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A Statistical Approach Using L_{25} Orthogonal Array Method to Study Fermentative Production of Clavulanic Acid by *Streptomyces clavuligerus* MTCC 1142

Parag S. Saudagar and Rekha S. Singhal*

Food and Fermentation Technology Department, University Institute of Chemical Technology, Matunga, Mumbai 400 019, India, E-mail: rekha@udct.org

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Abstract

Clavulanic acid is a naturally occurring antibiotic produced by *Streptomyces clavuligerus*. The present work reports on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142 using one-factor-at-a-time and L_{25} orthogonal array. The one-factor-at-a-time method was adopted to investigate the effect of media components (i.e., carbon source, nitrogen source and inoculum concentration) and environmental factors such as pH for clavulanic acid production. Production of clavulanic acid by *Streptomyces clavuligerus* was investigated using seven different carbon sources (*viz.* glucose, sucrose, modified starch, rice-bran oil, soybean oil, palm oil, and glycerol) and six different nitrogen sources (*viz.* peptone, yeast extract, ammonium chloride, ammonium carbonate, sodium nitrate and potassium nitrate). A maximum yield of 140 µg/mL clavulanic acid was obtained in the medium containing soybean oil as a carbon source and yeast extract as nitrogen source. Subsequently, the concentration of soybean flour, soybean oil, dextrin, yeast extract and K_2HPO_4 were optimized using L_{25} orthogonal array method. The final optimized medium produced 500 µg/mL clavulanic acid at the end of 96 h as compared to 140 µg/mL before optimization. Synthesis of precursor molecules as a metabolic driving force is of considerable importance in antibiotic synthesis. Attempts to increase the clavulanic acid synthesis by manipulating the anaplerotic flux on C_3 and C_5 precursors by supplementing the medium with arginine, ornithine, proline, valine, leucine, isoleucine, pyru-

*Author to whom all correspondence and reprint requests should be addressed.

vic acid and α-ketoglutarate were successful. Supplementing the optimized medium with 0.1 *M* arginine and 0.1 *M* leucine increased the yield of clavulanic acid further to 1100 μg/mL and 1384 μg/mL respectively.

Index Entries: Clavulanic acid; β-lactamases; fermentation; L_{25} orthogonal array; anaplerotic flux; amino acid precursors.

Introduction

Widespread use of β-lactam class of antibiotics such as penicillin and cephlosporins to combat bacterial infections has led to the emergence of bacterial strains resistant to these drugs. Resistance to β-lactam is conferred upon bacteria via β-lactamases. To overcome the acquired resistance, antibiotics can be co-administered with clavulanic acid, a potent inhibitor of bacterial β-lactamases *(1)*. Clavulanic acid is a naturally occurring antibiotic produced by *Streptomyces clavuligerus*. Clavulanic acid binds irreversibly with the enzyme to give a stable complex *(2)*, thereby making it inactive. The β-lactamases inhibitory activity of clavulanic acid is related to its 3R, 5R stereochemistry. The combined effective action of the β-lactamase inhibitor and antibacterial agent, make clavulanic acid very important both clinically and economically. Many investigators have attempted to obtain optimal submerged cultures for antibiotic production from different fungi such as erythromycin from *Saccharospora erythraea (3,4)* and cephamycin from *Streptomyces clavuligerus (5)*. To the best of our knowledge, the nutritional requirements and environmental conditions for submerged culture of *S. clavuligerus* for clavulanic acid production using orthogonal matrix is not demonstrated.

Medium optimization by the one-factor-at-time method involves changing one variable (nutrients, pH, temperature, etc.) while fixing the others at a certain arbitrary levels *(6)*. Because most industrial experiments usually involve a significant number of factors, a full factorial design results in a large number of experiments. To reduce the number of experiments to a practical level, only a small set from all the possibilities is selected. Taguchi constructed a special set of general design guidelines for factorial experiments that cover many applications *(6)*. The method uses a special set of arrays called orthogonal arrays, which stipulate the way of conducting the minimal number of experiments, and give the full information of all the factors that affect the performance parameter. Although there are many standard orthogonal arrays available, each of the arrays is meant for a specific number of independent design variables and levels. The additive assumption of the Taguchi design implies that the individual or main effects of the independent variables on performance parameter are separable. Under this assumption, the effect of each factor can be linear, quadratic, or may have higher order, but the model assumes the absence of any cross product effects (interactions) among the individual factors. That means the effect of independent variable 1 on performance parameter does not depend on the different level settings of any other independent vari-

