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Statistical Optimization of Cyclosporin A Production on a Semi-Synthetic Medium Using *Tolypocladium inflatum* MTCC 557

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Abstract: Statistical technique was used to optimize the fermentation conditions for maximum production of cyclosporin A (CyA) using *T. inflatum* MTCC 557. Various carbon and nitrogen sources were screened for the maximum production of CyA. A response surface methodology (RSM) involving 4 factors and 5 levels was adopted to acquire the best medium for the production of CyA. Thus polynomial model was created to correlate the relationship between four variables and CyA yield. The optimal combination of media components included (in g/l) glucose 58.46 casein peptone 8.66, KH_2PO_4 4.48 and KCl 3.23 that gave maximum production of 134.5 mg/l CyA which was 2.7-fold more than the basal media. Subsequently, the effect of inoculum age, inoculum size and amino acids on the production of CyA was studied.

Key words: Submerged fermentation · Cyclosporin A · Response surface method · *Tolypocladium inflatum*

INTRODUCTION

Cyclosporin A (CyA), a cyclic undecapeptide, is one of the most commonly prescribed immunosuppressive drugs for the treatment of patients with organ transplantation, autoimmune diseases, including AIDS, owing to its superior T-cell specificity and low levels of myelotoxicity [1,2]. The Sandoz Pharmaceutical Company initially developed it as an antifungal antibiotic in the early 1970s [3]. It is produced non-ribosomally from a multifunctional cyclosporin synthetase enzyme complex by a filamentous fungus *Tolypocladium inflatum* [4,5].

The production of cyclosporin in submerged fermentation has been reported to vary with respect to the production strain, fermentation conditions and the nutrient composition of the culture medium. Many attempts such as strain improvement [6, 7], immobilization [8], solid-state fermentation [9] and enzymatic synthesis [4] have also been made to optimize CyA production.

All naturally occurring cyclosporins are neutral cyclic peptides consisting of 11 amino acids some of which are unusual such as a C9 amino acid sarcosine, one or two D-amino acids and several N-methylated amino acids. Kobel and Traber [10] and Lee and Agathos [11] reported the directed synthesis of CyA and several analogues in the fermentation where the composition and titer of each analogue produced were strongly determined by the externally supplemented amino acids.

The effect of different media components as carbon and nitrogen sources [6, 12] and environmental factors such as pH and inoculum density [13] on CyA production has been studied. But, to the best of our knowledge, there is scarcity of literature on optimizing the medium constituents for the production of CyA in a submerged culture by using RSM. RSM is used to evaluate the relative significance of variables in the presence of complex interactions with limited number of experiments. RSM has been successfully employed for optimization of medium constituents for the production of metabolites [14,15].

Accordingly, the objectives of the present study were to evaluate different media components by using one factor-at-a-time by *T. inflatum* MTCC 557 and optimizing the concentration of selected ingredients to get the maximum production of CyA and creating a polynomial model equation to correlate the relationship between the variables using RSM. Finally, the model was assessed experimentally to verify its accuracy. Effect of different constituent amino acids was also evaluated further to increase the yields.

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MATERIALS AND METHODS

Materials: Glucose, sucrose, maltose, glycerol, fructose, galactose, starch, sorbitol mannitol, xylose, yeast extract, casein peptone, beef extract, bactopeptone, soybean meal, malt extract, ammonium sulphate, sodium nitrate, agar and urea were procured from Himedia Ltd, Mumbai. Salts like magnesium sulphate, sodium chloride, zinc chloride, manganese chloride, cobaltous chloride, ferric chloride, zinc sulphate and solvents like acetonitrile, n-butyl acetate, sodium hydroxide, concentrated hydrochloric acid and sulphuric acid were purchased from S.D. Fine Chem Ltd. Mumbai. 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.

Microorganisms: Strains of *T. inflatum* MTCC 989, *T. inflatum* MTCC 557 (indicated as *Beauveria nivea* in the MTCC catalog), *T. inflatum* NCIM 1283, were procured from MTCC, Chandigarh and NCIM, Pune. *T. inflatum* NRRL 18950 was a gift sample from ARS Culture Collection, United States. The cultures were maintained on agar slants containing malt extract 2 % and yeast extract 0.4 % (MYA), pH 5.4 at 4°C after growing it for 12 days at 24°C. The strains were screened for the maximum production of CyA.

