that the enzyme activity had a complex relationship with the independent variables which encompassed both first-order and second-order polynomials and might have more than one maximum point. The effects of glucose, peptone and groundnut meal fractions are shown in Figs. 1–3. Analyzing the contour plots for enzyme activity was the best way to evaluate the relationships between responses, variables and interactions.

Fig. 5. showed the contour plots of enzyme activity as a function of the interactions of any couple of variables by holding the other at low value. All three plots in Fig. 5 showed similar relationships with respect to the effect of each variable. Enzyme activity increased at high levels of glucose and peptone fraction under sufficient groundnut meal fraction. However, when glucose fraction was more than 6.5 %, enzyme activity started to decrease (Fig. 1). It was found that glucose fraction of 5–8 % and peptone fraction of 0.2–0.7 % led to the highest enzyme activity (Fig. 5). Peptone fraction was the most significant factor influencing enzyme activity, which increased as its concentration increased. However, if peptone fraction was higher than 0.45 %, enzyme activity would decrease. The reasons might be that too much peptone could cause the restriction of enzyme production.

### Verification of the regression models

The optimum values of the selected variables were obtained by solving the regression Eq. 2, which gave the following results in terms of coded values:  $X_1=0.000$ ,  $X_2=0.000$  and  $X_3=0.000$  with the corresponding enzyme activity of 353 U/mL. The actual values obtained by putting the respective optimum values in Eq. 1 were glucose 6.5 %, peptone 0.45 %, and groundnut meal 1 %. The response surfaces based on these coefficients, obtained with one variable kept at central level and the other two varying within the experimental range, showed the stationary ridge shape in the surface plots (Figs. 5A and B).

The model predicted that the maximum enzyme activity (353 U/mL) could be obtained by using the above optimum conditions. The verification of the results using the optimized conditions was accomplished by carrying out the experiments under the optimal conditions, *i.e.* glucose fraction 6.5 %, peptone fraction 0.45 %, groundnut meal fraction 1 % and keeping the other 5'-phosphodiesterase production parameters at the previous values. The maximum enzyme activity (353 U/mL) was obtained under these optimized parameters, and these experimental findings were in agreement with the model predictions.

To confirm the optimal conditions, a set of four replicate experiments with the optimal combination of substrates and concentrations was carried out.

### Production of 5'-phosphodiesterase

The time course of cultivation of *Penicillium citrinum* in the production medium is shown in Fig. 6. The pH of the medium gradually decreased, reached 4.2 at optimal enzyme levels and then gradually increased with time. 5'-phosphodiesterase activity was low up to 18 h, then gradually increased, reached a maximum after 49 h and

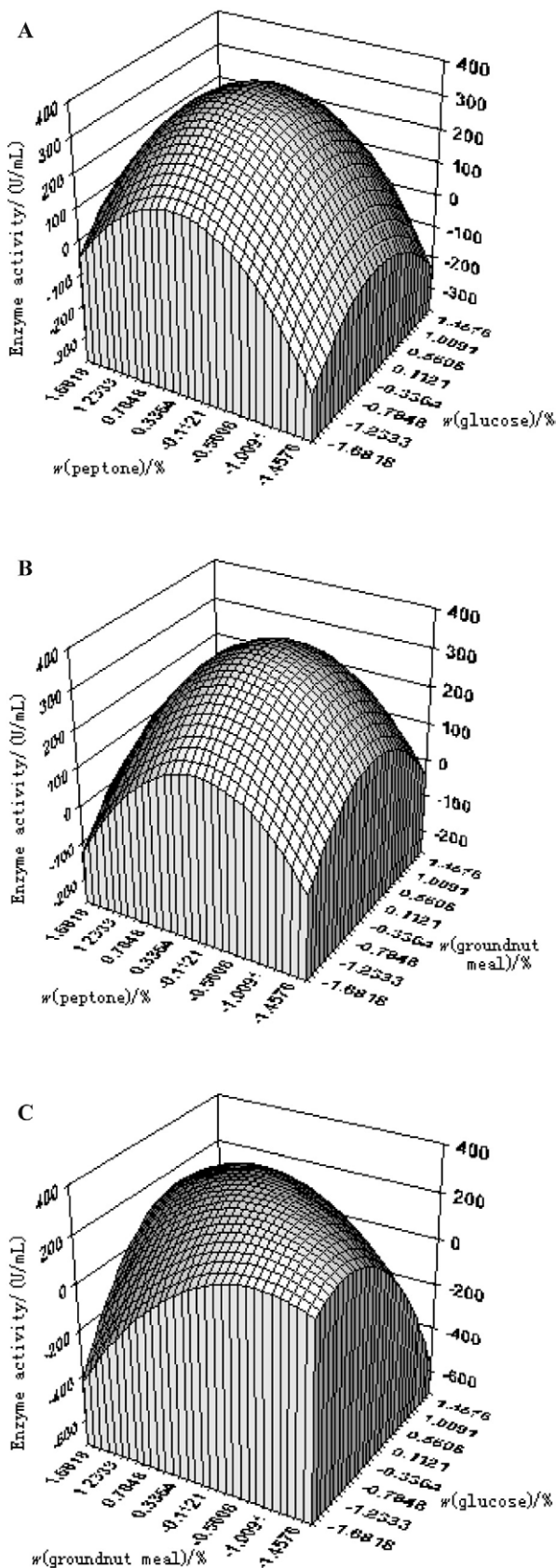


Fig. 5. Contour plots of enzyme activity as a function of the interactions of two variables by keeping the other at 0 level: interactions of peptone and glucose fraction (A), interactions of peptone and groundnut meal fraction (B), and interactions of groundnut meal and glucose fraction (C)

finally decreased. The concentration of biomass increased greatly between 16 and 40 h, and stopped growing after 40 h of incubation. The glucose was exhausted after 54 h.

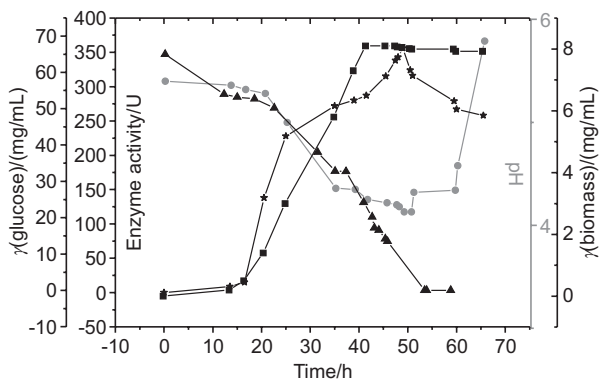


Fig. 6. Profile of 5'-phosphodiesterase's production ▲ glucose fraction, ★ 5'-phosphodiesterase, ■ biomass, ● pH

## Conclusions

To the best of our knowledge, there is not enough information concerning optimum nutritional requirements for 5'-phosphodiesterase production from *Penicillium citrinum*. Using the one-factor-at-a-time, orthogonal array method, and factorial central design, it was possible to optimize nutritional components of the medium to achieve higher 5'-phosphodiesterase activity. Three optimization techniques used in this work could be widely practical for other processes to optimize growth and production conditions. The optimized medium obtained in this study was a chemically defined medium and if compared to complex media, has some advantages for further studies such as preparation of 5'-nucleotides.

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