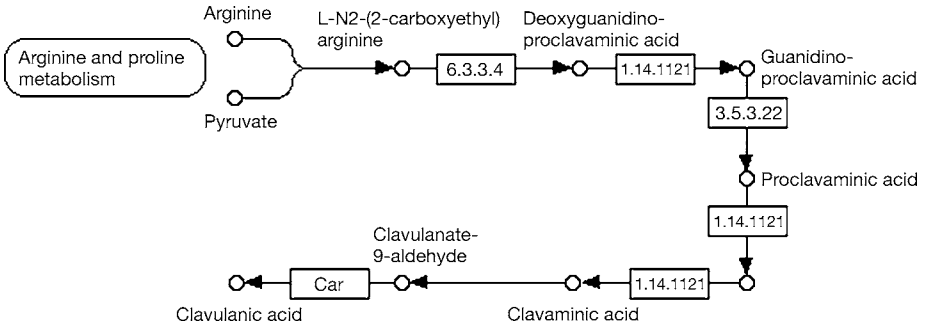

Fig. 1. Biosynthetic pathway for clavulanic acid *(21)*.

ables and *vice versa*. The crux of the orthogonal arrays method lies in choosing the level combinations of the input design variables for each experiment.

The present work deals with optimization of the process for clavulanic acid production by *S. clavuligerus* MTCC 1142 using a statistically based experimental design. In the first step, the one-factor-at-a-time method was used to investigate the effect of media constituents such as carbon source, nitrogen source, and inoculum concentration. Subsequently, in the second step, concentration of the medium components was optimized using an orthogonal matrix method. The biosynthesis of clavulanic acid (Fig. 1**)** has not been fully elucidated, although many intermediates and enzymes involved in the biosynthetic pathway have been identified *(7,8)*. An attempt is made to increase the yield of clavulanic acid by supplementing the fermentation medium with different amino acids.

Materials and Methods

Media Components

Glucose, sucrose, glycerol, yeast extract, ammonium carbonate, ammonium chloride, sodium nitrate, potassium nitrate, potassium dihydrogen phosphate, L-arginine, l-proline, L-pyruvic acid, L-ornithine, and α-ketoglutarate were purchased from M/S Hi-Media Limited, Mumbai, India. Soybean oil, palm oil and rice bran oil were purchased from Nature Fresh Limited, Mumbai.

Maintenance of the Microbial Culture and Fermentative Production of Clavulanic Acid

The MTCC 1142 strain of *S. clavuligerus* was procured from MTCC, Chandigarh, India. Clavulanic acid production was carried out in two stages. In the first stage, cells were grown in the seed culture, and in the second stage, seed culture was inoculated into the fermentation medium for clavulanic acid production. *S. clavuligerus* MTCC 1142 was maintained

on slants of a defined medium containing 0.4% yeast extract, 1% malt extract, 0.4% glucose and 2% agar with a pH adjusted to 7.2 ± 0.2. The slants grown at 25°C for 4 d were used for inoculation into a seed culture medium (1% soybean flour, 2% dextrin, 0.2% soybean oil, pH was adjusted to 7.0 ± 0.2). For the preliminary studies 2% of seed culture grown for 48 h in an incubator shaker at 25°C and at 200 rpm was used for inoculation into the production medium.

Fermentative Production of Clavulanic Acid

The medium designed by Lee et al. *(9)* was modified and used in the present study. Composition of the medium used was 1.1% soybean flour, 1.2% rice bran oil, 0.6% dextrin, 0.25% peptone and 0.25% KH_2PO_4 with a pH adjusted to 7.0 ± 0.2. Effect of different carbon and nitrogen sources was studied using the one-factor-at-time method. All the initial fermentation runs used 2% inoculum at 25°C in an incubator shaker at 200 rpm for 96 h. L_{25} orthogonal array was further used to optimize the concentrations of the best carbon source and nitrogen source.

Optimization of Fermentation Medium Using the One-Factor-at-a-Time Method

Effect of Different Carbon Sources

In the medium selected, rice bran oil was substituted with six different carbon sources viz. glucose, sucrose, modified starch, soybean oil, palm oil, and glycerol at 0.6–4.8% and studied for clavulanic acid production. Fifty milliliters of the autoclaved medium (pH 7.0 ± 0.2) was inoculated with 2% seed culture of *S. clavuligerus* MTCC 1142 and incubated for 96 h in an incubator shaker at 200 rpm and at 25°C.

