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The Improvement of Cephalosporin C Production by Fed-batch Culture of *Cephalosporium acremonium* M25 Using Rice Oil

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Abstract The objective of this study is to improve cephalosporin C (CPC) production by optimization of medium and culture conditions. A statistical method was introduced to optimize the main culture medium. The main medium for CPC production was optimized using a statistical method. Glucose and corn steep liquor (CSL) were found to be the most effective factors for CPC production. Glucose and CSL were optimized to 2.84 and 6.68%, respectively. CPC production was improved 50% by feeding of 5% rice oil at day 3rd and 5th day during the shake flask culture of *C. acremonium* M25. The effect of agitation speeds on CPC production in a 2.5-L bioreactor was also investigated with fed-batch mode. The maximum cell mass (54.5 g/L) was obtained at 600 rpm. However, the maximum CPC production (0.98 g/L) was obtained at 500 rpm. At this condition, the maximum CPC production was improved about 132% compared to the result with batch flask culture.

Keywords: cephalosporin C, *Cephalosporium acremonium*, optimization, statistical method, rice oil

INTRODUCTION

Since their introduction into clinical practice in the 1960s, cephalosporins became key agents in the antibacterial armamentarium with widespread use because of their favorable pharmacokinetic activity and safety profile [1]. Over the years modifications of the basic cephem structure have led to the evolution of several generations of cephalosporins, which are classified according to their spectrum of activity. Cephalosporins are produced by *Cephalosporium acremonium* and *Streptomyces clavuligerus*.

Cephalosporin C (CPC) fermentation by *C. acremonium* is characterized by morphological differentiation and repression by glucose. Glucose represses the enzyme responsible for the production of CPC. However, it is a rapidly metabolized carbon source and allows rapid cell growth. On the other hand, sugars such as sucrose and lactose are less rapidly metabolized than glucose, but do not appreciably repress the formation of CPC. Thus a combination of glucose with either sucrose or lactose is a useful feedstock in these fermentations [2]. The most recent works reporting the CPC production process deal with media containing two carbon sources, glucose and sucrose [3]. Vicik *et al.* [4] worked with a fed-batch process where glucose and methionine were added slowly. The yield improvement obtained by them is explained by the combined addition of sucrose and methionine. However, sucrose is intrinsically a slowly metabolized sugar, and it is not clear if that improvement is due to this aspect or to the methionine feeding [5]. It was also reported that CPC was produced by *Acremonium chrysogenum* using a complex medium with glucose and different plant oils as the major carbon sources [6]. The biochemical background of the beneficial effect of oils on CPC production is still not fully understood. Production of antibiotics with complex media has the disadvantage of high energy cost, because of high viscosity and formidable process monitoring, but the advantage of low raw material cost and high productivity. Also, in complex media, the strong variation of the pH-values is partly suppressed by the high buffer capacity of the medium [7].

Rice oil from rice (*Oryza sativa*) bran, which is the main by-product from milling in rice-processing countries, has high oil, protein and carbohydrate content [8]. In this study, rice oil was fed to culture broth of *C. acremonium* M25 to improve CPC production. The objective of this study is to improve CPC production by optimization of medium and culture conditions. A statistical method was introduced to optimize the main culture medium. To improve the CPC production, a rice oil feeding strategy was established.

MATERIALS AND METHODS

Strain

In our previous work, *C. acremonium* ATCC 20339

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was mutagenized with UV light and a mutant of *C. acremonium*, strain M25, was finally selected by an agar-diffusion method [9]. This strain was used for CPC production in this work.

Media and Culture Conditions

The stock cultures were maintained by transferring the organism monthly on potato dextrose agar slants. The basal seed medium consisted of 2.5% sucrose, 1.0% glucose, 2.5% corn steep liquor (CSL, for industrial use) and 0.4% $(NH_4)_2SO_4$. To improve the morphological differentiation, 3.0% soybean meal, 1.0% cotton seed flour and 0.5% $CaCO_3$ were added to the basal seed medium [9]. The main medium consisted of 2.84% glucose, 0.8% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.5% K_2HPO_4, 0.5% DL-methionine and 0.4% trace element solution [10]. To improve the CPC production, 6.68% CSL was added to the main medium [11]. Sugars and $(NH_4)_2SO_4$ were sterilized separately from other components. The pH was adjusted to 7.0 with 1 N NaOH prior to sterilization. Calcium carbonate ($CaCO_3$) was added after pH adjustment. Seed and main cultures were carried out in 250-mL Erlenmeyer flasks containing 15 mL of medium. The flask cultures were operated at 300 rpm, 27°C, on a rotary shaking incubator. The fed-batch fermentation in the stirred-tank fermenter (2.5 L) was carried out at 27 °C. The operating volume was 1.5 L with an air flow rate of 1.0 vvm, and 5% (v/v) rice oil was fed at 3rd and 5th day into the bioreactor culture broth.

