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ORIGINAL PAPER

Production of cephamycin C by *Streptomyces clavuligerus* NT4 using solid-state fermentation

Baburao Bussari · Parag S. Saudagar · Nikhil S. Shaligram · Shrikant A. Survase · Rekha S. Singhal

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Abstract Cephamycin C is an extracellular broad spectrum β-lactam antibiotic produced by *Streptomyces clavuligerus*, *S. cattleya* and *Nocardia lactamdurans*. In the present study, different substrates for solid-state fermentation were screened for maximum cephamycin C production by *S. clavuligerus* NT4. The fermentation parameters such as substrate concentration, moisture content, potassium dihydrogen phosphate, inoculum size and ammonium oxalate were optimized by response surface methodology (RSM). The optimized conditions yielded 21.68 ± 0.76 mg gds^{-1} of cephamycin C as compared to 10.50 ± 1.04 mg gds^{-1} before optimization. Effect of various amino acids on cephamycin C production was further studied by using RSM, which resulted in increased yield of 27.41 ± 0.65 mg gds^{-1}.

Keywords Cephamycin C · *Streptomyces clavuligerus* NT4 · Solid-state fermentation · RSM · Amino acids

Introduction

Since the discovery of cephalosporin C, a number of microbial species have been isolated which produced a variety of related antibiotic products. In all, over 1,000 naturally occurring or chemically modified antibiotic products have been reported, of which over 100 are commercially produced. One important class of microbially produced compounds, the cephamycins, was separately isolated in the early 1970s by Merck and Co., Inc., Germany and by Eli Lilly and Co., IN, USA. The first member of this family to be isolated was cephamycin C. It is produced by *Nocardia lactamdurans* as well as several other actinomycetes [1] and *Streptomyces clavuligerus* [2]. β-Lactamase resistance and the nature of the producing species distinguish the cephamycins from the cephalosporins. A characteristic methoxy substitution at the 7α position of the cepham nucleus distinguishes between cephamycins and cephalosporins. Much similar to cephalosporins, cephamycin C forms a series of compounds that are finding increasing use worldwide with a strong possibility of it replacing the currently used antibiotics in the near future.

In statistical-based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization [3, 4]. RSM has been used successfully in the optimization of bioprocesses [5]. The application of these design techniques in fermentation process results in improved product yields, reduced process variability, closer confirmation of the output response (product yield or productivity) to nominal and target requirements and reduced development, and overall costs [6]. Central composite rotatable design (CCRD) is the most widely used response surface design. Although rotatability is a desirable property of a central composite design, a face-centered design can be used when there is a difficulty in extending the star points beyond the experimental region defined by the upper and lower limits of each factor [7].

The principle amino acids that get incorporated directly into β-lactam antibiotics involved are those, which comprise the Arnestein tripeptide: cysteine, valine and α-aminoadipic acid (α-AAA) [8]. There are two major

B. Bussari · P. S. Saudagar · N. S. Shaligram · S. A. Survase · R. S. Singhal (✉)
Food Engineering and Technology Department,
Institute of Chemical Technology, University of Mumbai,
Matunga, Mumbai 400 019, India
e-mail: rekha@udct.org

pathways by which antibiotic-producing microorganism incorporate sulphur into cysteine and ultimately into β-lactam products. One way is by reduction of inorganic compounds such as sulphate or thiosulphate. An alternative pathway is the conversion of methionine into cysteine prior to the formation of the Arnestein tripeptide. In most commercial antibiotic-producing strains, one of these two pathways predominates [8].

Sulphur metabolism in cephamycin-producing actinomycetes has been investigated by Inamine and Birnbaum [9–11]. Cysteine, methionine and inorganic thiosulphate, the major sources of sulphur for antibiotic production, have all been found to inhibit growth of *N. lactamdurans*. However, Inamine and Birnbaum [9] found thiosulphate addition to the culture subsequent to growth to dramatically stimulate cephamycin production. This concept has been used in commercial production of cephamycins.

There is scarcity of literature on cephamycin C production by using solid-state fermentation (SSF). Moreover, SSF is advantageous due to low production cost and high productivity. This paper deals with optimization of cephamycin C production by SSF using *S. clavuligerus* NT4. In the first step, agricultural wastes were evaluated for the maximum production of cephamycin C. The effects of additional media constituents such as organic and inorganic nitrogen sources were also investigated. Subsequently, in the second step, RSM was applied to optimize media components and culture conditions, inoculum volume and moisture content. Furthermore, the effect of different precursors was studied on cephamycin production using RSM.

Materials and methods

Media components

Glucose, yeast extract, agar, malt extract, soybean meal, mycological peptone, ammonium sulphate, ammonium chloride, *p*-dimethyl aminobenzaldehyde, chitin, urea, sodium carbonate, NaOH and corn steep liquor were all procured from Himedia Ltd, Mumbai. Salts like magnesium sulphate, potassium dihydrogen phosphate, sodium chloride, zinc chloride, manganese chloride and solvents like HPLC grade acetonitrile, methanol, ethanol, concentrated HCl and concentrated H_2SO_4, etc., were purchased from Merck India Ltd, Mumbai. All solvents used were of AR grade except HPLC grade acetonitrile. Standard cephamycin C (authentic sample) was obtained as a gift sample through the kind courtesy of Merck Research Laboratories, Rahway, NJ, USA. Cottonseed meal (CSM) was a gift sample from Central Institute for Research on Cotton Technology (CIRCOT), Matunga, Mumbai. Sesame oil cake (SOC), wheat bran (WB), soy flakes (SFL), soy flour (SF), soy grit (SG), tamarind seed powder (TMP), rice stock (RS), cotton hull (CH/COH), wheat *rawa* (WR), rice bran (RB), saw dust (SD) and amaranth stack waste (ASR) were collected from local market. Pall 0.2-μm membrane filter (Ultipor® N_{66} ® Nylon 6, 6 membrane) was purchased from Pall Sciences, Pall Pharmalab filtration Pvt Ltd, Mumbai.

