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Statistical Optimization for Improved Production of Cyclosporin A in Solid-State Fermentation

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This work evaluates the effect of different amino acids on production of Cyclosporin (CyA) production in solid-state fermentation that was previously optimized for different fermentation parameters by one factor at-a-time for the maximum production of CyA by *Tolypocladium inflatum* MTCC 557. Based on the Plackett–Burman design, glycerol, ammonium sulfate, $FeCl_3$, and inoculum size were selected for further optimization by response surface methodology (RSM). After identifying effective nutrients, RSM was used to develop mathematical model equations, study responses, and establish the optimum concentrations of the key nutrients for higher CyA production. It was observed that supplementation of medium containing (% w/w) glycerol, 1.53; ammonium sulfate, 0.95; $FeCl_3$, 0.18; and inoculum size 6.4 ml/5g yielded a maximum of 7,106 mg/kg as compared with 6,480 mg CyA/kg substrate using one factor at-a-time. In the second step, the effect of amino acids on the production of CyA was studied. Addition of L-valine and L-leucine in combination after 20 h of fermentation resulted in maximum production of 8,166 mg/kg.

Keywords: Solid-state fermentation, cyclosporin A, wheat bran flour, coconut oil cake, *Tolypocladium inflatum*

Cyclosporin A (CyA), a cyclic undecapeptide antibiotic intracellularly produced by *Tolypocladium inflatum* [2], is a drug with broad-spectrum pharmacological properties including antifungal [6], antiparasitic [27], anti-inflammatory [25], and immunosuppressive activities [18]. It is also used as a second-line drug in autoimmune diseases like rheumatoid arthritis, uveitis, bronchial asthma, and inflammatory bowel disease [10, 24]. Fungi, when cultivated on a solid substrate, grow under conditions that are similar to their natural habitat, and hence are able to produce the metabolites more efficiently than in submerged fermentation [8].

Development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium, and trace element sources. The optimization of nutritional and environmental conditions plays an important role in developing bioprocesses and improving their performance. Nutritional requirement can be manipulated by the conventional or statistical methods [12]. The conventional method involves changing one independent variable at a time, while keeping others at a fixed level. Statistical methods are more rapid and reliable, shortlist significant nutrients, help in understanding the interactions among the nutrients at various concentrations, and reduce the total number of experiments resulting in saving time and resources.

The fungal production of peptide and depsipeptide antibiotics may be directed and enhanced by amino acid components of the antibiotic molecule. Kobel and Traber [11] reported the directed synthesis of CyA and several analogs during fermentation when supplemented with different amino acids. The biosynthesis of CyA and analogs appears to proceed *via* a nonribosomal mechanism involving the sequential activation of all amino acids, their *N*-methylation, and eventual peptide formation by a multifunctional enzyme, CyA synthatase [28].

In a previous study, we optimized the fermentation conditions by using conventional methods. This paper reports a detailed study on optimization of CyA production by solid-state fermentation (SSF) using a statistical approach. Most important nutrients influencing the yield of CyA were selected by using Plackett–Burman design. Response surface methodology (RSM) was further used to determine the optimum concentrations of the key nutrients for higher production of CyA. Subsequently, the effect of amino acids on the production of CyA was also evaluated.

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Materials and Methods

Materials

Glucose, maltose, glycerol, yeast extract, agar, malt extract, mycological peptone, casein peptone, and ammonium sulfate were procured from Himedia Ltd, Mumbai, India. Salts like magnesium sulfate, sodium chloride, zinc chloride, cobaltous chloride, ferric chloride, zinc sulfate, sodium hydroxide, and solvents like acetonitrile, *n*-butyl acetate, concentrated hydrochloric acid, and sulfuric acid were purchased from S. D. Fine Chem. Ltd., Mumbai, India. All solvents used were of AR grade except acetonitrile, which was of HPLC grade. Standard CyA (authentic sample) was a gift sample through the kind courtesy of RPG Life Sciences Ltd., Mumbai, India.

Wheat bran flour (WBF) and coconut oil cake (COC) were collected from a local market. Pall 0.2-μm membrane filter (Ultipor N_{66} Nylon 6, 6 membranes) was purchased from Pall Sciences, Pall Pharmalab filtration Pvt Ltd, Mumbai, India.