2.4.2. Effect of Different Nitrogen Sources

Peptone was substituted with five different nitrogen sources (viz. yeast extract, ammonium carbonate, ammonium chloride, sodium nitrate and potassium nitrate in the concentration range of 0.125–1.0%) and studied for clavulanic acid production in the similar way as described previously.

Effect of Inoculum Size

The optimized fermentation medium was supplemented with different concentrations of the seed culture medium (2%, 4%, 6% 8% and 10%) and studied for clavulanic acid production.

Optimization Using the L_{25}-Orthogonal Array

The design for the L_{25}-orthogonal array was developed and analyzed using "MINITAB 13.30" software. Table 1 depicts the L_{25}-orthogonal array design, which was used in the present study. All experiments were performed in at least triplicate.

Table 1
L_{25} Orthogonal Array for Clavulanic Acid[a] Production

	Components					Media constituents g/100mL					
RUN	A	B	C	D	E	A	B	C	D	E	Yield µg/mL
1	1	1	1	1	1	1.1	0.4	0.2	0.250	0.050	103.90 ± 2.0
2	1	2	2	2	2	1.1	0.8	0.4	0.500	0.100	83.28 ± 4.0
3	1	3	3	3	3	1.1	1.2	0.6	1.000	0.200	100.78 ± 2.3
4	1	4	4	4	4	1.1	1.6	0.8	1.500	0.250	135.15 ± 3.1
5	1	5	5	5	5	1.1	2.0	1	2.000	0.300	168.75 ± 2.6
6	2	1	2	3	4	2.2	0.4	0.4	1.000	0.250	131.25 ± 2.0
7	2	2	3	4	5	2.2	0.8	0.6	1.500	0.300	155.46 ± 0.9
8	2	3	4	5	1	2.2	1.2	0.8	2.000	0.050	144.53 ± 3.2
9	2	4	5	1	2	2.2	1.6	1.0	0.250	0.100	137.50 ± 2.5
10	2	5	1	2	3	2.2	2.0	0.2	0.500	0.200	153.90 ± 3.0
11	3	1	3	5	2	4.4	0.4	0.6	2.000	0.100	164.84 ± 2.4
12	3	2	4	1	3	4.4	0.8	0.8	0.250	0.200	217.96 ± 2.0
13	3	3	5	2	4	4.4	1.2	1.0	0.500	0.250	237.50 ± 1.6
14	3	4	1	3	5	4.4	1.6	0.2	1.000	0.300	229.68 ± 1.0
15	3	5	2	4	1	4.4	2.0	0.4	1.500	0.050	187.50 ± 1.8
16	4	1	4	2	5	6.6	0.4	0.8	0.500	0.300	219.53 ± 2.1
17	4	2	5	3	1	6.6	0.8	1.0	1.000	0.050	256.25 ± 2.3
18	4	3	1	4	2	6.6	1.2	0.2	1.500	0.100	303.90 ± 2.4
19	4	4	2	5	3	6.6	1.6	0.4	2.000	0.200	293.75 ± 2.9
20	4	5	3	1	4	6.6	2.0	0.6	0.250	0.250	322.65 ± 1.4
21	5	1	5	4	3	8.8	0.4	1.0	1.500	0.200	504.75 ± 3.4
22	5	2	1	5	4	8.8	0.8	0.2	2.000	0.250	416.40 ± 3.0
23	5	3	2	1	5	8.8	1.2	0.4	0.250	0.300	377.34 ± 1.5
24	5	4	3	2	1	8.8	1.6	0.6	0.500	0.050	337.50 ± 2.6
25	5	5	4	3	2	8.8	2.0	0.8	1.000	0.100	327.34 ± 0.5

[a]Experimental data are mean ± S.D. of triple determination.
A, soybean flour; B, soybean oil; C, dextrin; D, yeast extract; E, K_2HPO_4

Effect of pH on Clavulanic Acid Production

Effect of pH on clavulanic acid production was studied by varying the initial pH of the fermentation medium optimized using L_{25} orthogonal array in the pH range of 5.0–8.5.

Effect of Amino Acids on Clavulanic Acid Production

The biosynthetic pathway (Fig. 1) for the clavulanic acid synthesis is not clearly understood. The effect of variation of anaplerotic flux on the C3 (pyruvate) and C5 (arginine) precursor on clavulanic acid production by *S. clavuligerus* was determined by supplementing the production media with different amino acids (*viz.* l-arginine, L-ornithine, l-proline, L-valine, L-leucine), pyruvic acid and α-ketoglutarate at 0.05–2 *M* and studied for clavulanic acid production as described previously.