Microorganism and medium

The strain *S. clavuligerus* MTCC 1142 was procured from Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. An NTG mutant of the strain, developed in our laboratory that gave enhanced production of cephamycin C, was used for the study. This mutant strain was named as *S. clavuligerus* NT4. The *S. clavuligerus* NT4 was maintained on a medium having the following composition (in g l^{-1}) yeast extract 4, malt extract 10, dextrose 4 and agar 20 and pH 7.2–7.4; the slants were subcultured every 15 days.

Fermentation

Five grams of solid substrate was placed in 250 ml Erlenmeyer flasks and 2 ml salt solution (0.1% NaCl, 0.1% $MgSO_4$, 0.5% NH_4NO_3, 0.2% KH_2PO_4) [12] and 5 ml distilled water was added to produce moisture content of 65%. The flasks were then autoclaved at 121°C, 15 lbs for 20 min. Two milliliters of spore suspension (2×10^6 spores/ml) was added and incubated at 25 ± 2°C for 7 days.

Selection of the substrate

Different agricultural wastes such as CSM, SOC, WB, SFL, SF, SG, TMP, RS, CH/COH, WR, RB, SD, ASR were checked for their suitability for cephamycin C production. Different combinations of these substrates were also evaluated such as CSM and SOC (1:1), CSM and WB (1:1), CSM and SFL (1:1), CSM and SF (1:1) and CSM and SG (1:1). The substrate supporting maximum production of cephamycin C was selected for the further study. Once the fermentation was completed, a small amount ($\approx$1 g) of the fermented matter was taken for estimation of biomass.

Effect of supplementation of inorganic nitrogen sources

In addition to CSM, different inorganic nitrogen sources such as ammonium chloride, ammonium sulphate, ammonium oxalate, *tri*-ammonium citrate, sodium nitrate,

potassium nitrate, calcium nitrate, *di*ammonium hydrogen phosphate, ammonium *di*hydrogen phosphate, thiourea and urea were added at 0.06 M as 2 ml of salt solution by replacing 0.5% NH_4NO_3 and evaluated for cephamycin C production.

Effect of supplementation of organic nitrogen sources

In addition to CSM, different complex organic nitrogen sources such as yeast extract, soy bean meal, mycological peptone, beef extract, soya peptone, *P*-soyatose powder (soybean base with 85% protein), casein peptone, meat peptone, peptone type I, proteose peptone, corn steep liquor, peptone and malt extract were added at 0.5% as 2 ml of salt solution by replacing 0.5% NH_4NO_3 and evaluated for cephamycin C production.

Optimization of media by RSM

A CCRD for five independent variables was used to obtain the combination of values that optimizes the response within the region of three-dimensional (3D) observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, Design Expert Version 6.0.10 trial version (State Ease, Minneapolis, MN, USA). The components (independent variables) selected for the optimization were moisture content, amount of the substrate (CSM), potassium dihydrogen phosphate, inoculum size and ammonium oxalate. Regression analysis was performed on the data obtained from the design experiments.

To examine the combined effect of five different medium components (independent variables), on maximum cephamycin C production, a central composite factorial design (factorial portion 2^{5-1} with 10 stars points where $\alpha\pm$ is equal to square root of k and $k = 4$) of 26 plus 6 centre points leading to a total of 32 experiments were performed. The coded and actual values of independent variables are given in Table 1. The experiments were carried out in triplicate. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The effect of variables and their interactions and all the coefficients were calculated by the software package Expert Version 6.010.

Effect of metabolic precursors on cephamycin C production by RSM

Based on the biosynthetic pathway of cephamycin C in *S. clavuligerus*, four amino acids (L-lysine hydrochloride, L-valine, L-cystine and DL-methionine) were selected for their effect on the production of cephamycin C using RSM.

Analytical determination

Fermented matter was extracted with 0.1% (v/v) Tween-80 in distilled water, at room temperature (28 ± 2°C) on an orbital shaker at 180 rpm for 2 h. The extract was centrifuged at 10,000*g* and 1 ml of supernatant was extracted with 9 ml of methanol to remove proteins. This extract was filtered using Whatman filter paper (No. 1) and then using Pall 0.2-µm membrane filter (Ultipor® N_{66} ® Nylon 6, 6 membrane). Cephamycin C was estimated by HPLC [14]. Jasko HPLC system fitted with a reverse phase column Waters Sperisorb® ODS (C_{18} octadecyl silane, 250 × 4.6 mm ID). The mobile phase used was 60% 0.05 M KH_2PO_4 (pH 3.0) and 40% acetonitrile. A total of 20-µl extract (the centrifuged and deproteinized extract) was injected and eluted at a flow rate of 0.8 ml min^{-1}. Cephamycin C was detected at 253 nm (UV detection).