Microorganism

Tolypocladium inflatum MTCC 557 (indicated as *Beauveria nivea* in the MTCC catalog), was procured from MTCC, Chandigarh, India. The culture was maintained on MYA slants containing malt extract 2%, yeast extract 0.4%, and agar 2%, pH 5.4, at 4°C, after growing it for 12 days at 25°C.

Preparation of the Seed Inoculum

The organism was subcultured onto a fresh MYA slant and incubated at 25±2°C. After 12 days, to a fully grown slant, 10 ml of sterile saline containing 0.1% Tween 20 was added and mixed well. One ml of this saline containing approximately 10^8–10^9 spores was added to 50 ml of medium composed of malt extract 2%, yeast extract 0.4%, pH 5.4, taken in a 250-ml flask and incubated at 180 rpm for 72 h at 25±2°C. This was used as the seed for SSF.

Fermentation

The fermentation conditions as optimized in our earlier study [26] were used for further optimization. Five grams substrate in total (combination of HWBF and COC at 1:1) was placed in 250 ml Erlenmeyer flasks and distilled water added so as to get an initial moisture content of 70%. The flasks were then autoclaved for 20 min at 121°C/15 psi. After cooling the flasks to 29±2°C, substrate was inoculated with seed culture and the contents in the flask were thoroughly mixed and incubated at 25±2°C. All the experiments were performed in triplicate.

Statistical Media Optimization

Statistical media optimization involves initial screening of the components by using Plackett–Burman experimental design to understand the significance of their effect on the CyA formation, and then optimization of a few significant components using RSM.

Screening of Important Nutrient Components

This study was done by Plackett–Burman design for screening medium components with respect to their main effects and not their interaction effects [17]. A total of 11 variables (variables, k=11) including nine nutrients (comprising three carbon, three nitrogen, three minerals) and two dummy variables were screened in a total of 12 experiments using a Plackett–Burman design as shown in Table 1. Components were used at two concentrations, which corresponded to H (high level) and L (low level). The carbon and nitrogen sources were used at 5% as H level and 1% as L level. The minerals were used at a concentration that corresponded to H (high level) and L (low level) kept zero. High (+) and low (–) concentrations for the different variables were selected on the basis of some preliminary experiments (data not shown). The experiments (Table 1) were designed using the software Design Expert Version 6.0.10 trial version (State Ease, Minneapolis, MN, U.S.A.). The SSF medium consisted of 5 g of solid substrate with the potential nutrients as per the design. Moisture was maintained at 70% by supplement before autoclaving. The contents were autoclaved for 20 min at 121°C/15 psi. After cooling, flasks were inoculated with a specified volume of inoculum and incubated at 25°C for 9 days.

The numbers of positive signs and negative signs per trial are (k+1)/2 and (k–1)/2, respectively. Each column contains an equal number of positive and negative signs. Thus, each row represents a trial run and each column represents an independent (assigned) or dummy (unassigned) variable. The effect of each variable was determined by the following equation:

Table 1. Plackett–Burman design with coded values along with observed results for CyA production.

Sr. No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	D_1	D_2	CyA mg/kg[a]
1	1	–1	1	–1	–1	–1	1	1	1	–1	1	4,735±46
2	1	1	–1	1	–1	–1	–1	1	1	1	–1	4,982±36
3	–1	1	1	–1	1	–1	–1	–1	1	1	1	4,044±46
4	1	–1	1	1	–1	1	–1	–1	–1	1	1	5,728±42
5	1	1	–1	1	1	–1	1	–1	–1	–1	1	4,874±39
6	1	1	1	–1	1	1	–1	1	–1	–1	–1	4,089±56
7	–1	1	1	1	–1	1	1	–1	1	–1	–1	6,081±54
8	–1	–1	1	1	1	–1	1	1	–1	1	–1	4,204±35
9	–1	–1	–1	1	1	1	–1	1	1	–1	1	5,102±42
10	1	–1	–1	–1	1	1	1	–1	1	1	–1	5,614±41
11	–1	1	–1	–1	–1	1	1	1	–1	1	1	6,389±56
12	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	5,746±57

