STATISTICAL BASED MEDIA OPTIMIZATION AND PRODUCTION OF CLAVULANIC ACID BY SOLID STATE FERMENTATION IN JACKFRUIT SEED POWDER AS NOVEL SUBSTRATE USING STREPTOMYCES CLAVULIGERUS MTCC 1142

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Abstract

Statistics based optimization, Plackett–Burman design (PBD) and response surface methodology (RSM) were employed to screen and optimize the media components for the production of clavulanic acid from Streptomyces clavuligerus MTCC 1142, using solid state fermentation. jackfruit seed powder was used as both the solid support and carbon source for the growth of Streptomyces clavuligerus MTCC 1142. Based on the positive influence of the Pareto chart obtained from PBD on clavulanic acid production, five media components – yeast extract, beef extract, sucrose, malt extract and ferric chloride were screened. Central composite design (CCD) was employed using these five media components- yeast extract 2.5%, beef extract 0.5%, sucrose 2.5%, malt extract 0.25% and ferric chloride nutritional factors at three levels, for further optimization, and the second order polynomial equation was derived, based on the experimental data. Response surface methodology showed that the concentrations of yeast extract 2.5%, beef extract 0.5%, sucrose 2.5%, malt extract 0.25% and ferric chloride 2.5% were the optimal levels for maximal clavulanic acid production (19.37 mg /gds) which were validated through experiments.

KEYWORDS

Plackett–Burman design, Central composite design, Streptomyces clavuligerus MTCC 1142, clavulanic acid, jackfruit seed powder, solid state fermentation

1. INTRODUCTION

Antibiotics, spectacularly decrement of death and impairment from infectious diseases, exert a great act on the health care of our society. Unfortunately, the preposterous use of antibiotics caused a buildup of antibiotics resistance against pathogenic microorganisms, and now, it has become a very critical global public health problem [1]. Most of the pathogenic microorganisms expresses and releases antibiotic hydrolyzing enzymes. These enzymes inactivates antibiotics results in antibiotic resistance [2]. Clavulanic acid, kind of such specific β-lactamase inhibitor produced by S*treptomyces clavuligerus,* were first discovered in UK in 1974 [3- 4]. Therefore, there is a growing demand for more specific and perspective β-lactamase inhibitors.

Clavulanic acid is commonly produced using submerged fermentation due to its clear advantages in invariable Clavulanic acid production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost intensiveness for medium components [5]. In this context, solid-state fermentation has gained renewed importance and bright consideration from researchers payable to its consideration in current developments in biomass energy conservation, in solid waste treatment and in its application to produce antibiotics. Production of these secondary metabolites using agro-industrial residue under solid-state fermentation conditions accommodate diverse advantages in productivity, cost-effectiveness in labor, time and medium nutrients in addition to environmental advantages like less effluent production, waste minimization, etc. [6]. There is only one report describing use of agro-industrial residue for the production of clavulanic acid, e.g. wheat bran, wheat rawa, husk, straw, sugercane bagasse and brick powder [7]. However, this production characteristic would have to offer an aggressive advantage over extant product.

In antibiotics bioprocess, designing a suitable medium for maximum production is of critically considerable because the medium nutritional components significantly alter the product yield. This can be achieved using solid fruit materials as substrates [8]. Jackfruit seed powder is obtained after removing the fruit pulp from jackfruit, which is normally used as a steamed and eaten as a snack or used in some local dishes. The chemical constituents of jackfruit seed powder include (% w/w): carbohydrate (66.86); protein (14.02); crude fat (4.08) and crude fiber (1.8) and Ash (2.54) [9-10]. Considering its cheap cost and availability, an effort was made to Jackfruit seed powder as a solid substrate for the production of clavulanic acid from *Streptomyces clavuligerus* MTCC 1142.

The customary one-at-a-time optimization approach is clear and convenient, but it fails often. Statistical experimental design provides an effective strategy to optimize the medium components. Placket Burmen design (PBD) is especially appropriate to account for identify the most important components in the medium formula. A association of nutritional factors generating a certain optimum response can be identified through Placket Burmen design and the application of response surface methodology (RSM). The statistical method is more acceptable and competent than other standard one-at-a-time optimization approaches because it can study many variables concurrently with a lower number of observations, thus saving time and cost [11]. RSM is a well-known practical methods in the optimization of medium constituents and other

particular variables accountable for the production of *Bacillus* species [12] and olivanic acid [13].

The objective of this work was to optimize the fermentation medium by statistical operating procedure to amplify the clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142 using jackfruit seed powder, which is a low cost solid substrate.

2. MATERIALS AND METHODS

2.1 Substrate

Jackfruit seed (*Artocarpus heterophyllus*), procured from a local market of Dharwad, karanataka, India. It was dried at 60 °C for 72 h to reduce the moisture content to around 5%, and ground to the desired size (2 mm) and was used as the solid substrate.

2.2 Microorganism and growth conditions

Streptomyces clavuligerus MTCC 1142, obtained from Microbial Type Culture Collection, Chandigarh,, India, was used throughout the study. The culture was maintained on YMG slants having the composition (%): yeast extract 0.4, malt extract 1, glucose 0.4 and agar agar 2.0. The pH of the medium was adjusted to 7.0-7.4 and culture was incubated at 25 °C for 96 h. Sub culturing was carried out once in 2 weeks and the culture was stored at 4 °C.

2.3 Inoculum preparation

The *Streptomyces* strain was cultivated in a medium containing glycerol 2%, bacteriological peptone 1% and malt extract 1% per liter of distilled water with pH adjusted to 6.8.7.2. The cells were cultivated in this medium at 25 °C on a shaker at 200 rpm for 48 h [14].