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 milliliter 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 hours at 25±2°C.

Fermentation: In the present study, semi-synthetic medium (SSM) developed by Agathos *et al.* [16] was used as basal medium for optimization of CyA production by *T. inflatum* MTCC 557. The composition of medium used was (in g/l) glucose 50, peptone 10, K_2HPO_4 5 and KCl 2.5. pH was adjusted to 5.7 ± 0.2 and 5 ml seed culture was used to inoculate 50 ml of sterile production medium. The fermentation was carried out at 25±2°C for 14 days at 180 rpm.

The Screening Stage: One Factor at-a-time: Four different *T. inflatum* strains were screened for the maximum production of CyA using the basal medium. To evaluate the effect of different carbon sources on the production of CyA, glucose in the basal medium was replaced with different carbon sources, *viz*., glycerol, sucrose, maltose, fructose, galactose, soluble starch, sorbitol, mannitol and xylitol at 50 g/l. Different nitrogen sources at 10 g/l were tested for their effect on CyA production. They included bacto-peptone, casein peptone, yeast extract, soybean meal, corn steep liquor and malt extract. To study the effect of pH on CyA production, fermentation runs were carried out at initial pH varying from 2.7 to 7.7.

Superior Optimization Stage: RSM: 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).

The medium components (independent variables) selected for the optimization were glucose, casein peptone, KH_2PO_4 and KCl. The experimental design showing the coded as well as actual values of independent variables is shown in Table 1. Regression analysis was performed on the data obtained from the design experiments. The second order polynomial coefficients were calculated to estimate the responses of the dependent variable. Response surface plots were also obtained using Design Expert Version 6.0.10.

Effect of Slant Age, Inoculum Age and Inoculum Size: It was observed that slants which were incubated for 10 days or more were well sporulated; using such slants for seed inoculation resulted in higher CyA production, although the difference in yields was found to be statistically insignificant. Fermentation media was inoculated with seed culture of different age (24-96 h) and different size (5-20 % v/v).

Effect of Amino Acids: The effect of different amino acid members of CyA molecule on drug production was evaluated by supplementing the fermentation media. The amino acids tested were L-valine, L-leucine, DL-valine, L-methionine, L-aminobutyric acid and glycine, screened individually (4 g/l) as well as in combination with others. The time of addition of amino acids (0 to144 h) was also optimized to further increase the yield.

Table 1: The CCRD matrix of independent variables in coded as well as actual form with their corresponding response

Std	A: Glucose	B: Casein Peptone	C:KH_2PO_4	D: KCl	CyA[a], mg/l
1	+1 (70)[b]	+1 (10)[b]	+1 (6)[b]	-1 (2)[b]	59±7
2	+1 (70)	+1 (10)	-1 (3)	-1 (2)	66±6
3	+1 (70)	-1 (6)	+1 (6)	+1 (4)	92±7
4	-1 (40)	+1 (10)	-1 (3)	+1 (4)	69±9
5	+1 (70)	-1 (6)	-1 (3)	+1 (4)	70±7
6	-1 (40)	-1 (6)	+1 (6)	-1 (2)	60±4
7	-1 (40)	+1 (10)	+1 (6)	+1 (4)	50±6
8	-1 (40)	-1 (6)	-1 (3)	-1 (2)	43±7
9	-1.68 (29.77)	0 (8)	0 (4.5)	0 (3)	57±6
10	1.68 (80.22)	0 (8)	0 (4.5)	0 (3)	89±5
11	0 (55)	-1.68 (4.63)	0 (4.5)	0 (3)	52±6
12	0 (55)	1.68 (11.36)	0 (4.5)	0 (3)	95±9
13	0 (55)	0 (8)	-1.68 (1.97)	0 (3)	89±8
14	0 (55)	0 (8)	1.68 (7.02)	0 (3)	96±8
15	0 (55)	0 (8)	0 (4.5)	-1.68 (1.31)	102±9
16	0 (55)	0 (8)	0 (4.5)	1.68 (4.68)	95±7
17	0 (55)	0 (8)	0 (4.5)	0 (3)	130±8
18	0 (55)	0 (8)	0 (4.5)	0 (3)	132±5
19	0 (55)	0 (8)	0 (4.5)	0 (3)	134±9
20	0 (55)	0 (8)	0 (4.5)	0 (3)	133±8
21	0 (55)	0 (8)	0 (4.5)	0 (3)	132±7