Biomass estimation in SSF

This was determined by the procedure of Sakurai et al. [13] and Blix [14]. To 0.5 g of the dry fermented matter in a test tube, 2 ml of conc. H_2SO_4 (98%, density 1.84 g ml^{-1}, 36 N, 18 M) was added and kept for 24 h. It was diluted with distilled water to get 1 N H_2SO_4 (achieved by addition of 70 ml distilled water), sealed and autoclaved for 1 h. The mixture was cooled, filtered and the filtrate neutralized with 1 N NaOH to pH 7.0. The total volume was measured. To 1 ml of this solution, 1 ml of acetyl acetone reagent (1 ml of acetyl acetone in 50 ml 0.5 N Na_2CO_3) was added, sealed, and kept in a boiling water bath for 20 min. It was cooled, and then 6.0 ml ethanol and 1 ml of Ehrlish reagent (2.67 g of *p*-dimethyl aminobenzaldehyde dissolved in 1:1 mixture of analytical grade ethanol and conc. HCl and made up to 100 ml) was added, and the mixture incubated at 65°C for 10 min. After cooling, the optical density was measured at 530 nm against a reagent blank (using 1 ml of distilled water instead of 1 ml of sample solution) [15]. *N*-acetyl glucosamine was calculated as follows:

$$\text{Amount of } N-\text{glucosamine content} = \{\text{concentration} \times \text{total volume of solution after neutralization}/(\text{initial dry weight of fermented matter})\}.$$

Table 1 The central composite rotatable design (CCRD) matrix of independent variables in coded form with their corresponding response by experiments and predicted

Run	Variables[b]					Cephamycin C yield (mg gds^{-1})	
	A	*B*	*C*	*D*	*E*	Experimental[a]	Predicted
1	–1 (4)	–1 (0.1)	–1 (0.25)	–1 (2.0)	1 (70.0)	16.91 ± 0.31	16.99
2	1 (6)	–1 (0.1)	–1 (0.25)	–1 (2.0)	–1 (55.0)	13.34 ± 0.21	13.36
3	–1 (4)	1 (0.3)	–1 (0.25)	–1 (2.0)	–1 (55.0)	12.12 ± 0.16	12.00
4	1 (6)	1 (0.3)	–1 (0.25)	–1 (2.0)	1 (70.0)	17.35 ± 0.15	17.41
5	–1 (4)	–1 (0.1)	1 (0.75)	–1 (2.0)	–1 (55.0)	14.26 ± 0.16	14.24
6	1 (6)	–1 (0.1)	1 (0.75)	–1 (2.0)	1 (70.0)	13.53 ± 0.09	13.68
7	–1 (4)	1 (0.3)	1 (0.75)	–1 (2.0)	1 (70.0)	16.45 ± 0.15	16.46
8	1 (6)	1 (0.3)	1 (0.75)	–1 (2.0)	–1 (55.0)	13.42 ± 0.16	13.36
9	–1 (4)	–1 (0.1)	–1 (0.25)	1 (4.0)	–1 (55.0)	13.41 ± 0.12	13.40
10	1 (6)	–1 (0.1)	–1 (0.25)	1 (4.0)	1 (70.0)	16.91 ± 0.56	17.09
11	–1 (4)	1 (0.3)	–1 (0.25)	1 (4.0)	1 (70.0)	16.27 ± 0.54	16.32
12	1 (6)	1 (0.3)	–1 (0.25)	1 (4.0)	–1 (55.0)	15.81 ± 0.25	15.76
13	–1 (4)	–1 (0.1)	1 (0.75)	1 (4.0)	1 (70.0)	14.61 ± 0.18	14.75
14	1 (6)	–1 (0.1)	1 (0.75)	1 (4.0)	–1 (55.0)	13.39 ± 0.17	13.45
15	–1 (4)	1 (0.3)	1 (0.75)	1 (4.0)	–1 (55.0)	14.61 ± 0.62	14.53
16	1 (6)	1 (0.3)	1 (0.75)	1 (4.0)	1 (70.0)	13.25 ± 0.12	13.35
17	–2 (3)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	15.81 ± 0.34	15.83
18	2 (7)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	15.7 ± 0.25	15.52
19	0 (5)	–2 (0)	0 (0.5)	0 (3.0)	0 (62.5)	12.84 ± 0.15	12.59
20	0 (5)	2 (0.4)	0 (0.5)	0 (3.0)	0 (62.5)	13.05 ± 0.16	13.15
21	0 (5)	0 (0.2)	–2 (0)	0 (3.0)	0 (62.5)	13.81 ± 0.32	13.74
22	0 (5)	0 (0.2)	2 (1.0)	0 (3.0)	0 (62.5)	11.71 ± 0.24	11.61
23	0 (5)	0 (0.2)	0 (0.5)	–2 (1.0)	0 (62.5)	15.32 ± 0.18	15.31
24	0 (5)	0 (0.2)	0 (0.5)	2 (5.0)	0 (62.5)	15.73 ± 0.25	15.60
25	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	–2 (47.5)	13.79 ± 0.34	13.97
26	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	2 (77.5)	18.28 ± 0.64	17.95
27	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.52 ± 0.16	13.60
28	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.66 ± 0.13	13.60
29	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.58 ± 0.12	13.60
30	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.57 ± 0.15	13.60
31	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.58 ± 0.16	13.60
32	0 (5)	0 (0.2)	0 (0.5)	0 (3.0)	0 (62.5)	13.56 ± 0.18	13.60

A = cottonseed meal (CSM) (g), B = KH_2PO_4 (%), C = ammonium oxalate (%), D = inoculum size (ml) and E = moisture content (%)

