J Biochem Tech (2015) 6(3): 977-981
ISSN: 0974-2328

Statistical medium optimization for the production of cephalosporin-c acylase by *Pseudomonas diminuta*

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Received: 09 December 2014 / Received in revised form: 09 August 2015, Accepted: 22 November 2015, Published online: 15 January 2016

Abstract

Media composition for Cephalosporin-C acylase (CPC acylase) was optimized using a stepwise strategy. Out of the fifteen nutrients, five key ingredients viz. glucose, beef extract, glutamic acid, adipic acid and sodium chloride were identified for best medium composition by applying Plackett-Burman (PB) statistical design. Results indicated that above factors negatively affects the CPC acylase production i.e. increase in the concentration of these factors decreases the acylase production. The optimal concentration of key ingredients was determined using Central Composite Design (CCD) and the results obtained were fitted with a second order polynomial model. By applying the optimized conditions, a model was designed which predicted the CPC acylase activity as 9.56 U/mL which was in close resemblance to the experimental value of 11.2 U/mL.

Keywords: Cephalosporin-C acylase, Medium optimization, Response surface methodology, 7 -aminocephalosporanic acid, *Pseudomonas diminuta*

Introduction

Beta lactam antibiotics such as semisynthetic cephalosporins have made a significant contribution in resisting bacterial infections. They are broad spectrum antibiotics with low toxicity and are resistant to beta lactamases (Ren et al. 2014). 7- aminocephalosporanic acid (7-ACA), which is the key intermediate for the synthesis of semisynthetic cephalosporins, is produced from cephalosporin C (CPC) by two methods. CPC can be hydrolyzed to 7-ACA either chemically or enzymatically using enzyme CPC acylase [EC.3.5.1.11] (Gaurav et al. 2007). The two step enzymatic method is preferred as it is more environmental friendly (Ren et al. 2014). But the bottleneck is low productivity and high cost associated with the large scale industrial production of CPC acylase. Any fermentation process is significantly influenced by various parameters (Meiying et al. 2001 and Meiying et al. 2002). It is well known that formulation of cultivation media is of utmost importance for any biotechnology based industrial process because it ultimately affects the product yield and economy of the whole process (de Souza, et al. 2006). Conventional methods used for media formulation are labour extensive and time consuming due to large number of experiments. They depend on single factor optimization and do not study interaction between various factors (Moyo et al. 2003; Adinarayana et al. 2003). Considering the above drawbacks, more recently, this approach has been replaced with statistical screening methodologies. Plackett-Burman (PB) design and Response Surface Methodology (RSM) are two of such methods (Wang and Lu 2004; Adinarayana and Ellaiah 2002). PB design is a good tool for determining the key ingredients for medium composition in a multivariable system (Plackett and Burman 1946). Process optimization tool such as RSM is then applied for determining the exact concentration (Liu et al. 2003; Chakravarti et al. 2002; Francis et al. 2003). Thus, these two methods complement each other to statistically optimize the process and develop a model.

In the present work, stepwise optimization was done which includes (1) screening of nutrients, (2) highlighting the components which significantly affect CPC acylase activity by using PB design and (3) optimizing the concentration of significant nutrients by Central composite design (CCD).

Materials and Methods

Microorganism

Pseudomonas diminuta NCIM 2865, procured from National Collection of Industrial Microorganism, N.C.L., Pune, India was cultivated at 28 ± 2 ^{0}C for 18 hr in a medium (pH=7) which was composed of (per litre) 10 g peptone, 10 g beef extract, 5 g sodium chloride and 20 g glucose.

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Production medium and condition

The enzyme cephalosporin-C acylase enzyme was produced at the same temperature and pH *i.e*. 28 ± 2 ^{0}C and pH 7.0 respectively, for 40 hr in a medium composed of (per litre) 10g each of peptone and yeast extract, 5 g each of sodium chloride and mono sodium glutamate and 0.5 g of glucose (Shimizu et al. 1975). The inoculum level was maintained at 5% (v/v). After completion of the incubation period, the cells were harvested by centrifugation at 10,000 rpm for 15 minutes. The cell mass, thus obtained, was washed with 0.01 M phosphate buffer (pH=7) and stored at 4 ^{0}C for further use.

Optimization procedure and experimental design

Plackett-Burman (PB) is a widely used statistical tool for screening the key factors prior to optimization using Response Surface Methodology (RSM). Once the key components are identified using PB design, Central composite design was done for the optimization of the concentration of key components to maximize CPC acylase activity.