Quantification of Clavulanic Acid in the Broth

Clavulanic acid in the fermentation broth was estimated spectrophotometrically as described by Bird et al. *(10)*, and used by Kwon and Kim *(11)* and Mayer and Deckwer *(12)*. The assay method is based on the reaction of clavulanic acid with imidazole that results in formation of 1-(4-aza-8-hydroxy-6-oxo) oct-2-en-l-oyl-imidazole, which shows absorbance at 312 nm. The method is applicable to clavulanate concentrations as low as 2 μg/mL and is specific for intact clavulanate in presence of degradation products. Fermentation broth was clarified by centrifugation at 10,000*g* at 4°C for 25 min. To the supernatant in test tubes, 2 mL of imidazole reagent (8.25 g imidazole in 100 mL water, pH adjusted to 6.8 ± 0.02 with 5 *M* HCl) was added. The tubes were stoppered, and contents were mixed by vortexing on a cyclomixer, and subsequently immersed in water bath at 30°C for 12 min. The tubes were then removed and cooled rapidly to 20°C. The optical density at 312 nm was measured in 1-cm cells using mixture of 1 mL water and 2 mL imidazole reagent as reference. Augmentin (intravenous injection containing 100 mg of clavulanic acid and 500 mg of amoxicillin) was used as the standard.

Growth of S. clavuligerus *MTCC 1142*

Because the culture broth contained insoluble components in soybean flour, it was impossible to measure the optical density or dry cell weight. Therefore cell concentration was correlated to intracellular nucleic acid concentration using diphenylamine (DPA) method as developed by Burton *(13)*. Heating with strong acid converts the deoxyribose of DNA to furfural. DPA reacts with furfural to give a deep blue coloured compound providing a measure of nucleic acids present. Fermentation broth was clarified by centrifugation at 10,000*g* at 4°C for 25 min. The supernatant was separated and to the cell pellet so obtained, 5 mL of citrate-phosphate buffer, pH 6.8 was added. The cell suspension was then sonicated for 20 min using Branson probe sonifier 450 to disrupt the cells. The suspension was centrifuged at 16,000*g* at 4°C for 25 min. To 1 mL supernatant in the test tube, 2 mL DPA reagent (1.5 g DPA in 100 mL glacial acetic acid and 15 mL of concentrated sulfuric acid; 0.5 mL of 2% ethanal was added to the DPA reagent on the day of the test) was added and vortexed to get an uniform solution. The tubes were immersed in a boiling water bath for 10 min and then checked for absorbance at 595 nm.

Results and Discussion

Optimization Using One-Factor-at-a-Time

Figure 2 documents the effect of different carbon sources on clavulanic acid production. The medium was supplemented with lipids and carbohydrates as carbon sources. Carbohydrates are the simplest energy sources for growth and secondary metabolite production. However, rapid catabo-

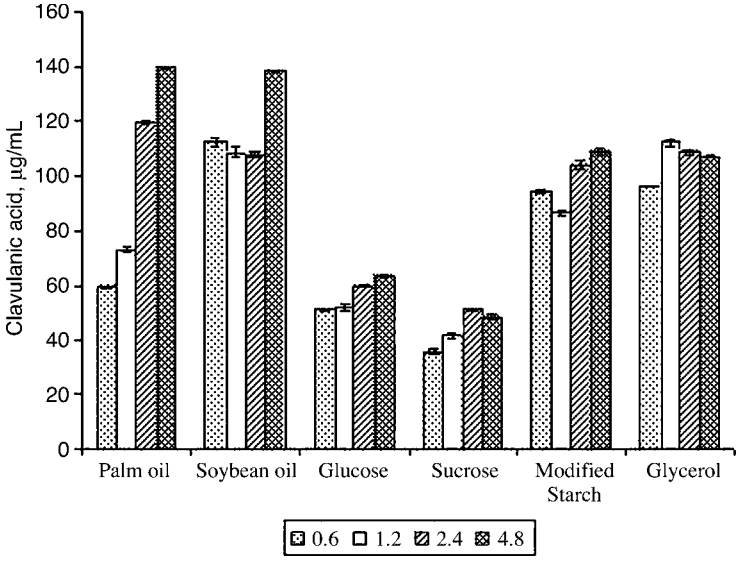

Fig. 2. Effect of different carbon sources on clavulanic acid production (experimental data are mean ± S.D. of triple determination) in *Streptomyces clavuligerus* MTCC 1142.

lism of glucose, sucrose and modified starch resulted in an increase in the biomass (Fig. 3) but a decrease in clavulanic acid production (Fig. 2). These results are in accordance to those reported by Revilla et al. *(14)* for penicillin production in *P. chrysogenum* and Aharonowitz and Demain *(15)* for cephalosporin biosynthesis in *S. clavuligerus*.