[a] Results are mean ± SD of three determinations

[b] Values in parentheses are uncoded variables

Results and discussion

Optimization of fermentation medium using one factor-at-a-time method

Selection of the substrate

Experiments were performed using different agricultural wastes as solid substrates to check their suitability for cephamycin C production (Fig. 1). CSM supported maximum cephamycin C of 10.50 ± 1.04 mg g^{-1} of dried substrate [gds, i.e., g of dried fermented matter (DFM)] among all substrates used. Hence, for further study, CSM was used as the substrate. SOC, WB and combination of SG and CSM gave 7.99 ± 0.23, 7.92 ± 0.41 and 8.66 ± 0.87 mg gds^{-1} cephamycin C, respectively. Among the substrates evaluated, RB and SD showed low growth as well as low production of cephamycin C (0.61 ± 0.08 and 0.14 ± 0.01 mg gds^{-1} for RB and SD, respectively). Kota and Sridhar [16] reported a maximum cephamycin C

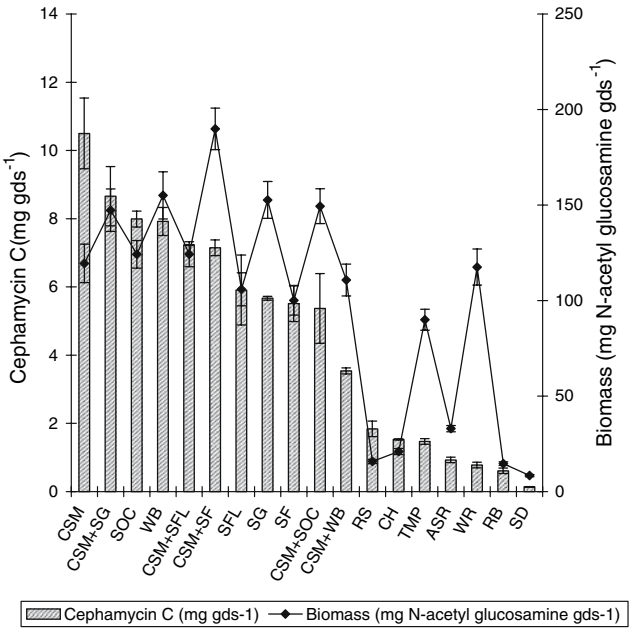

Fig. 1 Evaluation of various agricultural wastes for cephamycin C and biomass production by solid-state fermentation. Fermentation was carried out at an initial moisture content of 65% and temperature of 25 ± 2°C

production of 7.0 and 5.2 mg g^{-1} of substrate with wheat *rawa* and WB, respectively.

Biomass estimation in SSF for different substrates

The biomass on each solid substrate during cephamycin C production was also evaluated (Fig. 1). Combination of CSM:SF (1:1) yielded a maximum biomass of 189.89 ± 4.93 mg of *N*-acetyl glucosamine g^{-1} of dried substrate among all substrates used. SG and WB supported growth of 152.67 ± 2.12 and 155.08 ± 4.65 mg of *N*-acetyl glucosamine gds^{-1}, respectively. Among these substrates, RB and SD showed low biomass growth as well as low production of cephamycin C.

Growth curve and production profile of cephamycin C using CSM

The growth curve and production profile of the cephamycin C on CSM was studied with respect to time (Fig. 2). The production of the cephamycin C, a secondary metabolite, was observed in the beginning of stationary growth phase, from third day of fermentation (2.65 ± 0.16 mg gds^{-1}) and reached a maximum on day 6 (10.5 ± 0.24 mg gds^{-1}). It then decreased slightly up to day 8 (9.85 ± 0.15 mg gds^{-1}). This could be because the organism might have reached the death phase. All the subsequent studies were carried out

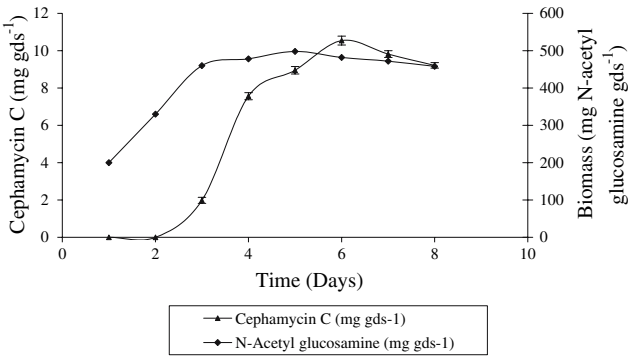

Fig. 2 Production profile of cephamycin C by *Streptomyces clavuligerus* NT4 using cottonseed meal as substrate

with the fermentation for 6 days. Kota and Sridhar [16] reported the production of cephamycin C in solid substrate to begin from the third day of fermentation (6 mg g^{-1}), reach a maximum on the fifth day (15 mg g^{-1}), and then decrease slightly up to 30th day (9.5 mg g^{-1}).

Effect of supplementation of inorganic nitrogen sources

Addition of inorganic nitrogen sources to CSM was checked for its effect on cephamycin C production (Fig. 3). Of these, ammonium oxalate gave a maximum yield (15.34 ± 0.87 mg gds^{-1}) of cephamycin C, and potassium nitrate the least (4.18 ± 0.09 mg gds^{-1}). Kota and Sridhar [16] reported an increase in cephamycin C production to 10 mg g^{-1} by supplementing nutrients as cottonseed deoiled cake (5 g) + corn steep liquor (1 g) + sunflower deoiled cake (1 g) to wheat *rawa* (10 g) as substrate.