Pre-selection of nutrients using Plackett-Burman design

Two different carbon sources (lactose and glucose), three nitrogen sources (tryptone, peptone and beef extract), two amino acids (glutamic acid and glycine), two inducers (adipic acid and glutaric acid) and six minerals (magnesium sulphate, sodium chloride, ammonium chloride, ammonium nitrate, potassium dihydrogen phosphate and sodium nitrate) were pre-tested. All the above mentioned variable were screened by conducting twenty-four experiments where each variable was tested at two different levels *i.e*. (+) high level and (-) low level. Design matrix is shown in Table 1.

All the experiments were performed in duplicate for the mean calculation. Response was measured in terms of acylase activity.

Central Composite Design (CCD)

Central Composite Design (CCD) was done to optimize the concentration of key components selected from PB design, for maximum activity. In the CCD, 2^5 factorial was designed with thirty two experiments. All the experiments were performed in duplicate for the mean calculation. The variables and their values at various levels are given in Table 2. The lower and upper limits of all the five variables were same as in PB design as shown in Table 1. The experimental design was analyzed by RSM. The quadratic equation for the variables is;

$$Y_{i} = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_i x_{ii}^2 + \Sigma\beta_{ij} x_i x_j$$

Where Y_i is the predicted response, $x_i x_j$ are input variables, which influence response variable Y; β_0 is the offset term; β_i is the i^{th} linear coefficient; β_{ii} the i^{th} quadratic coefficient and β_{ij} the ij^{th} interaction coefficient

Data analysis

Minitab statistical software 13.20 was used for carrying out all experimental design and result analysis.

Cell disruption

The cell mass stored at 4 ^{0}C was taken out and subjected to lysozyme-HCl treatment for cell wall lysis. Aqueous solution of 5.0 mg of lysozyme-HCl was mixed well with the 5.0 mL

Table 1: Plackett-Burman experimental design matrix with CPC acylase production

Run order	Variables[a]/levels[b]															
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**Activity**
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.1
2	+	-	-	-	-	+	-	+	-	-	+	+	-	-	+	7.6
3	+	+	-	-	-	-	+	-	+	-	-	+	+	-	-	3.4
4	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+	5.7
5	+	+	+	+	+	-	-	-	-	+	-	+	-	-	+	4.1
6	+	-	-	+	+	-	-	+	+	-	+	-	+	+	+	8.2
7	+	+	+	-	-	-	-	+	-	+	-	-	+	+	-	4.8
8	+	+	-	-	+	+	-	+	-	+	+	+	+	+	-	4.7
9	-	+	-	+	-	-	+	+	-	-	+	+	-	+	-	5.8
10	-	+	+	-	-	+	+	-	+	-	+	+	+	+	+	3.9
11	+	-	+	+	+	+	+	-	-	-	-	+	-	+	-	5.6
12	-	-	+	+	-	+	-	+	+	+	+	+	-	-	-	6.7
13	+	+	-	+	-	+	+	+	+	+	-	-	-	-	+	3.6
14	-	+	+	+	+	+	-	-	-	-	+	-	+	-	-	4.5
15	+	+	+	+	-	-	-	-	+	-	+	-	-	+	+	4.3
16	-	+	-	+	+	+	+	+	-	-	-	-	+	-	+	6.2
17	-	+	-	-	+	+	-	-	+	+	-	+	-	+	+	5.0
18	-	+	+	-	+	-	+	+	+	+	+	-	-	-	-	6.5
19	-	-	+	+	-	-	+	+	-	+	-	+	+	+	+	5.4
20	+	-	+	-	+	+	+	+	+	-	-	-	-	+	-	7.8
21	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	5.2
22	+	-	-	+	+	-	+	-	+	+	+	+	+	-	-	7.2
23	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	6.0
24	+	-	+	-	-	+	+	-	-	+	+	-	+	-	+	6.9

[a]A lactose at a high level of 10 g/L and a low level of 5 g/L; B glucose at a high level of 10 g/L and a low level of 5 g/L; C tryptone at a high level of 6 g/L and a low level of 3 g/L; D peptone at a high level of 6 g/L and a low level of 3 g/L; E beef extract at a high level of 6 g/L and a low level of 3 g/L; F glutamic acid at a high level of 0.4 g/L and a low level of 0.2 g/L; G glycine at a high level of 0.4 g/L and a low level of 0.2 g/L; H adipic acid at a high level of 0.1 g/L and a low level of 0.05 g/L; I glutaric acid at a high level of 0.1 g/L and a low level of 0.05 g/L; J magnesium sulphate at a high level of 0.1 g/L and a low level of 0.05 g/L; K sodium chloride at a high level of 0.1 g/L and a low level of 0.05 g/L; L ammonium chloride at a high level of 0.6 g/L and a low level of 0.3 g/L; M ammonium nitrate at a high level of 0.6 g/L and a low level of 0.3 g/L; N potassium dihydrogen phosphate at a high level of 0.6 g/L and a low level of 0.3 g/L; I sodium nitrate at a high level of 0.4 g/L and a low level of 0.2 g/L.
b(+) High concentration of variable; (-) low concentration of variable.

activated cell suspension and incubated at 37 ^{0}C for 1 hr which marks the completion of cell lysis.