Analytical Methods

The dry cell weight of mycelium was measured as follows; 10 mL of culture broth was centrifuged at 12,000 × *g* for 10 min, and filtered through a preweighted Whatman glass-microfiber filter GF/C. After being washed twice with distilled water, the cells were dried at 95°C to a constant weight prior to measuring the dry weight.

The amount of glucose and sucrose was measured by using a modified DNS method [12].

CPC was measured by high performance liquid chromatography (HPLC) using a reverse phase column of μ Bondapak C-18 and 254 nm UV detector. The mobile phase was an acetonitrile-phosphate buffer. The elution mixture was a 98% phosphate buffer and a 2% acetonitrile with a flow rate of 0.9 mL/min. Cephalosporin C zinc salt (Sigma, USA) was used as standard.

Experimental Design

Fractional factorial designs were employed to optimize main medium. Using analysis of variance (ANOVA) and response surface methodology (RSM), the optimal concentration of main medium which had a significant effect on CPC production was determined. The variables were coded according to the Equation:

$$x_i = (X_i - X_0)/\Delta X \qquad i = 1, 2, 3 \ldots, j \tag{1}$$

Table 1. Level of the variables tested in fractional factorial design

		Coded values		
	Symbol	-1	0	1
Sucrose	A	2%	3.6%	5.2%
Glucose	B	1%	2.7%	4.4%
$(NH_4)_2SO_4$	C	0.4%	0.8%	1.2%
DL-methionine	D	0.2%	0.5%	0.8%
CSL	E	2%	5%	8%

0.3% KH_2PO_4, 0.5% K_2HPO_4, 0.4% trace element solution [9]

where x_i is the coded (dimensionless) value of the variable X_i, X_0 the value of X_i at the centre point, and ΔX the step change.

The behavior of the system was explained by the following second degree polynomial equation:

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \tag{2}$$

where y is the predicted response, β_0 the offset term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} the interaction effect. SAS program (SAS Institute Inc., USA) was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation.

RESULTS AND DISCUSSION

Optimization of the Main Medium Using Statistical Methods

From preliminary experiments made in order to examine the effects of different components on CPC production, five factors which play the most important role in the CPC synthesis were chosen. These were the sucrose, glucose, $(NH_4)_2SO_4$, DL-methionine and CSL. Table 1 shows the values of variables at different levels of the fractional factorial design (FFD). The experimental design and the results of the FFD are illustrated in Table 2. The CPC production varied in a range of 0~0.423 g/L. On the basis of these experimental values, statistical testing was carried out using analysis of variance (ANOVA).

As shown in Table 3, when the F test was applied on each factor for CPC production, three variables (sucrose, glucose, and CSL) and interaction between glucose and CSL were statistically significant at a 1% level of significance. So, the effect of each component on CPC production was investigated. Especially, in the case of glucose, the F- and P-values for CPC production were 815.03 and 0.0001, respectively. From this result, it was concluded that glucose was the most effective component on CPC production.

To determine the optimal concentrations of glucose and CSL, statistical analysis by RSM was performed on

Table 2. Experimental designs and results for analysis of variance (ANOVA)

RUN	A	B	C	D	E	CPC Production (g/L)
1	-	-	-	-	+	0.319
2	+	-	-	-	-	0.349
3	-	+	-	-	-	0.2
4	+	+	-	-	+	0.274
5	-	-	+	-	-	0.393
6	+	-	+	-	+	0.349
7	-	+	+	-	+	0.185
8	+	+	+	-	-	0
9	-	-	-	+	-	0.378
10	+	-	-	+	+	0.393
11	-	+	-	+	+	0.349
12	+	+	-	+	-	0
13	-	-	+	+	+	0.363
14	+	-	+	+	-	0.378
15	-	+	+	+	-	0
16	+	+	+	+	+	0.274
17	0	0	0	0	0	0.393
18	0	0	0	0	0	0.423
19	0	0	0	0	0	0.408
20	0	0	0	0	0	0.423