[a]Results are mean ± SD of three determinations

[b]values in the parenthesis are real values of selected variables in g/l

Analytical Determinations: CyA extraction and estimation: The CyA extraction from the culture broth was carried out according to the method of Agathos *et al.* [16]. A 10 ml of culture broth was extracted with equal volume of n-butyl acetate. Before extracting the sample, a concentrated solution of NaOH was added to reach the concentration of 1N and heated at 60°C for 30 min. The mixed sample was kept on rotary shaker (180 rpm) for 24h. After centrifuging, the extract was filtered using Whatman filter paper (No.1) and then through Pall 0.2 ìm membrane filter (Ultipor® N_{66}® Nylon 6, 6 membranes) to give clear extract. One milliliter of the extract was evaporated under vacuum to dryness. The dried extract was dissolved in equal volume (1 ml) of HPLC grade acetonitrile. Twenty microliters of sample was analyzed for CyA content using HPLC (Jasko system) fitted with a reverse phase column Waters Sperisorb® ODS (C_{18} octadecyl silane, 250 X 4.6 mm ID) by the method described by Survase *et al.* [9]. 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.

Estimation of Biomass: 10 ml of broth was centrifuged at 10,000 rpm for 20 min washed twice with distilled water, centrifuged and taken on a preweighed Whatman filter paper. This was dried to a constant weight at 80°C.

RESULTS AND DISCUSSION

Screening Stage- One Factor At-a-time: Several strains were screened for the maximum production of CyA by using SSM reported by Agathos *et al.* [16] with 10 % inoculum of 72 h old seed at 25 ± 2°C for 14 days. *T. inflatum* MTCC 557 gave maximum production of 47.42 ± 2.31 mg/l, followed by 36.11 ± 1.25 mg/l and 34.80 ± 1.02 mg/l by *T. inflatum* NRRL 18950 and *T. inflatum* NCIM 1283, respectively and hence *T. inflatum* MTCC 557 was used for the further optimization studies. *T. inflatum* MTCC 989 gave lower titers of CyA.

Fig. 1 shows the effect of carbon sources on CyA and biomass production by *T. inflatum* MTCC 557. Of all the carbon sources, glucose supported the maximum production of 49.8 ± 1.98 mg/l followed by sucrose and maltose (45.52 and 44.56 mg/l, respectively). It was also observed that glycerol, sorbitol, starch and galactose supported only biomass growth and were not able to create the physiological condition to produce CyA. Glycerol produced maximum biomass (16.58 g/l measured as DCW) as compared to other carbon sources. Dreyfuss *et al.* [3] used glucose (40 g/l) as carbon source and reported to produce 180 mg/l of CyA using industrial strain of *T. inflatum*. Glucose has also been reported to be a better carbon source for CyA production

Table 2: 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	132.38	18370.31	0.61	639.21	< 0.0001
A	9.23	482.05	0.60	234.82	< 0.0001
B	12.71	914.20	0.60	445.35	< 0.0001
C	1.67	38.47	0.38	18.74	0.0669
D	-2.08	24.50	0.60	11.93	< 0.0001
A^2	-20.96	6567.02	0.37	3199.10	< 0.0001
B^2	-20.73	6424.92	0.37	3129.88	< 0.0001
D^2	-14.23	3028.82	0.37	1475.48	< 0.0001
C^2	-12.11	2193.44	0.37	1068.52	< 0.0001
AB	-8.68	250.08	0.79	121.82	0.0007
AC	1.96	31.00	0.51	15.10	< 0.0001
AD	15.42	788.31	0.79	384.02	0.0020
BC	-8.09	523.74	0.51	255.14	< 0.0001
BD	1.15	4.40	0.79	2.14	< 0.0001
CD	-0.90	6.60	0.51	3.21	0.0470

by Balakrishnan and Pandey [17] and Sallam *et al.* [18]. Abdel-fattah *et al.* [12] used three carbon sources as glucose (10g/l), sucrose (20g/l) and starch (20g/l) in combination to give maximum CyA (110mg/l) production using *T. inflatum* DSMZ 915. Agathos *et al.* [16] reported use of sorbose (30g/l) to produce maximum CyA (105 mg/l) using *T. inflatum* ATCC 34921. Margaritis and Chahal [19] developed fructose based medium for the production of CyA using *Beauveria nivea.* They used fructose to minimize the catabolite repression and oxygen limitation in the pallets formed during the production stage to get maximum CyA yields.