Enzyme assay

For determining cephalosporin-C acylase activity, production of 7-ACA was measured. The sample of 5.0 mg/mL of cephalosporin-C in phosphate buffer (0.1 M) and known volume of enzyme solution was incubated at 37 ^{0}C at 100 rpm for 3 hr. The reaction was quenched by acetic acid (4% v/v). The solution was centrifuged. The cell free supernatant was used for measuring the amount of 7-ACA. Cephalosporin-C acylase activity was determined by Marrelli method (Marrelli,1968). Enzyme activity was expressed as unit per mil liter (U/mL).

Results and Discussion

Plackett-Burman Experimental Design

The impact of different nutrient components on the cephalosporin-C acylase production was evaluated by Plackett-Burman design which is shown in Table 1. The effect of different nutrient components was determined by T-values and P-values as shown in Figure 1. The larger the magnitude of T-test and smaller the P-values, higher is the significance of corresponding coefficient.

As is evident from the P-value (<0.05) obtained from regression analysis, five nutrients (out of fifteen nutrient supplements studied) *i.e*. glucose, beef extract, glutamic acid, adipic acid and sodium chloride were found to have significant effect on acylase production. As a carbon source, glucose was shown to have significant effect than lactose. Similarly, as a source of nitrogen, beef extract has shown greater influence on acylase production as compared to tryptone and peptone. Glutamic acid, adipic acid and sodium chloride showed more impact on acylase production as compared to their other counter parts used in the present study. Hence, these five components were identified as key components for acylase production. Plackett-Burman design experiments showed wide variation in terms of activity with different levels which emphasizes the importance of optimization for higher acylase production. It also necessities further optimization of concentration of key components.

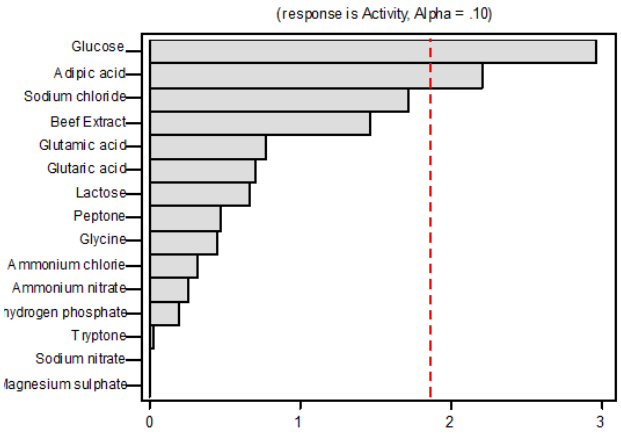

Figure 1: Pareto chart to show the variables effects lactose, glucose, tryptone, peptone, beef extract, glutamic acid, glycine, adipic acid, glutaric acid, magnesium sulphate, sodium chloride, ammonium chloride, ammonium nitrate, potassium dihydrogen phosphate, sodium nitrate on response CPC acylase activity for *P. diminuta*.

Central composite design (CCD)

Table 2: Central composite design (CCD) experimental plan for medium composition of CPC acylase production and results of CCD