$X_1, \ldots, X_9$ are independent variables and D_1 and D_2 are dummy variables.
[a]Results are mean±SD of at least three determinations

$$E(x_i)=2(\Sigma M_i^+ - M_i^-)/N$$

where $E(x_i)$ is the concentration effect of the tested variable. M_i^+ and M_i^- are the total production from the trials where the variable (x_i) measured was present at high and low concentrations, respectively; and N is the number of trials. Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff}=\Sigma(E_d)^2/n$$

where V_{eff} is the variance of the concentration effect, E_d is the concentration effect for the dummy variable, and n is the number of dummy variables. The standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (*P*-value) of each concentration effect was determined using the Student's *t*-test:

$$t(x_i)=E(x_i)/SE$$

where $E(x_i)$ is the effect of variable x_i.

Optimization of Concentrations of the Selected Medium Components Using RSM

RSM was used to determine the optimum nutrient concentrations for the production of CyA. A central composite rotatable design (CCRD) for four independent variables was used to obtain the combination of values that optimizes the response within the region of three-dimensional 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, U.S.A.).

The medium components (independent variables) selected for the optimization were glycerol, ammonium sulfate, $FeCl_3$, and inoculum size. Regression analysis was performed on the data obtained from the design experiments.

Coding of the variables was done according to the following equation:

$$x_i=(Xi-Xcp)/\Delta Xi,\ i=1, 2, 3, \ldots, k \quad (1)$$

where is the x_i, dimensionless value of an independent variable; Xi is the real value of an independent variable; Xcp is the real value of an independent variable at the center point; and ΔXi is the step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

The experiments were carried out in triplicate, which was necessary to estimate the variability of measurements (*i.e.*, the repeatability of the phenomenon). Replicates at the center of the domain in three blocks permit the checking of absence of bias between several sets of experiments. The relationship of the independent variables and the response was calculated by the second-order polynomial equation:

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i X_i+\sum_{i=1}^{k}\beta_{ii}X_iX_j+\sum i\sum j\,\beta_{ij}X_iX_j \quad (2)$$

Y is the predicted response; β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficient; β_{ij} the cross-product coefficient; and k is the number of factors.

The second-order polynomial coefficients were calculated using the software package Design Expert version 6.0.10 to estimate the responses of the dependent variable. Response surface plots were also obtained using Design Expert version 6.0.10.

Effects of Amino Acids

The effects of different amino acid members of the CyA molecule on drug production was evaluated by supplementing the fermentation media with different amino acids such as L-valine, L-leucine, DL-valine, L-methionine, L-aminobutyric acid, and glycine. They were screened individually [4% (w/w)] as well as in combination with each other. The time of addition of amino acids (0 to 144 h) was also optimized to further increase the yield.

Production Profile of CyA and Growth Curve of *T. inflatum* MTCC 557

To study the production profile of CyA and growth curve of *T. inflatum* MTCC 557, three flasks were taken each day for a period of 12 days and processed to determine CyA content and biomass content. All other conditions were maintained as previously described.

Analytical Determinations

Biomass was determined by the procedures of Sakurai *et al.* [20] and Blix [4]. 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, 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 of 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 of 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 of 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) were added, and the mixture was 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:

Amount of *N*-acetyl glucosamine content={concentration×total volume of solution after neutralization/(initial dry weight of fermented matter)}

CyA extraction and estimation was carried as reported by Survase *et al.* [26]. CyA content was analyzed using HPLC (Jasko System) fitted with a reverse-phase column Waters Sperisorb ODS (C_{18} octadecyl silane, 250×4.6 mm ID). The mobile phase consisted of acetonitrile and water in the ratio 70:30 with a flow rate of 1 ml/min. The temperature of the column was maintained at 70°C and the HPLC profile was monitored at 210 nm.

RESULTS AND DISCUSSION

Optimization by Plackett–Burman Design

Plackett–Burman design was adopted to select the most significant medium components. High and low limits of variables are shown in Table 2. The experimental design protocol along with responses of different experimental trials is shown in Table 1.