Table 3. Statistical analysis of factors through analysis of variance

Factor	CPC Production	
	F - value	P - value
A	176.60	0.0008
B	**815.03**	**0.0001**
C	31.03	0.0114
D	1.32	0.3339
E	**197.84**	**0.0008**
AB	12.36	0.0390
AC	25.48	0.0150
AD	1.94	0.2580
AE	30.64	0.0116
BC	50.44	0.0057
BD	5.77	0.0957
BE	**276.95**	**0.0005**
CD	3.67	0.1514
CE	0.02	0.8981
DE	58.13	0.0047

(A: sucrose, B: glucose, C: $(NH_4)_2SO_4$, D: DL-methionine, E: CSL)

Table 4. Experimental designs and results for determination of optimal concentration of glucose and CSL

Runs	Glucose	CSL	CPC Production (g/L)
1	-1	-1	0.170
2	1	-1	0
3	-1	1	0.185
4	1	1	0.319
5	-1.414	0	0
6	0	-1.414	0
7	1.414	0	0
8	0	1.414	0.334
9	0	0	0.408
10	0	0	0.408
11	0	0	0.438

the basis of experiment design given in Table 4. Using these results, the following second order polynomial equations relating to the CPC production explained the experimental data.

$$\text{CPC production} = 0.418 - 0.005X - 0.188X^2 + 0.101Y - 0.104Y^2 + 0.076XY$$

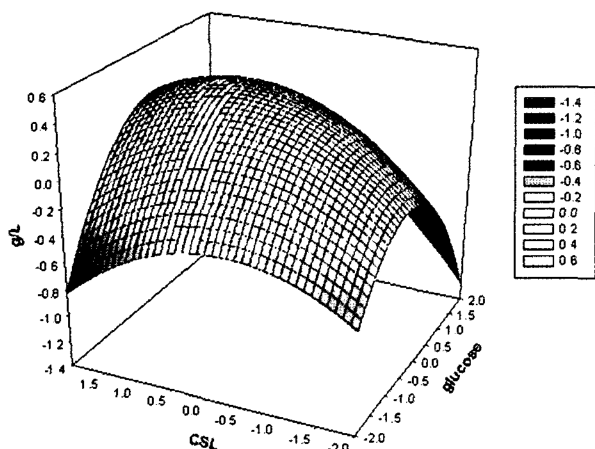
(Where X = concentration of glucose,
Y = concentration of CSL)

The contour plots on CPC production obtained from the calculated response surface are represented in Fig. 1. According to these results, optimal glucose and CSL concentrations were calculated to be 2.84% (coded value; 0.08) and 6.68% (coded value; 0.56), respectively. The maximum value of CPC production predicted from the model was 0.443 g/L.

Optimization of Main Culture Conditions

Fig. 2 shows time courses of CPC production in shake flask culture using an optimized main medium. The maximum CPC production (0.423 g/L) was obtained at 3rd day, and then CPC production was gradually decreased. The maximum CPC production was similar to that predicted by statistical analysis. But, as glucose depleted, cell mass and CPC production gradually decreased. From this result, it was thought that a second carbon source is necessary to maintain cell growth and CPC production after glucose depletion.

Many researchers reported that a fed batch culture was more effective than a batch culture for CPC production [2,13,14]. So, the effect of feeding of carbon sources during the main culture was investigated. As carbon sources, glucose, soybean oil and rice oil were chosen. And each carbon source was fed at day 3rd and 5th day at a concentration of 3% (v/v). Glucose feeding at 3rd and 5th day increased cell mass, but CPC production was lower than that obtained in the batch culture (data not



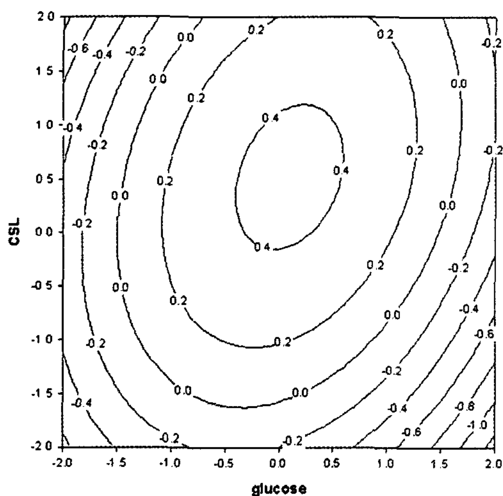


Fig. 1. Effect of glucose and CSL on CPC production.