Addition of a low-solubility carbon source such as oil is a method of avoiding carbon catabolite regulation. In the present study, lipids were found to act as better carbon sources for production of clavulanic acid by *S. Clavuligerus.* It can be seen from Fig. 2 that 4.8% soybean oil and 4.8% of palm oil supported maximum production of 139 µg/mL of clavulanic acid. 1.2% glycerol and 2.4% rice bran oil gave a yield of 111.5 µg/mL and 120 µg/mL of clavulanic acid, respectively. The addition of oil to a growth medium is also preferred on an energy basis, as typical oil contains approx 2.25 times the energy of glucose on a weight-to-weight basis *(15)*.

Figure 4 documents the effect of different nitrogen sources on clavulanic acid production. Of the six nitrogen sources selected, the inorganic nitrogen sources, such as ammonium chloride, ammonium carbonate, sodium nitrate, and potassium nitrate, did not have a major effect on clavulanic acid. Organic nitrogen sources such as peptone and yeast extract had a major impact on clavulanic acid production. Yeast extract at 0.5% supported maximum yield of 112 µg/mL clavulanic acid.

Clavulanic acid production increased with increasing the inoculum concentration and was optimized to 4%. There was no further increase in the yield with further increasing the inoculum concentration to 6%, 8% and 10%.

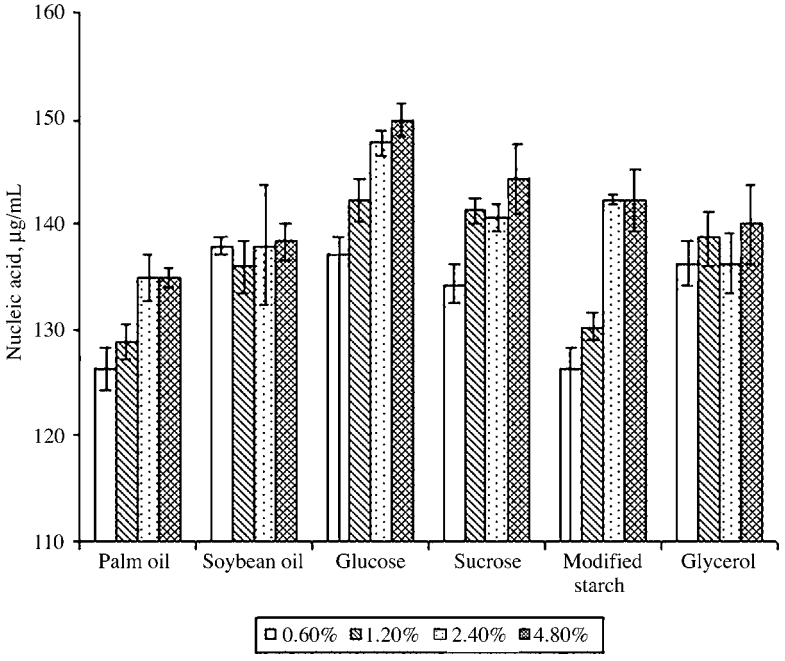

Fig. 3. Effect of different carbon sources on the growth (experimental data are mean ± S.D. of triple determination) of *Streptomyces clavuligerus.*

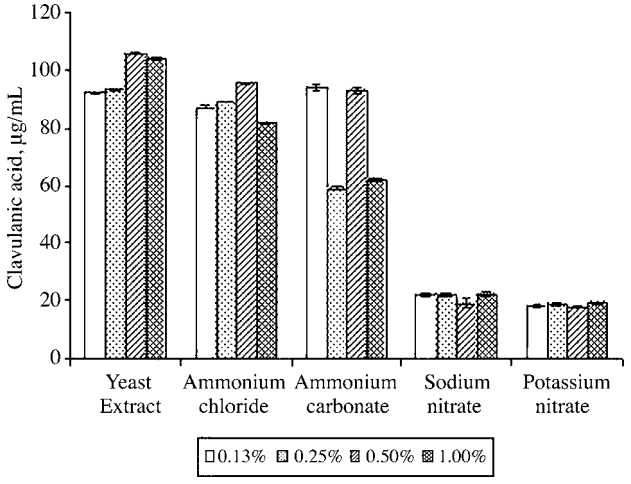

Fig. 4. Effect of different nitrogen sources on clavulanic acid production (experimental data are mean ± S.D. of triple determination) in *Streptomyces clavuligerus* MTCC 1142.