Table 2 shows ANOVA for the results of the Plackett–Burman design. Contrast coefficients allow the determination

Table 2. Assigned concentrations of variables at different levels in Plackett–Burman design and ANOVA of the results for production of CyA by *T. inflatum* MTCC 557.

Factor	Level, %		Mean square	Coefficient estimate	*F*-Value	*P*-Value
	Lower	Higher				
Maltose (X_1)	1	5	19,8661	−128.67	35.08	0.0273
Maltodextrin (X_2)	1	5	37,408	−55.83	6.60	0.1239
Glycerol (X_3)	1	5	1,219,856	−318.83	215.43	0.0046
Bactopeptone (X_4)	1	5	10,443	29.5	1.84	0.3074
Casein peptone (X_5)	1	5	2,739,896	−477.83	483.89	0.0021
Ammo. sulfate (X_6)	1	5	1,626,560	368.17	287.27	0.0035
$FeCl_3$ (X_7)	0	0.25	405,536	183.83	71.62	0.0137
$ZnSO_4$ (X_8)	0	0.15	557,283	−215.5	98.42	0.0100
$CoCl_2$ (X_9)	0	0.05	18,565	−39.33	3.27	0.2119

of the effect of each constituent. A high contrast coefficient either positive or negative indicates that a factor has a large impact on titer; whereas a coefficient close to zero means that a factor has little or no effect. The *P*-value is the probability that the magnitude of a contrast coefficient is due to random process variability and it serves as a tool for checking the significance of each of the coefficients. A low *P*-value indicates a "real" or significant effect. The significance of each variable was determined by applying the Student's *t*-test [14].

Analysis of the *P*-value showed that among the variables tested, maltose, glycerol, ammonium sulfate, casein peptone, $FeCl_3$, and $ZnSO_4$ had significant effects on CyA production ($P<0.05$) (Table 2). Agathos *et al.* [2] previously reported sorbose, sucrose, and glucose to be promising carbon sources for the volumetric production of CyA in SmF. Ramana Murthy *et al.* [19] also reported millet, sorghum flour, and $FeCl_3$ to be the most promising medium components for CyA production, whereas soluble starch had an inhibitory effect on CyA yield. Abdel-fattah *et al.* [1] reported the use of glucose, sucrose, and starch as carbon sources, with ammonium sulfate as nitrogen source, to support maximum production of CyA. Dreyfuss *et al.* [6] used a combination of $NaNO_3$ and casein peptone as a nitrogen source for the production of CyA using an industrial strain of *T. inflatum*.

Table 3. CCRD matrix of independent variables in actual form with their corresponding response.

Std.	Glycerol (% w/w)	Ammo. sulfate (% w/w)	$FeCl_3$ (% w/w)	Inoculum size (ml/5 g)	CyA[a] (mg/kg)
1	3	1.5	0.35	3	5,300±92
2	3	1.5	0.15	3	5,465±68
3	3	0.5	0.35	7	6,142±98
4	1	1.5	0.15	7	6,275±62
5	3	0.5	0.15	7	5,458±99
6	1	0.5	0.35	3	6,207±89
7	1	1.5	0.35	7	5,295±102
8	1	0.5	0.15	3	5,991±95
9	0.31	1	0.25	5	6,573±85
10	3.68	1	0.25	5	4,422±79
11	2	0.15	0.25	5	6,887±92
12	2	1.84	0.25	5	5,997±86
13	2	1	0.08	5	6,328±96
14	2	1	0.41	5	6,232±68
15	2	1	0.25	1.63	5,919±78
16	2	1	0.25	8.36	6,585±99
17	2	1	0.25	5	7,039±87
18	2	1	0.25	5	7,045±91
19	2	1	0.25	5	7,030±79
20	2	1	0.25	5	7,025±89
21	2	1	0.25	5	7,036±97

[a]Results are the mean of three determinations.

Table 4. Analysis of variance (ANOVA) for the experimental results of the central-composite design (Quadratic Model).