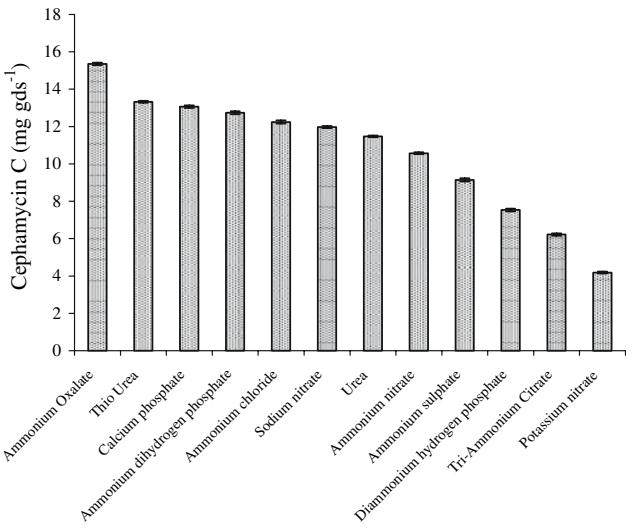

Fig. 3 Evaluation of inorganic nitrogen sources on cephamycin C production by *S. clavuligerus* NT4. Ammonium nitrate was replaced with various inorganic nitrogen sources at 0.06 M

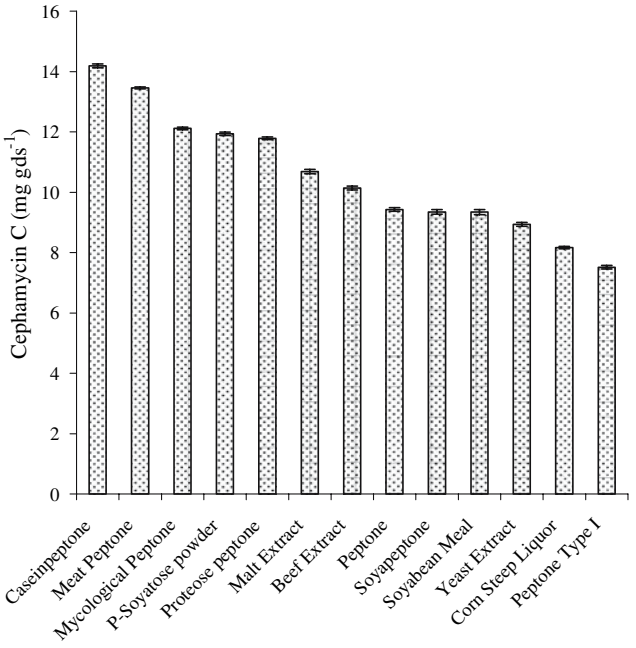

Fig. 4 Evaluation of organic nitrogen sources on cephamycin C production by *S. clavuligerus* NT4. Ammonium nitrate was replaced with various organic nitrogen sources at 0.5%

Effect of supplementation of organic nitrogen sources

Different complex nitrogen sources were added to CSM to check for their effect on cephamycin C production. A total of 0.5% of organic nitrogen sources were added as 2 ml of salt solution (0.1% NaCl, 0.1% $MgSO_4$, 0.5% organic nitrogen source and 0.2% KH_2PO_4). Of these, casein peptone gave maximum yield (14.19 ± 0.86 mg gds^{-1}) of cephamycin C production and corn steep liquor the lowest (8.16 ± 0.07 mg gds^{-1}) (Fig. 4). Among the screened nitrogen sources, ammonium oxalate gave the maximum yield and was therefore used as additional nitrogen source in the salt solution.

Response surface methodology

The CCRD gave quadratic model for the given set of experimental results. Equation (3) represents the mathematical model relating the production of cephamycin C with the independent process variables, A to E and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert. The experimental and predicted values of yields of cephamycin C are given in Table 1.

The results were analyzed by using ANOVA, i.e., analysis of variance suitable for the experimental design used. The ANOVA of the quadratic model indicates that the model is significant. The Model F value of 107.53 implies the model to be significant and is calculated as the ratio of mean square regression and mean square residual. Model P value (Prob $> F$) is very low (<0.0001), again signifying the model to be significant.

The P values were used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Values of $P < 0.050$ indicate the model terms to be significant. The coefficient estimates and the corresponding P values suggests that, among the test variables used in the study, B, C, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, CD, CE and DE (where A = cottonseed meal, B = KH_2PO_4, C = ammonium oxalate, D = inoculum size and E = moisture content) are significant model terms. C, E, A^2, C^2, D^2, E^2, AC, AE, CE and DE ($P < 0.0001$) have the largest effect on cephamycin C production, followed by CD, B^2, B, AD and AB. The variables A and D were found to be insignificant, and so were the mutual interaction between BC, BD and BE.

The corresponding second-order response model for Eq. (2) that was found after analysis for the regression was

$$\begin{aligned}&\text{Cephamycin C yield}\left(\text{mg gds}^{-1}\right)\\&= 13.604 - (0.077 \times A) + (0.139 \times B) - (0.532 \times C)\\&+ (0.072 \times D) + (0.995 \times E) + \left(0.519 \times A^2\right)\\&- \left(0.189 \times B^2\right) - \left(0.251 \times C^2\right) + \left(0.444 \times D^2\right)\\&+ \left(0.621 \times E^2\right) + (0.148 \times A \times B) - (0.691 \times A \times C)\\&+ (0.159 \times A \times D) - (0.298 \times A \times E) + (0.059 \times B \times C)\\&+ (0.019 \times B \times D) - (0.011 \times B \times E)\end{aligned} \quad (3)$$

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.995, indicating 99.5% of the confidence level of the model to predict the response (cephamycin C yield). The "Pred R-Squared" of 0.871 is in reasonable agreement with the "Adj R-Squared" of 0.985. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 40.741 indicates an adequate signal.