The composition of the basal media used for L_{25} Orthogonal array was 4.8% soybean oil, 4.4% soy flour, 0.5%yeast extract, 0.6% dextrin and 0.25% KH_2PO_4.

Optimization Using L_{25} Orthogonal Array

Once the best carbon and nitrogen sources were selected, the medium was subjected to final optimization using L_{25} orthogonal array. The parameters optimized involved concentrations of soybean flour, soybean oil, dextrin, yeast extract, and K_2HPO_4. The concentrations for the media components were selected based on the basal media composition that was optimized using one factor at a time. Table 2 represents the response table for means (larger is better) and for signal to noise ratio obtained with L_{25} orthogonal array. The last two rows in the tables document the delta values and ranks for the system. Rank and delta values help to assess which factors have the greatest effect on the response characteristic of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristic average for a factor. A higher delta value indicates greater effect of that component. "Rank" orders the factors from the greatest effect (based on the delta values) to the least effect on the response characteristic. The order in which the individual components selected in the present study effect the fermentation process can be ranked as soybean flour > dextrin > yeast extract > K_2HPO_4 > soybean oil, suggesting that soybean flour had a major effect and soybean oil had least effect on clavulanic acid production by *S. clavuligerus*. Figures 5 and 6 represent the main effect plots for the system. Main effects plots show how each factor affects the response characteristic. A main effect is present when different levels of a factor affect the characteristic differently. MINITAB creates the main effects plot by plotting the characteristic average for each factor level. These averages are the same as those displayed in the response (Table 2). A line connects the points for each factor. When the line is horizontal (parallel to the *x* axis), then there is no main effect present. Each level of the factor affects the characteristic in the same way and the characteristic average is the same across all factor levels. When the line is not horizontal (parallel to the *x* axis), then there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the more the line is not parallel to the *x* axis), the greater the magnitude of the main effect. In the present study it can be seen that for each of the five variables at five levels, we find that one level increases the mean compared to the other level. This difference is a main effect, i.e., soybean flour at level 5, soybean oil at level 3, dextrin at level 5, yeast extract at level 4, and K_2HPO_4 at level 3, shows a main effect. These levels also represent the optimal concentrations of the individual components in the medium.

Response tables can also be used to predict the optimal levels of each component used in the study. To obtain the optimized levels or composition of each factor, the intuitive analysis based on statistical calculations is shown in Table 2. Table 3 documents the final medium for clavulanic acid production by *S. clavuligerus.* To confirm these results, experiments were carried out using these nutrient concentrations and it was observed that the

Table 2
Response Table for Means and S/N Ratio

Level	A		B		C		D		E	
	Mean	S/N ratio	Mean	S/N ratio	Mean	S/N ratio	Mean	S/N ratio	Mean	S/N ratio
1	118.375	41.194	227.656	45.633	241.562	46.669	231.875	46.315	205.937	45.546
2	144.531	43.181	225.875	45.915	214.625	45.425	206.344	45.412	203.374	45.094
3	207.500	46.261	232.812	46.393	216.25	45.796	209.062	45.625	257.031	46.847
4	279.219	48.838	226.719	46.506	208.905	45.942	260.156	47.172	248.594	47.011
5	395.468	51.817	232.03	46.845	263.75	47.459	237.656	46.767	230.156	46.793
Delta	277.093	10.623	6.937	1.211	54.845	2.033	53.812	1.759	53.657	1.916
Rank	1		5		2		3		4	

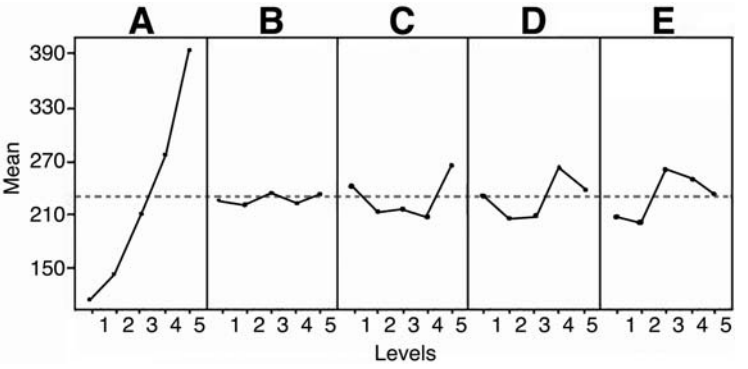

Fig. 5. Main effects plot for means.