Model	Coefficient estimate	Sum of Squares	Standard Error	F Value	Prob>F
Model	7,219.52	1.241E+7	21.07	365.61	<0.0001
A	-43.37	2.313E+6	20.70	954.46	<0.0001
B	-639.50	5.941E+5	20.70	245.09	<0.0001
C	-324.06	12,094.65	13.33	4.99	0.0669
D	-29.76	2.218E+5	20.70	91.50	<0.0001
A^2	198.00	5.328E+6	12.74	2,198.08	<0.0001
B^2	-597.08	7.755E+5	12.74	319.94	<0.0001
D^2	-227.79	1.533E+6	12.74	632.35	<0.0001
C^2	-320.25	1.631E+6	12.74	672.76	<0.0001
AB	-330.33	98,178.70	27.04	40.51	0.0007
AC	172.13	2.058E+5	17.40	84.89	<0.0001
AD	160.37	66,051.84	27.04	27.25	0.0020
BC	-141.18	5.228E+5	17.40	215.68	<0.0001
BD	-255.63	7.138E+5	27.04	294.50	<0.0001
CD	-464.12	15,051.13	17.40	6.21	0.0470

Optimization by RSM

Based on the Plackett–Burman design, glycerol (A), ammonium sulfate (B), $FeCl_3$ (C), and inoculum size (D) were selected for further optimization by RSM. To examine the combined effect of these independent variables on CyA production, 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=3) of 16 plus 5 center points leading to a total of 21 experiments were performed. A CCRD matrix of independent variables in the form of actual values, along with responses of each experimental trial, is given in Table 3. The results were analyzed by Design Expert version 6.0.10 and are cited in Table 4. The ANOVA of the quadratic regression model indicated the model to be significant ($P<0.05$).

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. P-values, along with the coefficient estimate, are given in Table 4. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Values of P less than 0.05 indicate model terms to be significant. The coefficient estimates and the corresponding P values suggested that among the test variables used in the study, A (glycerol), B (ammonium sulfate), D (inoculum size), A^2 (glycerol^2), B^2 (ammonium sulfate^2), C^2 ($FeCl_3$ ^{2}), and D^2 (inoculum size^2) were significant model terms. Interactions between glycerol and ammonium sulfate; glycerol and $FeCl_3$; glycerol and inoculum size; ammonium sulfate and $FeCl_3$; ammonium sulfate and inoculum size; and $FeCl_3$ and inoculum size were significant. Other interactions were found to be insignificant.

The corresponding second-order response model for Equation (3) that was found after analysis for the regression was CyA, mg/kg=-1,724.93+(1,356.59×glycerol)+(4,084.45×ammo. sulfate)+(18,704.31×$FeCl_3$)+(1,584.33×inoculum size)-(597.08×glycerol^2)-(911.17×ammo. sulphate^2)-(32,025.07×$FeCl_3^2$)-(82.58×inoculum size^2)+(344.25×glycerol×ammo. sulfate)+(1,603.75×glycerol×$FeCl_3$)-(70.59×glycerol×inoculum size)-(5,112.50×ammo. sulfate×$FeCl_3$)-(464.12×ammo. sulfate×inoculum size)-(16.87×$FeCl_3$×inoculum size).

The fit of the model was also expressed by the coefficient of regression R^2, which was found to be 0.99, indicating that 99.0% of the variability in the response (CyA yield) could be explained by the model.

Other parameters of ANOVA for response surface quadratic model were also studied. The "Pred R-Squared" of 0.86 is in reasonable agreement with the "Adj R-Squared" of 0.99. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 66.43 indicates an adequate signal.

Table 5. Predicted and experimental yields of CyA by various media combinations.

Sr. No.	Glycerol (% w/w)	Ammo. Sulfate (% w/w)	$FeCl_3$ (% w/w)	Inoculum size (ml/5 g)	CyA (mg/kg)	
					Predicted	Experimental[a]
1	1.53	0.95	0.18	6.40	7,157.42	7,106±59
2	1.56	0.63	0.29	4.55	7,088.14	7,048±35
3	1.55	0.75	0.27	4.75	7,002.53	7,006±48

[a]Results are the mean of three determinations.

The special features of the RSM tool, "contour plot generation" and "point prediction," were also studied to find the optimum value of the combination of the four media constituents for the maximum production of CyA. These predicted values were experimentally verified. Table 5 documents the predicted and experimental yields of CyA by various media combinations. It was observed that medium containing (% w/w) glycerol, 1.53; ammonium sulfate, 0.95; $FeCl_3$, 0.18; and inoculum size 6.4 ml/5 g yielded a maximum of 7,106 mg/kg of CyA.