Run order	X_1	X_2	X_3	X_4	X_5	Y (U/mL)
1	5.0	3.0	0.4	0.05	0.05	8.2
2	7.5	4.5	0.3	0.075	0.025	11.4
3	10.0	6.0	0.2	0.05	0.1	8.6
4	10.0	6.0	0.2	0.1	0.05	8.9
5	10.0	6.0	0.4	0.05	0.05	8.2
6	10.0	3.0	0.2	0.05	0.05	7.6
7	7.5	4.5	0.3	0.075	0.075	11.4
8	5.0	3.0	0.2	0.1	0.05	8.3
9	7.5	4.5	0.1	0.075	0.075	10.9
10	5.0	6.0	0.2	0.05	0.05	7.2
11	12.5	4.5	0.3	0.075	0.075	8.4
12	10.0	6.0	0.4	0.1	0.1	9.8
13	10.0	3.0	0.4	0.05	0.1	7.8
14	5.0	6.0	0.4	0.05	0.1	7.4
15	7.5	4.5	0.5	0.075	0.075	10.1
16	5.0	6.0	0.4	0.1	0.05	8.8
17	7.5	4.5	0.3	0.125	0.075	8.6
18	5.0	6.0	0.2	0.1	0.1	8.5
19	7.5	1.5	0.3	0.075	0.075	9.2
20	7.5	4.5	0.3	0.025	0.075	7.7
21	7.5	4.5	0.3	0.075	0.075	11.4
22	7.5	4.5	0.3	0.075	0.125	10.6
23	10.0	3.0	0.2	0.1	0.1	9.0
24	2.5	4.5	0.3	0.075	0.075	6.8
25	7.5	4.5	0.3	0.075	0.075	11.4
26	5.0	3.0	0.4	0.1	0.1	8.7
27	7.5	4.5	0.3	0.075	0.075	11.4
28	7.5	7.5	0.3	0.075	0.075	8.5
29	10.0	3.0	0.4	0.1	0.05	9.6
30	7.5	4.5	0.3	0.075	0.075	11.4
31	5.0	3.0	0.2	0.05	0.1	7.9
32	7.5	4.5	0.3	0.075	0.075	11.4

X_1 glucose; X_2 beef extract; X_3 glutamic acid; X_4 adipic acid; X_5 sodium chloride; Y CPC acylase activity

After identifying the key components using PB design, optimization of their concentration was done using central composite design (CCD). So, the next step was optimization of concentration of glucose, beef extract, glutamic acid, adipic acid and sodium chloride for medium optimization. 2^5 factorial design was employed with thirty-two experiments and corresponding acylase activity was given (Table 2). The second order polynomial equation for CPC acylase activity relates the production of acylase with independent process variables X_1 to X_5

$$\begin{aligned} Y = {} & -14 + 2.0X_1 + 2.0X_2 + 20.0X_3 + 196.0X_4 + 41.0X_5 \\ & - 0.0X_1^2 - 0.0X_2^2 - 27.0X_3^2 - 1378.0X_4^2 \\ & - 238.0X_5^2 + 0.0X_1X_2 - 0.0X_1X_3 \\ & + 1.0X_1X_4 + 1.0X_1X_5 - 0.0X_2X_3 \\ & + 1.0X_2X_4 + 3.0X_2X_5 + 47.0X_3X_4 \\ & - 77.0X_3X_5 - 10.0X_4X_5 \end{aligned}$$

Where Y is the response variable, acylase activity (U/mL) and X_1, X_2, X_3, X_4 and X_5 the values of independent variables, glucose, beef extract, glutamic acid, adipic acid and sodium chloride concentration (g/L) in the medium, respectively.

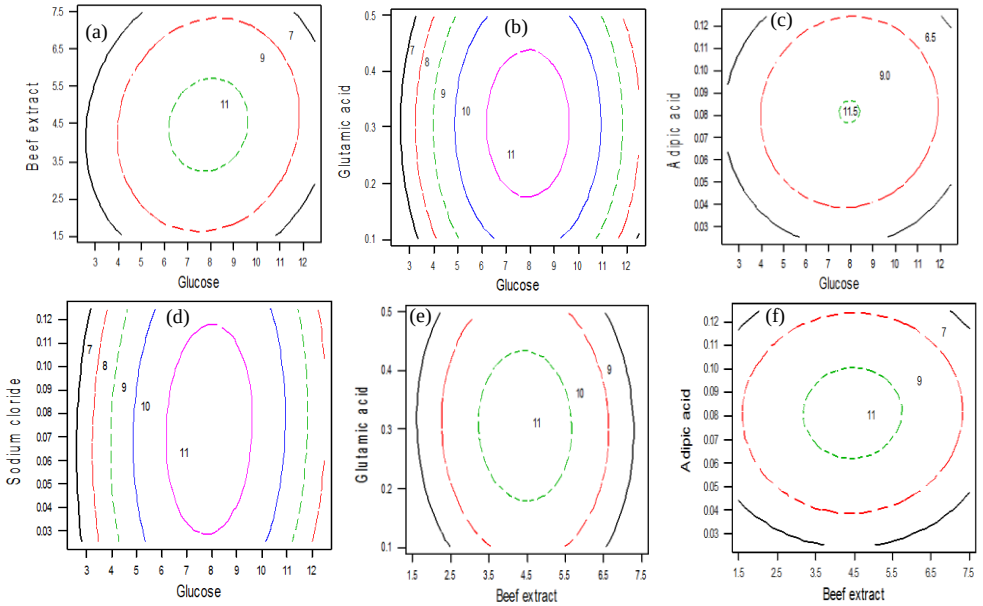

Figure 2: Contour plot of acylase activity (U/mL) vs : (a) glucose and beef extract; (b) glucose and glutamic acid ; (c) glucose and adipic acid ; (d) glucose and sodium chloride; (e) beef extract and glutamic acid; (f) beef extract and adipic acid.