Three-dimensional graphs were generated for the pairwise combination of the five factors while keeping the other three at their center point levels (Fig. 5). From the centre point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified.

"Contour plot generation" and "point prediction" were studied to find optimum value of the five independent variables for the maximum production of cephamycin C. These predicted values were experimentally verified. Table 2 documents the yields of cephamycin C by various

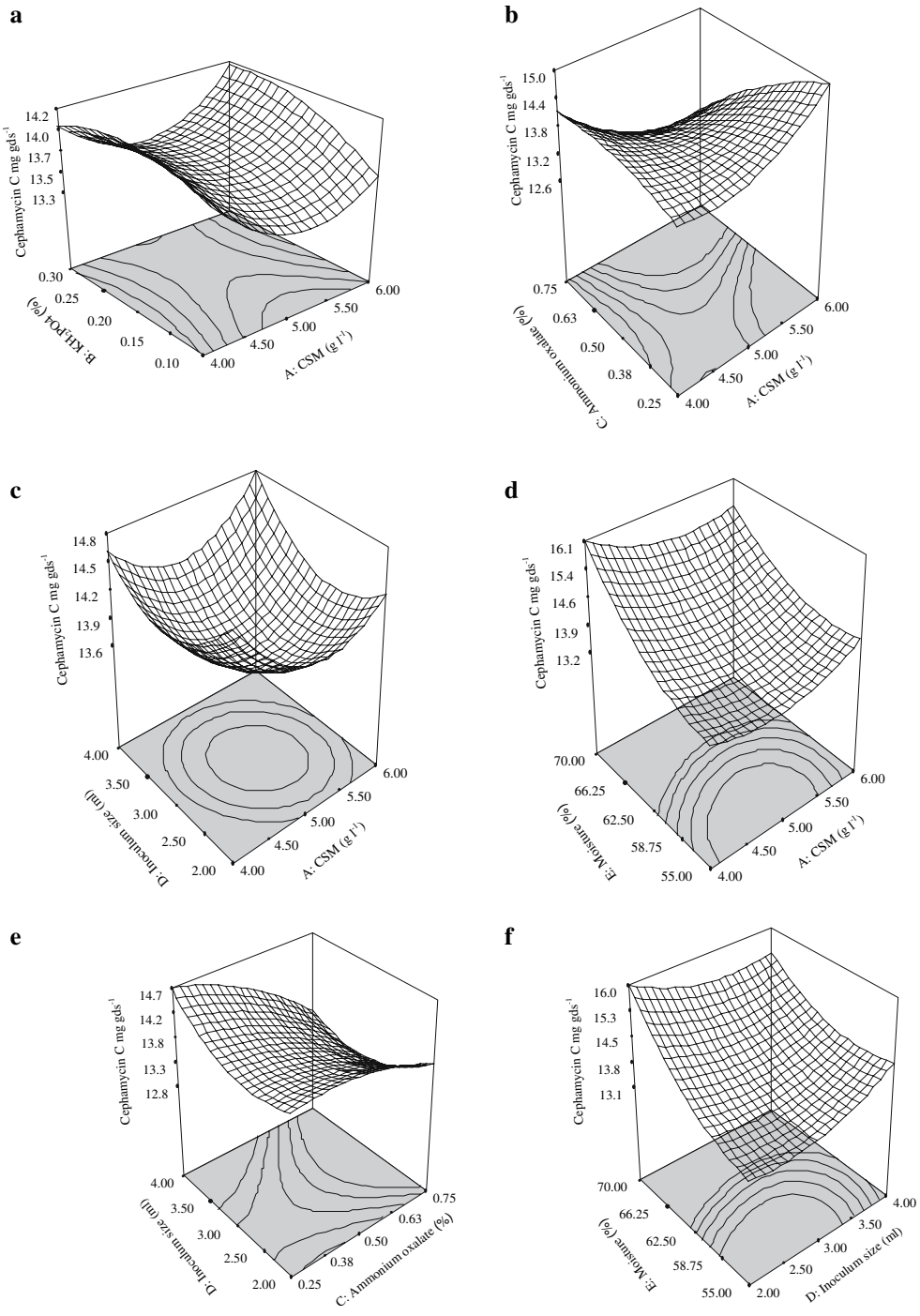

Fig. 5 Three-dimensional surface plot for cephamycin C production. **a** Effect of KH_2PO_4 and CSM when other variables are held at zero level; **b** effect of ammonium oxalate and CSM when other variables are held at zero level; **c** effect of inoculum size and CSM when other variables are held at zero level; **d** effect of moisture content and CSM when other variables are held at zero level; **e** effect of ammonium oxalate and inoculum size when other variables are held at zero level; **f** effect of moisture content and inoculum size when other variables are held at zero level

predicted media combination. The maximum production of cephamycin C obtained using the optimized medium was 21.68 ± 0.76 (mg gds^{-1}) with 6.27 g of CSM; 0.244% of KH_2PO_4; 0.08% of ammonium oxalate; 3.68 ml of inoculum size; and 76.375% of moisture contents. Kota and Sridhar [16] reported a maximum production of 15 mg g^{-1} of substrate of cephamycin C in solid substrate after optimization by one factor at a time.