Survase *et al.* [26] reported that supplementation of the solid substrate with 1% w/w of glycerol and ammonium sulfate produced maximum CyA. Isaac *et al.* [9] reported a higher spore density to give higher production of CyA in submerged fermentation using *T. inflatum* UAMH 2472. Ramana Murthy *et al.* [19] and Sallam *et al.* [21] used 72-h-old seed culture for maximum production of CyA. Ramana Murthy *et al.* [19] reported 6 ml of inoculum per 10 g of substrate to provide maximum CyA production.

Accordingly, three-dimensional graphs were generated for the pairwise combination of the four factors, while keeping the other two at their center point levels. Graphs for three significant interactions are given here to highlight the roles played by these factors (Fig. 1). From the central point of the contour plot, the optimal process parameters were identified.

Effects of Amino Acids

The biosynthesis of CyA and analogs is known to involve sequential activation of all amino acids, their *N*-methylation, and eventual peptide formation by a multifunctional enzyme, CyA synthatase. Fig. 2 shows the effect of different amino acids supplementation on CyA production. Of all the amino acids tested, L-valine produced the maximum (7,615 mg/kg) enhancing effect on CyA production, followed by L-leucine (7,512 mg/kg) and α-amino butyric acid (7,465 mg/kg). DL-Valine, on the other hand, did not increase the product titer as that of L-valine. We found that supplementing fermentation media with L-methionine reduced CyA production.

When added together, L-valine and L-leucine increased CyA production further to give 7,922 mg/kg (Fig. 3).