Among the nitrogen sources studied, casein peptone supported the maximum CyA production (83.56 mg/l) followed by bactopeptone (45.56 mg/l) (Fig 2). Casein peptone supported the maximum biomass production of 16.35 g/l measured as DCW. The production of CyA was least supported by yeast extract, malt extract and corn steep liquor. Abdel-fattah *et al.* [12] reported the use of ammonium sulphate as nitrogen source supporting maximum production. They also reported yeast extract to have positive effect on CyA production. Agathos *et al.* [16] screened different organic nitrogen sources as bactopeptone, soytone and corn steep liquor at various concentrations and reported bactopeptone at 10 g/l to give the maximum production of CyA. Balaraman and Nisha [20] used three nitrogen sources as casein acid hydrolysate (30g/l), malt extract (20g/l) and peptone (10g/l) in static fermentation to produce maximum CyA after 21 days fermentation using *Tolypocladium* sp. (VCRC F21 NRRL No.18950).

An initial pH of 5.7 supported maximum CyA (47 mg/l) and biomass (11.2 g/l as DCW) production. Most studies on fermentative production of CyA were carried out at pH 5.7.

Optimization Stage- RSM: The combined effect of four independent variables A: glucose (g/l); B: casein peptone (g/l); C: KH_2PO_4(g/l); D: KCl (g/l) for production of CyA was examined using RSM. The CCRD gave quadratic model for the given set of experimental results. Eq. (1) represents the mathematical model relating the production of CyA 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 6.0.10. The experimental values of yields of CyA 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 indicated the model to be significant (Table 2). The Model F-value of 639.21 implies the model to be significant and is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob>F) was 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 less than 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, A, B, C, D, A2, B2, C2, D^2, AB, AC, AD and BC are significant model terms whereas the mutual interaction BD and CD was significant. The second order response model found after analysis for the regression was:

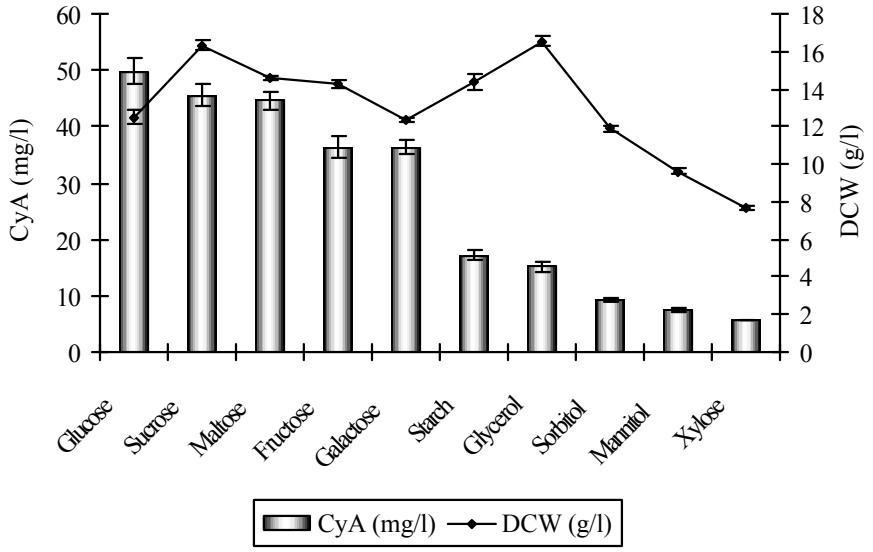

Fig. 1: Effect of various carbon sources on CyA production using *T. inflatum* MTCC 557