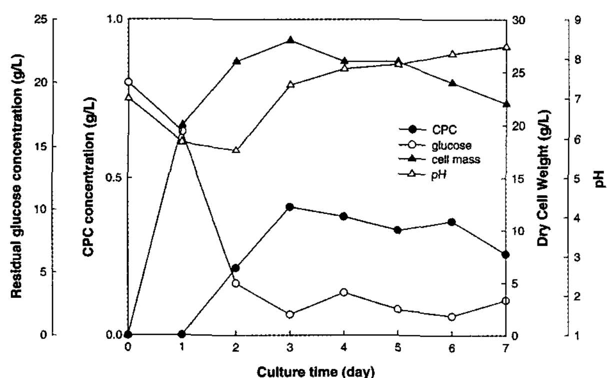


Fig. 2. Time courses of CPC production by batch culture of *C. acremonium* M25 with an optimized main culture medium. The flask culture was carried out at 300 rpm, 27°C, on a rotary shaking incubator.

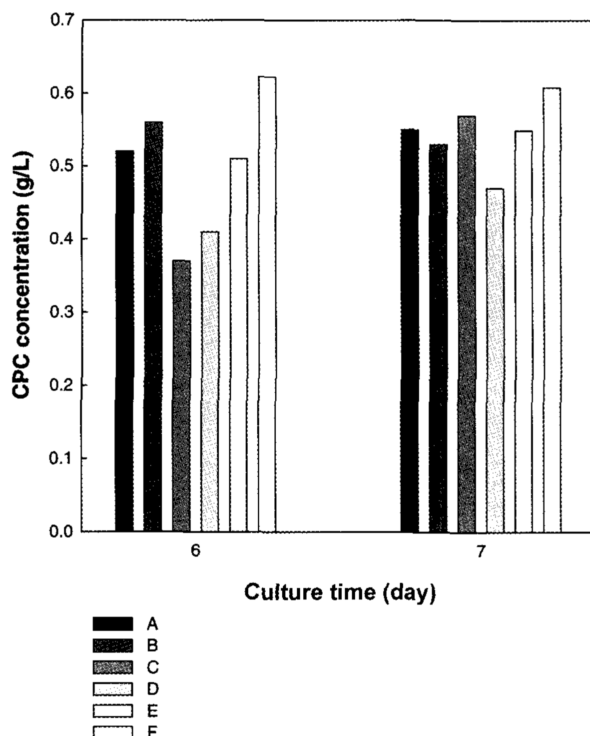


Fig. 3. Effect of soybean oil and rice oil feeding time on CPC production. A: 3% soybean oil was fed at 3rd day, B: 3% rice oil was fed at 3rd day, C: 3% soybean oil was fed at 5th day, D: 3% rice oil was fed at 5th day, E: 3% soybean oil was fed at 3rd and 5th day, F: 3% rice oil was fed at 3rd and 5th day.

shown). Fig. 3 shows the effect of soybean oil and rice oil feeding on CPC production. It was observed that cell mass and CPC production increased after soybean oil and rice oil feeding. Especially, when 3% rice oil was fed at 3rd and 5th day, respectively, CPC production (0.61 g/L) increased about 44% compared to that of batch culture. So, optimal rice oil concentration to feed was determined. At 3rd and 5th day during culture time, 1%, 3%, and 5% rice oil was fed to the culture broth. CPC production increased as rice oil concentration increased. Moreover, CPC productivity was the highest when 5% rice oil was fed. But, above 5% rice oil, unused oil remained and inhibited oxygen transfer to the culture broth (data not shown). Park *et al.* [15] reported similar results using soybean oil as the sole carbon source for cephamycin production. As a result of two steps of feeding of rice oil, CPC production increased significantly. So 5% rice oil was chosen as a feeding carbon source. And feeding time was determined to be 3rd and 5th day. Fig. 4 shows the time course of CPC production by a fed-batch culture of *C. acremonium* M25 in the shake flask. At 3rd and 5th day, 5% rice oil was fed to the culture broth. At 3rd day, glucose almost consumed and then 5% rice oil was fed. Because of the rice oil feeding, cell mass and CPC production increased at 4 days, and pH decreased quickly to pH 5.2 from pH 7.1. At 5th day, 5% rice oil was fed once again. Rice oil feeding at 5th day maintained cell mass