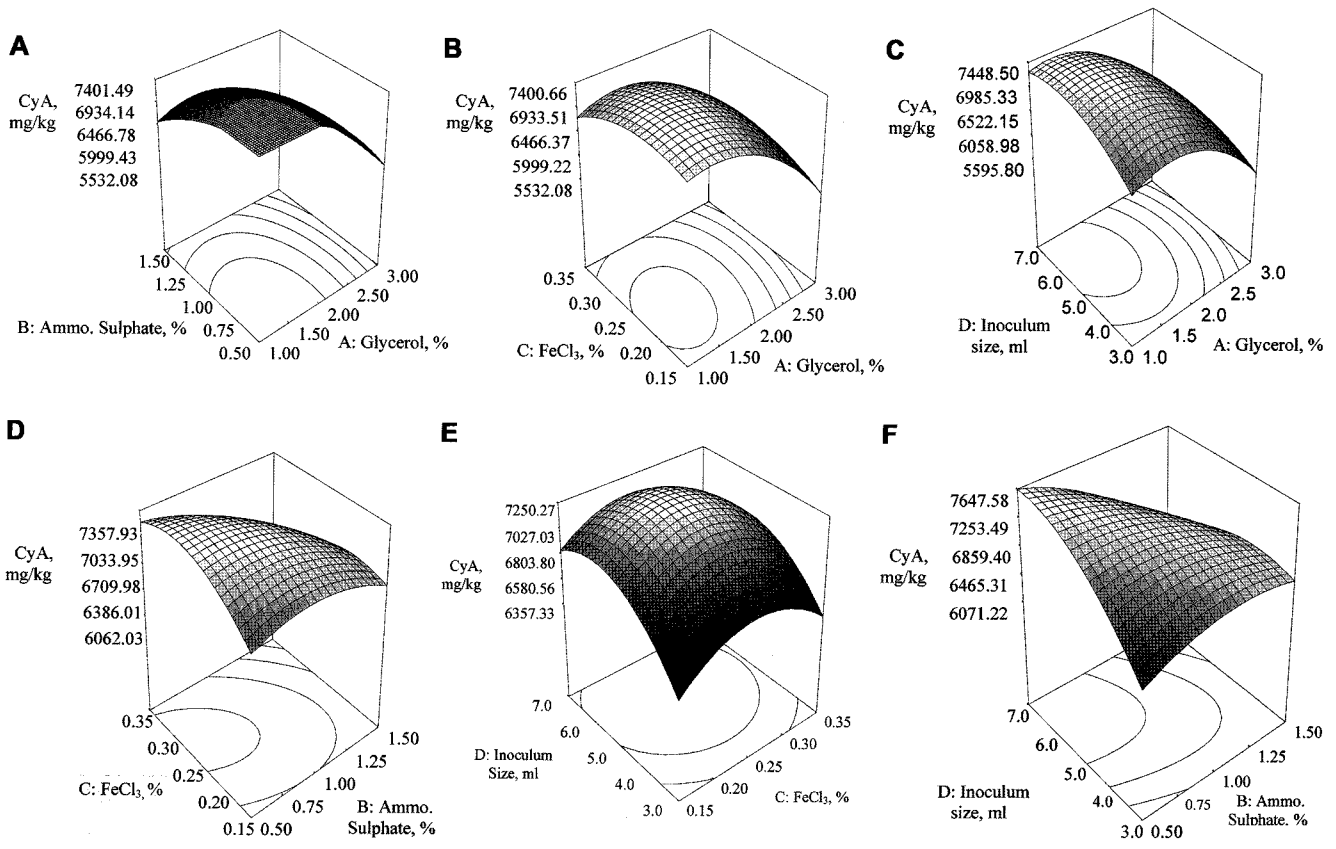

Fig. 1. 3D-surface plot for CyA production.
A. Effects of glycerol and $(NH_4)_2SO_4$ when other variables are held at zero level. **B**. Effects of glycerol and $FeCl_3$ when other variables are held at zero level. **C**. Effects of glycerol and inoculum size when other variables are held at zero level. **D**. Effects of $(NH_4)_2SO_4$ and $FeCl_3$ when other variables are held at zero level. **E**. Effects of $(NH_4)_2SO_4$ and inoculum size when other variables are held at zero level. **F**. Effects of $FeCl_3$ and inoculum size when other variables are held at zero level.

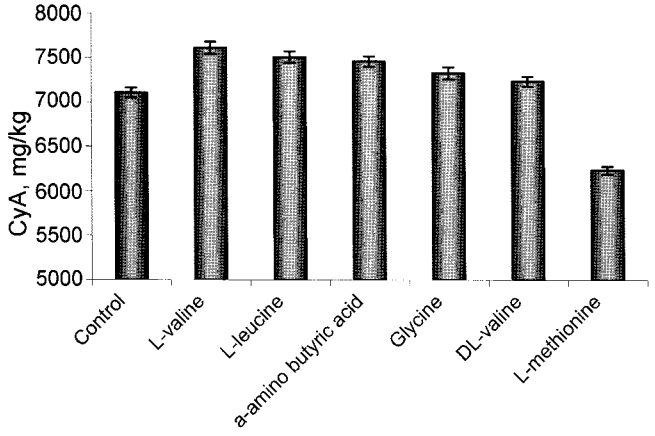

Fig 2. Effects of constituent amino acids on production of CyA using *T. inflatum* MTCC 557. All amino acids were added at 4% w/w.

Similar results were encountered by Lee and Agathos [13], Balakrishnan and Pandey [3], and Nisha *et al.* [16] in CyA biosynthesis. These two amino acids seem to act independently of each other and their modes of action are different. Bussari *et al.* [5] observed that inclusion of L-valine in addition to L-lysine, cystine, and DL-methionine stimulated cephamycin C production by *S. clavuligerus.* When L-methionine was added to medium supplemented with L-valine, the stimulatory effect of L-valine was found to be completely reversed. Zocher *et al.* [28] reported that methionine could not take part in the biosynthesis, as methylated amino acids interfere with the biosynthesis of cyclosporin *in vivo.*

The optimal amount of L-valine was also investigated. It was observed that the precursor role of L-valine was consistent after reaching a saturation level at 4% w/w initial L-valine concentration. A further increase in L-valine concentration did not result in an enhanced. Similar results were obtained by Balakrishnan and Pandey [3], where