The moisture content (*E*) played very significant role ($P < 0.0001$). With lower moisture content (47.5–55%), the available oxygen in the void volume is sufficient but the water content is not enough to support good metabolic activity and removal of the heat generation. This may account for lower cephamycin C production and biomass. As the moisture level increased from 62.5 to 77.5%, air present in the void volume was replaced by water, resulting

Table 2 Validation of the model obtained by RSM for the production of cephamycin C by *Streptomyces clavuligerus* NT4

Sr. no.	Factor *A*	Factor *B*	Factor *C*	Factor *D*	Factor *E*	Cephamycin C yield (mg gds^{-1})	
	CSM (g)	KH_2PO_4 (%)	Ammonium oxalate (%)	Inoculum size (2×10^6 spores/ml)	Moisture content (%)	Experimental [a]	Predicted
1	6.27	0.244	0.08	3.69	76.375	21.68 ± 0.76	21.60
2	6.80	0.174	0.3	1.77	77.05	21.65 ± 0.53	21.70
3	6.04	0.210	0.3175	2.67	77.05	20.15 ± 0.74	19.80
4	5.78	3.660	0.0225	3.45	77.05	20.78 ± 0.45	20.99

[a] Results are mean ± SD of three determinations

in decrease of available oxygen. The optimum moisture content was found to be 76.4%.

Ammonium oxalate (*C*), ($P < 0.0001$) played an important role in determining the product yield of cephamycin C. When the concentration of soluble ammonium oxalate was kept low, cephamycin C production was relatively high. As the residual concentration of ammonium increased, antibiotic accumulation decreased rapidly. In the present study, the optimum ammonium oxalate concentration was found to be 0.08%. Ammonium salts are known to be absorbed rapidly by many organisms during growth, and could be used to obtain high cell yields but would not support acceptable levels of antibiotic synthesis when used as the sole nitrogen source [8].

KH_2PO_4 (*B*) ($P = 0.0046$) has a great significance in cephamycin C fermentation. The optimum KH_2PO_4 concentration was found to be 0.244%. The role of phosphate in regular β-lactam antibiotic synthesis is less well understood than that of sulphur. Despite not being incorporated directly into the β-lactam nucleus, phosphate affects secondary metabolism in β-lactam-producing organisms. Elucidation of the precise interaction between phosphate metabolism and antibiotic biosynthesis has been complicated by the fact that phosphate is involved in a host of related and non-related metabolic pathways [8]. In general, three separate regulatory roles, viz. independently inhibition of enzyme action, regulation of protein expression and limit on the cell growth have been postulated for phosphate [17]. High level of exogenously added phosphate can repress the synthesis of phosphate enzymes [18].

Effect of metabolic precursors on cephamycin C production by RSM

In cephamycin-producing *Streptomyces* and *Nocardia*, incorporation of α-AAA into the Arnestein tripeptide is regulated in quite a different manner from fungi. In these organisms, α-AAA is a catabolic product of lysine degradation. As a result, lysine accumulation leads to a greater lysine breakdown. Presumably, the resultant higher levels of α-AAA resulting from lysine accumulation would increase the rate of incorporation into β-lactam antibiotics and leads to higher levels of accumulation. If α-AAA incorporation into the Arnestein tripeptide is the step that limits biosynthesis in a *Streptomyces* antibiotic fermentation, it should be possible to increase product yield by adding lysine to the production medium. Such stimulation has been observed with a number of cephamycin-producing organisms [8].

The media optimized as above was further examined for the combined effect of four different amino acids on cephamycin C production, again using RSM. A central composite factorial design (factorial portion 2^{4-1} with 8 stars points where $\alpha\pm$ is equal to square root of k and $k = 4$) of 16 plus 5 center points leading to 21 experiments were performed. The coded and actual values of independent variables are given in Table 3. Here, amino acids were added in the form of % (w/v) to the 2 ml salt solution having the optimized composition given by RSM. The CCRD gave quadratic model for the given set of experimental results. Equation (4) represents the mathematical model relating the production of cephamycin C with the independent process variables, *A* to *D* and the second-order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert. The experimental and predicted values of yields of cephamycin C are given in Table 3.

The results were analyzed by using ANOVA, i.e., analysis of variance suitable for the experimental design used. The ANOVA of the quadratic model indicates that the model is significant. The Model *F* value of 23.92 and Model *P* value (Prob > *F*) is very low (<0.0004) and implies that the model is significant.

The coefficient estimates and the corresponding *P* values suggested that, among the test variables used in the study, *B*, *C*, A^2, B^2, C^2, *AB*, *AD*, *BC* and *CD* (where *A* = L-lysine hydrochloride (%), *B* = L-valine (%), *C* = cystine (%) and *D* = DL-methionine (%) are significant model terms. *B*, *BC*, *CD* and C^2 (respective *P* values are 0.0002, 0.0005, 0.0005 and 0.0006) had the largest effect on cephamycin C production, followed by B^2, A^2, *C*, *AD* and

Table 3 The CCRD matrix of independent variables in coded form with their corresponding response from experiments and predicted