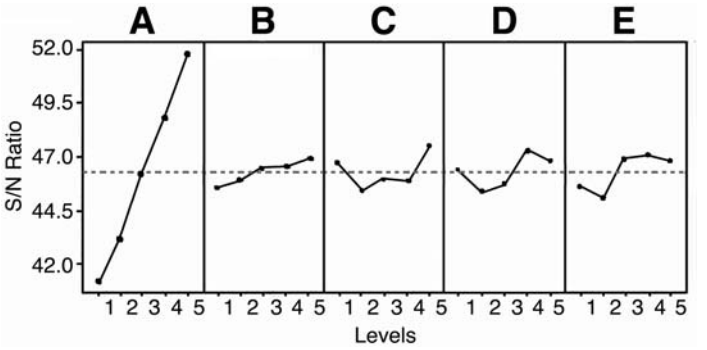

Fig. 6. Main effects plot for S/N ratio.

Table 3
Composition of the Final Optimized Medium for Clavulanic Acid Production Using *S. clavuligerus* MTCC 1142

Component	Quantity %
Soybean flour	8.8
Soybean oil	1.2
Dextrin	1
Yeast extract	1.5
K_2HPO_4	0.2
pH	7.0 ± 0.2
Inoculum size	4
Temperature	25°C

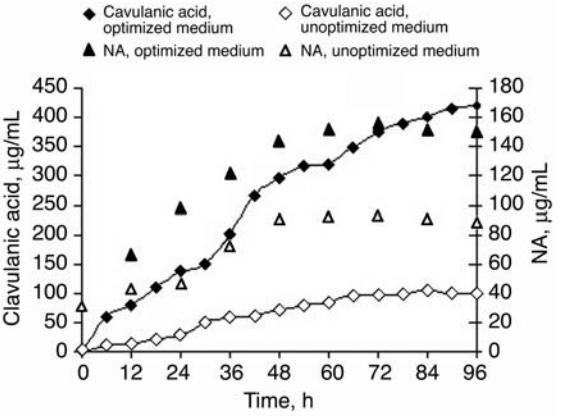

Fig. 7. Batch studies for clavulanic acid production in *Streptomyces clavulaigerus* MTCC 1142 .

mean value obtained was 500 µg/mL as compared to 493.53 predicted using MINITAB for the same composition. The final optimized medium produced 500 µg/mL clavulanic acids at the end of 96 h as compared to 140 µg/mL before optimization (Fig. 7). This implied that the selected conditions were the most suitable in practice.

Effect of pH

It can be seen from the Fig. 8 that pH plays an important role as it affects both growth as well as clavulanic acid production. It was observed that pH of 7.0 ± 0.2 supported maximum clavulanic acid production of 500 µg/mL, whereas a pH of 7.5 resulted in maximum cell growth (140 µg/mL nucleic acid). pH above 7.5 and below 6.0 decreased the yield of clavulanic acid to 200 µg/mL and 290 µg/mL, respectively, indicating degradation of clavulanic acid at higher and lower pH values.

Effect of Amino Acids on Production of Clavulanic Acid

Figure 9 illustrates the effect of varying concentrations (0.01 *M* to 0.2 *M*) of different amino acids on clavulanic acid production by *S. clavuligerus* MTCC 1142. It was observed that, all the amino acids except L-valine when supplemented in the medium increased the yield of clavulanic acid at optimal concentrations. At higher concentrations, a decrease in antibiotic production was observed, thus explaining the concentration dependent stimulation of clavulanic acid. L-arginine (0.1 *M*), L- proline (0.2M), DL-leucine (0.2 *M*), and pyruvic acid (0.1 *M*) had a greater stimulatory effect on clavulanic acid production as compared to L-ornithine (0.2 *M*), α-ketoglutarate (0.1 *M*), L-valine (0.1 *M*), dl-isoleucine (0.2 *M*). The possible reasons for the observed results can be explained with respect to the biosynthetic pathway for clavulanic acid production. It is well documented that pyruvate acts as C3 precursor, whereas arginine acts as the C5 precursor for clavulanic acid