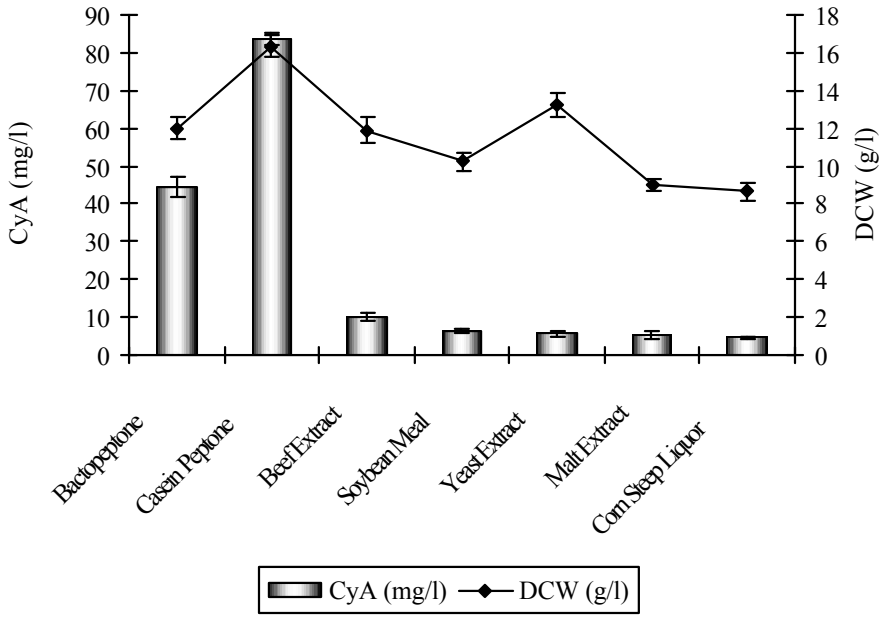

Fig. 2: Effect of various nitrogen sources on CyA production using *T. inflatum* MTCC 557