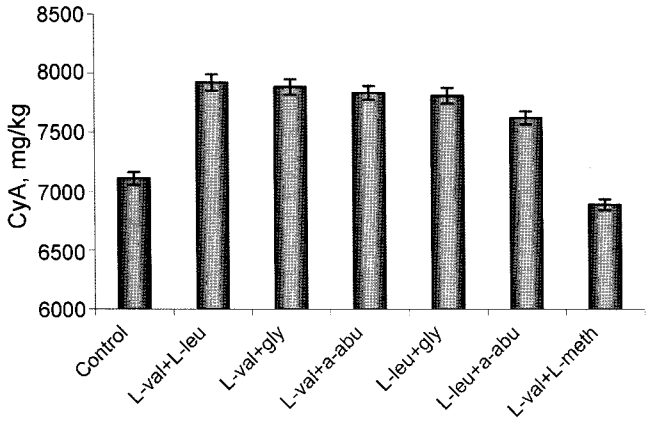

Fig 3. Effects of combination of amino acids on production of CyA using *T. inflatum* MTCC 557.

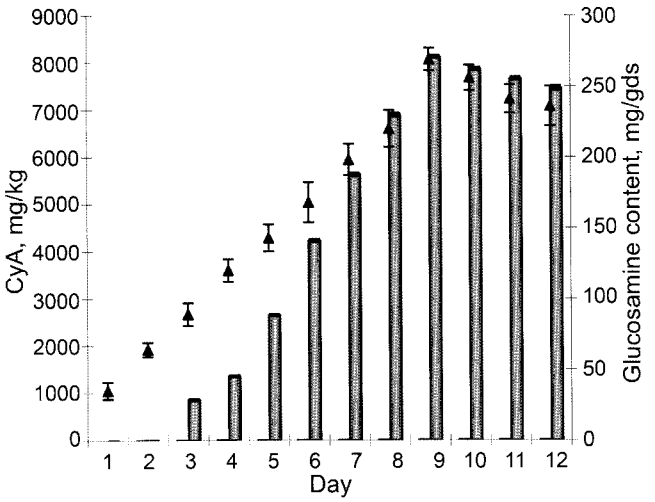

Fig 4. Production profile and growth curve of CyA production using *T. inflatum* MTCC 557.

addition of L-valine (4 g/l) was found to be optimum. The findings were also in accordance with the findings obtained with L-leucine in bacitracin fermentation by *B. licheniformis* [7].

The optimum time for addition of L-valine and L-leucine in combination for maximum product titer was found to be 20 h (data not shown). Addition of L-valine and L-leucine in combination after 20 h of fermentation resulted in maximum production of 8,166 mg/kg. Lee and Agathos [13] and Balakrishnan and Pandey [3] showed similar results, showing the significance of addition of amino acids after 18 h and 20 h of the fermentation.

In short, the constituent amino acids of the CyA molecule, such as L-valine, L-leucine, and α-amino butyric acid, may affect the production of the drug by assuming one or more roles such as precursor, inducer, and/or developmental regulator. Nisha *et al.* [16] reported that L-valine, L-leucine, and a-amino butyric acid showed an increase of 26%, 17%, and 16 %, respectively, in production of CyA when added in SSF.

The growth curve and production profile of the CyA were studied with respect to time (Fig. 4) using the optimized media. The production of the CyA was observed from the third day of fermentation (856±89 mg/kg) and reached a maximum on day 9 (8,123±32 mg/kg). It then decreased up to day 12 (7,460±49 mg/kg). This could probably be because the organism might have reached the death phase.

Ramana Murthy [19] reported that maximum production of CyA (5,043 mg/kg bran) was obtained with supplementation of a combination of millet flour, sorghum flour, $FeCl_3$, $ZnSO_4 \cdot 7H_2O$, and $CoCl_2 \cdot 6H_2O$ using *T. inflatum* DRCC 106. Sekar *et al.* [23] optimized fermentation conditions and reported maximum production of 1,400 mg/kg bran. Sekar and Balaraman [22] studied the effect of fermentation parameters such as thickness of solid substrate bed, type of

inoculum, size of inoculum, and relative humidity and reported a maximum production of 1,980 mg/kg bran. Survase *et al.* [26] reported a novel medium for the production of CyA with maximum production of 6,480 mg/kg substrate.

The present study showed that statistical methods gave the precise optimized media for CyA production. Work on strain improvements to further improve the yields of CyA is in progress.

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