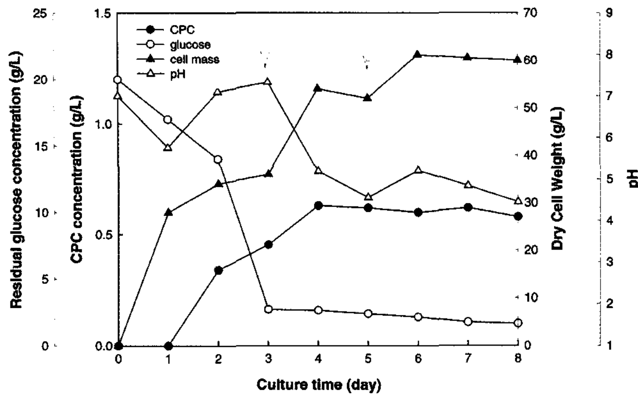


Fig. 4. Time courses of CPC production by fed-batch culture of *C. acremonium* M25 in the shake flask. The flask culture was carried out at 300 rpm, 27°C, on a rotary shaking incubator. Arrows represent 5% rice oil feeding at 3rd and 5th day, respectively.

and CPC production. Compared to a batch culture, higher cell mass (61.2 g/L) and CPC production (0.633 g/L) was obtained by a fed-batch culture using rice oil.

CPC Production in 2.5-L Bioreactor by Fed-batch Culture Using Rice Oil

CPC was produced by *C. acremonium* M25 in a 2.5-L bioreactor using an optimized medium and 5% rice oil feeding. It has been reported that oxygen transfer is a very important factor in many fermentation processes [16,17]. So, the effect of agitation speed on CPC production in a 2.5-L bioreactor was investigated. At different agitation speeds (200~600 rpm), changes of CPC production, cell mass, and residual glucose concentration were observed. As agitation speed increased, changes of CPC, cell mass, and residual glucose showed different tendency (Fig. 5). In the case of glucose consumption, at high agitation speeds (500~600 rpm), residual glucose decreased rapidly to 3rd day, but the glucose consumption rate was relatively low at low agitation speeds (200~300 rpm) (Fig. 5(a)). High cell mass was obtained as agitation speed increased, and the maximum cell mass concentration (54.5 g/L) was obtained at 600 rpm (Fig. 5(b)). As shown in Fig. 5(c), it proved that oxygen transfer had effect on CPC fermentation. At high agitation speeds (500~600 rpm), CPC production increased exponentially after 4 days when glucose and rice oil exhausted. The maximum CPC concentration (0.98 g/L) was obtained at 500 rpm. So, the optimal agitation speed in 2.5-L bioreactor is 500 rpm. Fig. 6 shows the time courses of CPC production by a fed-batch culture in the 2.5-L bioreactor. The glucose was almost consumed after 2 days. After rice oil feeding at 3 days, cell mass increased significantly to 46.9 g/L, and pH decreased rapidly to pH 6.05 from pH 7.65. Cell mass was lower than that of a fed-batch flask culture. This result shows that *C. acremonium* M25 differentiates more in a 2.5-L bioreactor culture than in a flask culture. CPC production increased after rice oil feeding at 5th day. The maximum CPC production was 0.98 g/L, which is about 54% greater than that obtained by a batch culture in the shake flask.