The Pareto chart (Figure 1) was an additional graphic used to display the effect of different variables and represents the estimated effect, in decreasing sequence of variables. Glucose, beef extract and adipic acids are more significant factors with a value of $P < 0.05$, for CPC acylase production. The enzyme activity increases with increase in glucose concentration up to an optimum concentration after which the enzyme activity decreases due to catabolite repression

Regression analysis (Table 3) shows that 3.2% of the total variations are not explained by the model *i.e.* determination coefficient is 96.8%. In the present study, the computed F-value is 16.56 whereas the tabulated F-value, $F_{20,11} = 2.6464$ at $\alpha = 0.05$ level so that the null hypothesis (H_0) is rejected. Having rejected the null hypothesis, it can be inferred that treatment differences are highly significant.
Graphical representation technique (2D contour plots and 3D response surface) is a widely used technique for optimizing the values of variables. The regression equation is graphically represented by 2D contour plots (Figures 2 a-f) with an aim to optimize the values of variables to get the maximum response. This representation helps in deciding the optimum conditions by visualizing the relation between the response and experimental levels of each variable and the type of interaction between them.

Table 3**:** Analysis of variance (ANOVA) for production of cephalosporin-C acylase using central composite design criterion.

Source	DF	Adj SS	Adj MS	F	P
Regression	20	65.0351	3.2518	16.56	0.000
Linear	5	13.6128	2.7226	13.87	0.000
Square	5	56.2201	11.2440	57.27	0.000
Interaction	10	1.6462	0.1646	0.84	0.606
Residual	11	2.1595	0.1963		
Lack of fit	6	2.1595	0.3599		
Pure error	5	0.0000	0.0000		
Total	31				

R, coefficient of correlation = 0.9838; R^2, coefficient of determination = 96.8%; SS, sum of square; MS, mean square; DF, degree of freedom; R^2(adj) = 90.9%

Figure 2a -2f depicts the contour plots showing the effects of two variables on the CPC acylase activity while other three variables are fixed at their optimal concentration. In Fig. 2a, an elliptical nature of the contour plot indicates that the interaction between the corresponding variable are significant. It shows that at low beef extract concentration and high glucose concentration (7.5 g/L) CPC acylase activity increases. But at high concentration of both the variables, decrease in enzyme activity is observed due to catabolite repression of glucose. The maximum activity obtained was 11.0 U/mL with low beef concentration (4.5 g/L). Fig. 2b reveals that at the lower level of Glutamic acid (which acts as free amino acid nitrogen source involved in enzyme synthesis) i.e. 0.3 g/L the maximum enzyme activity was observed as 11.0 U/mL. The synthesis of enzyme acylase increases with addition of inducers, in this case, adipic acid and was found to be maximum 11.5 U/mL at the concentration of 0.075 g/L of adipic acid (Fig. 2c).

Fig.2d showed that the enzyme activity increases from 7.2 to 11.4 U/mL with decrease in concentration of sodium chloride. The optimal level of sodium chloride concentration was 0.075 g/L at which the enzyme activity was 11.4 U/mL. In Fig.2e, the contour plots are not perfectly elliptical in nature. Thus indicates that there are fewer interactions among the independent variables. Fig.2f shows that with increase in concentration of beef extract and adipic acid, enzyme activity increases from 7.0- 11.0 U/mL.

The optimal combination of major constituents of media for acylase production evaluated from contour plots was as follows: glucose, 7.5 g/L; beef extract, 4.5 g/l; glutamic acid, 0.3 g/L; adipic acid, 0.075 g/L; sodium chloride, 0.075 g/L. The maximum activity of enzyme obtained by using the above optimized concentration of the variables was 9.56 U/mL. The maximum enzyme activities obtained experimentally were found to be 11.2 U/mL. This is clearly in close agreement with the model prediction.

Conclusion

Conventional processes for medium optimization are very time consuming and expensive. To overcome these problems, medium optimization for cephalosporin-C acylase production by *Pseudomonas diminuta* NCIM 2865 was done by response surface methodology in the present study. This method involves an experimental design followed by regression analysis and corresponding model generation. The result of current investigation reveals that this statistical method helps in finalizing the optimum levels of the most significant factors in minimum time with maximum accuracy.

Acknowledgment

One of the authors (Kumar Gaurav) is thankful to University Grant Commission (UGC), India for providing financial assistance as Senior Research fellow during Ph.D. work.

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