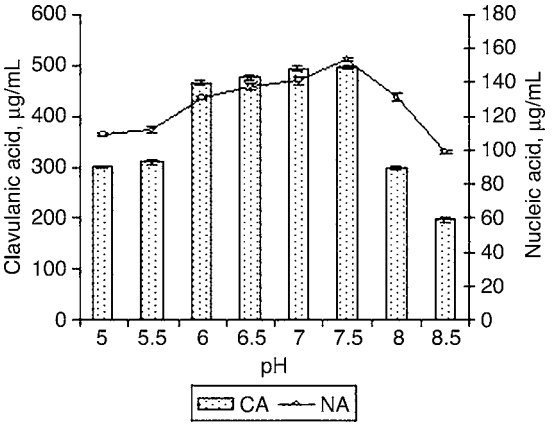

Fig. 8. Effect of pH (experimental data are mean ± S.D. of triple determination) on growth and clavulanic acid production in *Streptomyces clavuligerus* MTCC 1142.

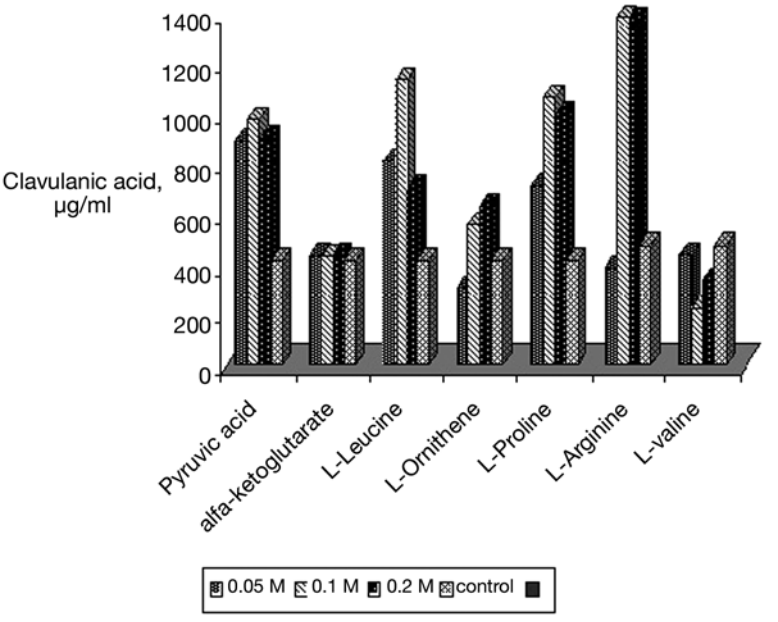

Fig. 9. Effect of stimulators (experimental data are mean ± S.D. of triple determination) on clavulanic acid production in *Streptomyces clavuligerus* MTCC 1142.

production *(17)*. Radiolabeled feeding experiments have indicated arginine and ornithine to be efficiently incorporated into clavulanic acid structure *(18,19)*. Addition of L-proline, l-leucine, pyruvic acid, and α-ketoglutarate decreased the anaplerotic flux on pyruvate (C3 precursor) for the synthesis of these amino acids, thus diverting the pathway to clavulanic acid production. L-leucine and L-arginine were found to be the most efficient precursor

for clavulanic acid production and increased the yield of clavulanic acid from 500 μg/mL to 1400 μg/mL and 1100 μg/mL (Fig. 7), suggesting maximum flux to flow through synthesis of L-leucine and L-arginine from pyruvate during the normal metabolism in *S. clavuligerus*. Supplementing the medium with L-valine decreased the yield of clavulanic acid, which may be attributed to a simultaneous increase in the yield of cephamycin (another β-lactam produced by *S. clavuligerus*), as L-valine is reported to be the precursor for cephamycin production *(20)*.

Conclusions

Using the one-factor at-a-time method and the orthogonal matrix method, it was possible to determine optimal operating conditions to obtain maximum production of clavulanic acid in *S. Clavuligerus*. Supplementing arginine, proline, leucine, α-ketoglutarate and pyruvic acid decreased the anaplerotic carbon flux of pyruvate to the synthesis of these amino acids, thus allowing more C3 precursor for clavulanic acid. Arginine, 0.1 *M* and 0.1 *M* leucine increase clavulanic acid production from 500 μg/mL to 1100 μg/mL and 1400 μg/mL, respectively.

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