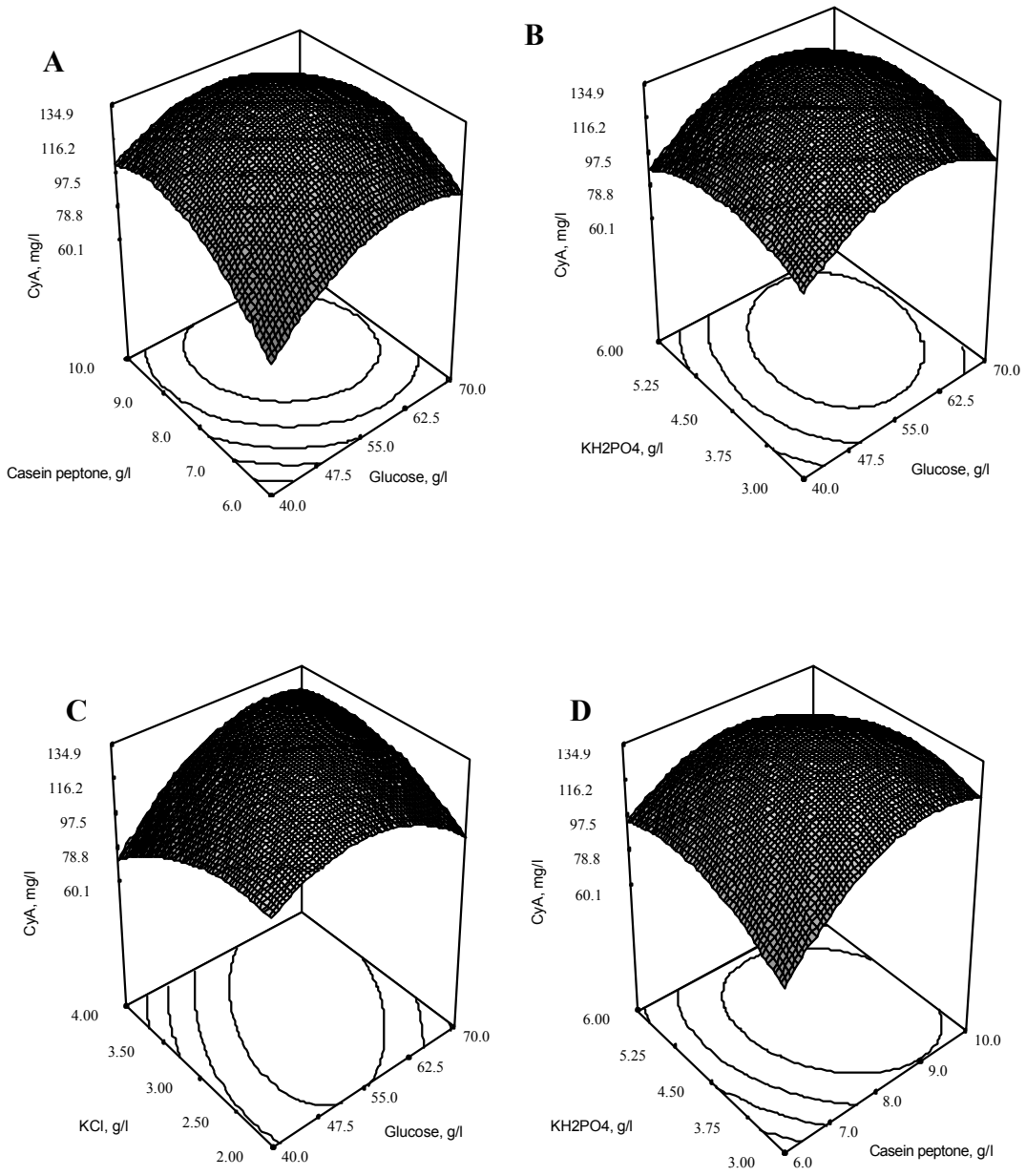

Fig. 3: 3D-surface plot for CyA production; A Effect of glucose and casein peptone when other variables are held at zero level; B Effect of glucose and KH_2PO_4 when other variables are held at zero level; C Effect of glucose and KCl when other variables are held at zero level; D Effect of casein peptone and KH_2PO_4 when other variables are held at zero level

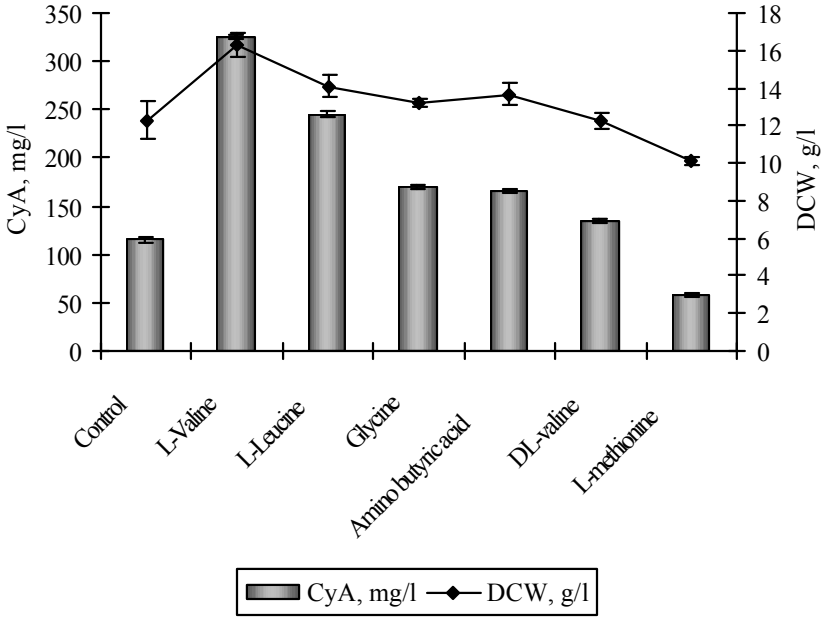

Fig. 4: Effect of different amino acids on production of CyA using *T. inflatum* MTCC 557

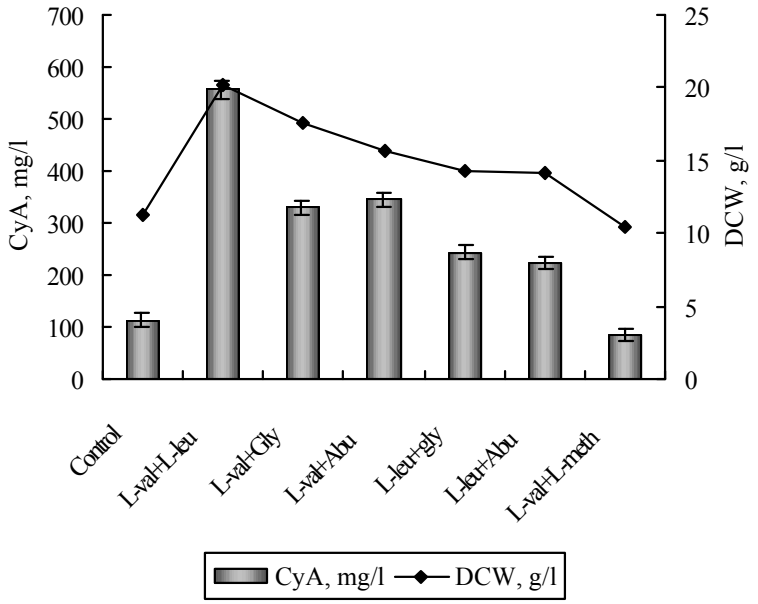

Fig. 5: Effect of combination of amino acids on production of CyA using *T. inflatum* MTCC 557