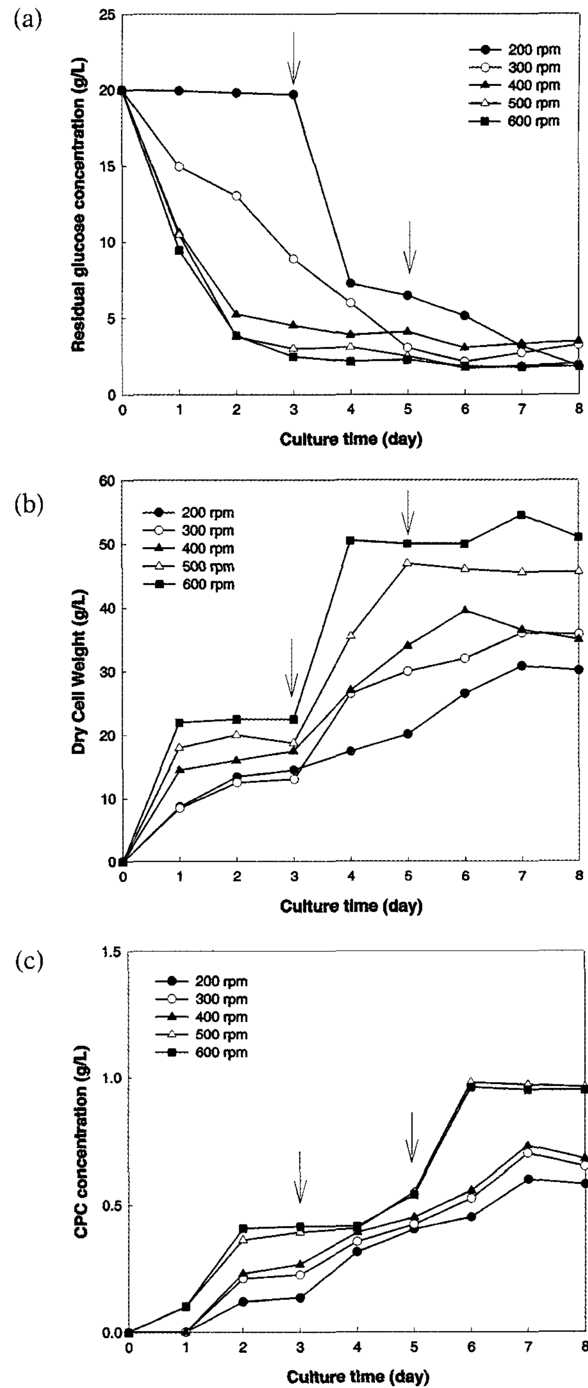


Fig. 5. Effect of agitation speeds on (a) residual glucose concentration, (b) cell mass and (c) CPC production in a 2.5-L bioreactor. Arrows represent 5% rice oil feeding at 3rd and 5th day, respectively.

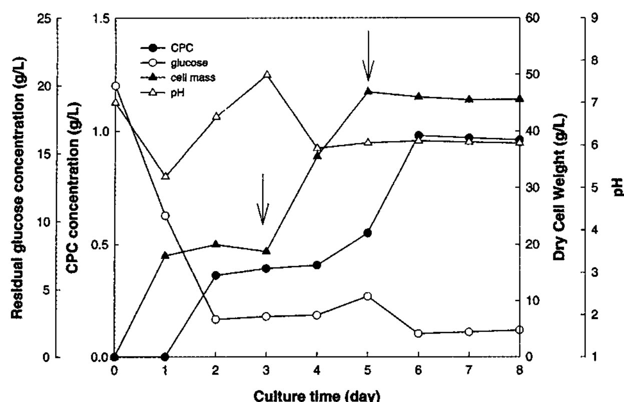


Fig. 6. Time courses of CPC production by a fed-batch culture of *C. acremonium* M25 in the 2.5-L bioreactor. The 2.5-L bioreactor culture was carried out at 27°C , with an air flow rate of 1.0 vvm and agitation speed as 500 rpm. Arrows represent 5% rice oil feeding at 3rd and 5th day, respectively.

CONCLUSION

The main medium for CPC production was optimized using a statistical method. Glucose and CSL were found to be the most effective factors on CPC production. Glucose and CSL were optimized to be 2.84% and 6.68%, respectively. To improve the CPC production, rice oil was fed as second carbon source. CPC production was improved 50% by feeding of 5% rice oil at 3rd and 5th day during the shake flask culture of *C. acremonium* M25. The effect of agitation speeds on CPC production in a 2.5-L bioreactor was investigated with fed-batch mode. The maximum CPC production (0.98 g/L) was obtained at 500 rpm. In the fed-batch 2.5-L bioreactor culture, the maximum CPC production was about 132% greater than that obtained in a batch flask culture. So, it is suggested that large scale production of CPC by *C. acremonium* M25 in the fed-batch culture using rice oil is possible.

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