Std run	Media components (%)[b]				Cephamycin C yield (mg gds^{-1})	
	A	*B*	*C*	*D*	Experimental[a]	Predicted
1	1 (3)	1 (0.9)	1 (1.5)	–1 (0.4)	23.38 ± 0.32	23.42
2	1 (3)	1 (0.9)	–1 (0.5)	–1 (0.4)	23.76 ± 0.52	23.94
3	1 (3)	–1 (0.3)	1 (1.5)	1 (1.2)	22.28 ± 0.25	22.32
4	–1 (1)	1 (0.9)	–1 (0.5)	1 (1.2)	24.57 ± 0.32	24.75
5	1 (3)	–1 (0.3)	–1 (0.5)	1 (1.2)	22.71 ± 0.16	22.89
6	–1 (1)	–1 (0.3)	1 (1.5)	–1 (0.4)	24.76 ± 0.09	24.80
7	–1 (1)	1 (0.9)	1 (1.5)	1 (1.2)	22.12 ± 0.54	22.16
8	–1 (1)	–1 (0.3)	–1 (0.5)	–1 (0.4)	22.94 ± 0.21	23.12
9	–2 (0)	0 (0.6)	0 (1.0)	0 (0.8)	22.23 ± 0.62	22.12
10	2 (4)	0 (0.6)	0 (1.0)	0 (0.8)	22.31 ± 0.23	22.19
11	0 (2)	–2 (0)	0 (1.0)	0 (0.8)	22.93 ± 0.45	22.82
12	0 (2)	2 (1.2)	0 (1.0)	0 (0.8)	25.42 ± 0.26	25.31
13	0 (2)	0 (0.6)	–2 (0)	0 (0.8)	25.01 ± 0.42	24.76
14	0 (2)	0 (0.6)	2 (2.0)	0 (0.8)	23.74 ± 0.21	23.76
15	0 (2)	0 (0.6)	0 (1.0)	–2 (0)	23.35 ± 0.47	23.24
16	0 (2)	0 (0.6)	0 (1.0)	2 (1.6)	23.34 ± 0.64	23.23
17	0 (2)	0 (0.6)	0 (1.0)	0 (0.8)	23.21 ± 0.23	23.09
18	0 (2)	0 (0.6)	0 (1.0)	0 (0.8)	23.05 ± 0.32	23.09
19	0 (2)	0 (0.6)	0 (1.0)	0 (0.8)	23.06 ± 0.24	23.09
20	0 (2)	0 (0.6)	0 (1.0)	0 (0.8)	23.11 ± 0.35	23.09
21	0 (2)	0 (0.6)	0 (1.0)	0 (0.8)	23.04 ± 0.31	23.09

A = L-lysine hydrochloride (%), B = L-valine (%), C = cystine (%) and D = DL-methionine (%)

[a] Results are mean ± SD of three determinations

[b] Values in parentheses are uncoded variables

AB. The interactions such as *A*, *D*, D^2 and *AC* were found to be insignificant.

The corresponding second-order response model for Eq. (2) that was found after analysis for the regression was

$$\begin{aligned}&\text{Yield of cephamycin } C\left(\text{mg gds}^{-1}\right)\\&= 23.095 + (0.02 \times A) + (0.622 \times B) - (0.249 \times C)\\&- (0.003 \times D) - \left(0.234 \times A^2\right) + \left(0.242 \times B^2\right)\\&+ \left(0.292 \times C^2\right) + \left(0.034 \times D^2\right) + (0.392 \times A \times B)\\&- (0.023 \times A \times C) + (0.479 \times A \times D) - (0.527 \times B \times C)\\&+ (0.302 \times B \times D) - (0.540 \times C \times D)\end{aligned} \quad (4)$$

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.982 indicating 98.2% of the confidence level of the model to predict the response (cephamycin C yield).

The predicted values by "contour plot generation" and "point prediction" were experimentally verified. The maximum production of cephamycin C obtained using the optimized medium was 27.41 ± 0.65 (mg gds^{-1}). Table 4 documents the yields of cephamycin C by various predicted media combination. There is no report available on the effect of amino acids on the production of cephamycin C by SSF. Although, Hallada et al. [19] have demonstrated a 50% increase in total cephamycin A and B production by *S. griseus* (NRRL 3912) when 0.20% L-lysine hydrochloride was added to submerged fermentation. Mendelowitz and Aharonowitz [20] observed that inclusion of valine in addition to lysine and DL-*meso*-diaminopimelate stimulated antibiotic production by *S. clavuligerus*. The optimum L-lysine hydrochloride concentration was found to be 3.86%, valine 0.84%, L-cystine 0.1% and DL-methionine 1.5%.

Concluding remarks

Cottonseed meal was found to be the best substrate for the production of cephamycin C by the mutant strain *S. clavuligerus* NT4 using SSF. The initial moisture content had significant effect on the growth and cephamycin C production. Supplementation of CSM with additional organic nitrogen, inorganic nitrogen sources and amino acids

Table 4 Validation of the model developed to study the effect of amino acids on the production of cephamycin C by *S. clavuligerus*

Sr. no.	Factor *A*	Factor *B*	Factor *C*	Factor *D*	Cephamycin C yield (mg gds^{-1})	
	L-Lysine hydrochloride (%)	L-Valine (%)	L-Cystine (%)	DL-Methionine (%)	Experimental[a]	Predicted
1	3.86	0.84	0.10	1.50	27.41 ± 0.65	28.51
2	2.79	1.18	0.15	0.70	27.18 ± 0.52	28.06
3	2.76	0.66	0.08	1.30	26.03 ± 0.43	26.63
4	2.61	1.16	1.15	1.42	25.72 ± 0.36	26.30

[a] Results are mean ± SD of three determinations

proved to be beneficial and increased the yield from 10.5 to 27.41 mg gds^{-1} after optimization of the cephamycin C production by RSM.

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