CyA (mg/l) =
-829.40 + 9.70 (glucose) + 115.63 (casein peptone) + 76.64 (KH_2PO_4) + 12.17 (KCl) - 0.09 (glucose$^{2)}$ - 5.18 (casein peptone$^{2)}$ - 6.32 ($KH_2PO_4^{2)}$ - 12.11 $(KCl)^2$ - 0.28 (glucose × casein peptone) + 0.088 (glucose × KH_2PO_4) + 1.02 (glucose × KCl) - 2.69 (casein peptone × KH_2PO_4) + 0.57 (casein peptone × KCl) - 0.61 (KH_2PO_4 × KCl)(1)

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 % of the confidence level of the model to predict the response (CyA yield). The "Pred R-Squared" of 0.98 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 73.27 indicates an adequate signal.

Accordingly, three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their center point levels. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified.

The special features of the RSM tool, "contour plot generation" and "point prediction" were also studied to find optimum value of the combination of the four media constitutes for the maximum production of CyA. The optimal combination of media components included (in g/l) glucose 58.46, casein peptone 8.66, KH_2PO_4 4.48 and KCl 3.23. The optimal composition was verified experimentally and compared with the data calculated from the model. The experimentally obtained CyA yield was 134.5 mg/l, whereas the predicted value from the polynomial model was 136.5 mg/l, thereby confirming the high accuracy of the model at more than 99 % under the investigated conditions.

Effect of Inoculum Age and Density: It was observed that 10 % v/v of 72 h old seed culture resulted in maximum CyA production of 137.4 mg/l (data not shown). Increased seed culture age and inoculum size did not increase the yields significantly. Isaac *et al.* [13] reported a higher spore density to give higher production of CyA in submerged fermentation using *T. inflatum* UAMH 2472. Ramana Murthy *et al.* [7] and Sallam *et al.* [18] used 72 h old seed culture for maximum production of CyA. The spore inoculum plays a critical role in the maximization of CyA production [21]. They reported that 3 % of the spore inoculum generated the highest level of CyA productivity in a 15-day *T. niveum* production culture. Less than 3 % spore inoculation in the production culture apparently induced a prolonged lag phase resulting in delayed mycelial growth, which eventually lowered CyA productivity. However, more than 3% of spore inoculation appeared to stimulate germination too profoundly in a fixed culture volume, thereby resulting in the limitation of both oxygen and nutrients.

Effect of Amino Acids: Fig. 4 shows the effect of different amino acids supplementation on CyA production. Of all the amino acids tested, L-valine produced the maximum CyA of 325 mg/l followed by L-leucine and glycine (245 mg/l and 170 mg/l, respectively). 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. Lee and Agathos [11] reported similar effect when sarcosine (n-methyl glycine) was added to medium.

When added together, L-valine and L-leucine increased drug production dramatically (556 mg/l) (Fig 5). These two amino acids seem to act independently and their mode of action is different. This effect was not observed with other amino acid combinations. Similar results were encountered by Lee and Agathos [11]; Balakrishnan and Pandey [17] and Nisha *et al.* [22] in CyA biosynthesis. When L-methionine was added to medium supplemented with L-valine, the stimulatory effect of L-valine was completely reversed. Zocher *et al.* [23] 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 and time of addition of L-valine was also investigated. It was observed that the precursor role of L-valine was consistent after reaching a saturation level at 6 g/l initial L-valine concentration. Maximum CyA production of 378 mg/l was observed at initial L-valine concentration of 6 g/l. A further increase in concentration did not enhance the yield further. The optimum time for L-valine addition for maximum product titre was found to be 20 h (Data not shown). When added after 20 h, CyA production of 745 mg/l was obtained. This could be due the utilization of amino acids as nitrogen source in early phase of fermentation.

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