2.4 Optimization for clavulanic acid production by one-variable-at-a-time approach

SSF was carried out in a 250 ml Erlenmeyer conical flask containing 10 g solid substrate (jackfruit seed powder). In the present article, the requirement of medium components including various carbon (1% [w/w], glucose, fructose, glycerol, sucrose, and maltose) and nitrogen sources (1% [w/w], yeast extract, malt extract, bacteriological peptone, soya peptone, and beef extract) and inorganic salts (1% [w/w], calcium chloride, ferric chloride, potassium di-hydrogen phosphate, sodium chloride, and manganese chloride) were optimized. The jackfruit seed powder substrate was moistened with distilled water. The contents were sterilized and inoculated with 0.2 ml of 48 h grown culture broth under sterile conditions. After 10 days, clavulanic acid was extracted with aqueous two-phase system of polyethylene glycol (PEG) and potassium phosphate [15].

2.5 Quantification of clavulanic acid by HPLC

Clavulanic acid was identified and analyzed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV UV–Vis detector and a CBM-101 Shimadzu HPLC/PC integrator. The column used was a Spherisorb 5µ ODS2 column (4.6 mm X 250 mm) from Waters (Mil-ford, MA, USA). Samples were eluted isocratically with a sonicated mixture of 60% (v/v) 50 mM KH_2PO_4 pH 3.08 and 40% (v/v) methanol at a flow rate of 0.8 mL/min, and analyzed in the UV detector at 312 nm [16]. Augmentin (intravenous injection containing 100 mg of clavulanic acid and 500 mg of amoxicillin) was used as the standard. The yield of clavulanic

acid was expressed in mg/gds.

2.6 Plackett-Burman design for the screening of important nutritional factors

Plackett-Burman design is used to screen out crucial nutritional factors. In this design (Table 2) total number of runs is constantly superior to total number of variables (medium nutritional factors) by one unit. Each variable delineated in high and a low level defines the upper and lower limits of the range covered by variables [17]. Experiments were performed with different combinations of high and low values of the variables and analyzed for their effect on the product formation. In the advance experiment, ten independent variables (shown in Table 1) were selected for the screening in twelve trials. The results obtained with standard experiments (data not shown) helped in the preference of independent variables. MINTAB, Trial version 16 (Minitab Inc., PA, USA), was used for experimental designs and statistical analysis of the experimental data. The effect of each variable was decided by following equation

$$E_{(X1)} = 2\frac{\sum M_{1H} - \sum M_{1L}}{N} \Rightarrow (1)$$

Where $E_{(X1)}$ is the concentration effect of the tried variable. M_{1H} and M_{1L} are the clavulanic acid yield (mg/gds) from the trials where the variable display at high and low concentration, respectively, and N is the total number of trials.

The variables with confidence levels greater than 95% were reasoned to affect the clavulanic acid production significantly. Five variables, which were found to be the most effective components for clavulanic acid production in PBD, were preferred for further medium optimization studies using CCD and RSM.

2.7 Optimization of the selected medium nutritional factors by RSM using CCD

Response surface designs are used to investigate non-linear relationships between independent (medium nutritional factors) and the dependent (clavulanic acid yield) variables. These associations help in selecting the concentrations of the medium nutritional factors producing maximum product. 2^{3-1} fractional factorial CCD intended by Box et al. (1978) [18]. Is the most approved and comprehensively used design to study the interaction effect of the medium nutritional factors [19].Total thirty two experiments with sixteen cube points, ten star points and six replicas of the central point were engaged to fit the second order polynomial model. Design along with range and the levels of the five preferred variables are shown in Table 3 and Table 4 . Following regression equation was developed by the application of RSM display an empirical association between the logarithmic values of clavulanic acid yield and the coded units of the test variables (medium nutritional factors).

$$Y = b_0 + b_1X_i + b_2 X_{ii} + b_3X_{iii} + b_4X_{iiii} + b_5X_{iiiii} + b_6X_i^2 + b_7X_{ii}^2 + b_8X_{iii}^2 + b_9X_{iiii}^2 + b_{10}X_{iiiii}^2 + b11X_iX_{ii} + b12X_iX_{iii} + b13X_iX_{iiii} + b14X_iX_{iiiii} + b15X_{ii}X_{iii} + b16X_{ii}X_{iiii} + b17X_{ii}X_{iiiii} + b18X_{iii}X_{iiii} + b19X_{iii}X_{iiiii} + b20X_{iiii}X_{iiiii} \Rightarrow (2)$$

Where Y is the dependent or response variable, b is the regression coefficient and X is the coded or un-coded level of the independent variables.

'Minitab 16 trial version (Minitab Inc., PA, USA)' was exploited for the graphical and statistical analysis of the data obtained from the CCD. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface/contour plots [20].

2.8 Validation of the experimental model

To validate the model equation, experiments were guided in triplicates for clavulanic acid production under optimum conditions predicted by the model.

3.0 RESULTS AND DISCUSSION

3.1 Jackfruit seed powder: a cheap substrate for clavulanic acid production

This article has showed that jackfruit seed powder can be used as a solid substrate for clavulanic acid production. The choosing of an model substrate for the production of any secondary metabolite is an important factor from an industrial point of view. A model substrate should be attainable during the year [21]. Reports on SSF of jackfruit seed powder substrate for the production of clavulanic acid are not available. Jackfruit seed powder substrate provender nutrients to the microbial culture and anchorage for the growing microbial cells. This is the first report on production of clavulanic acid from *Streptomyces clavuligerus* MTCC 1142, using statistical experimental design and RSM in optimization of its production under SSF.

3.2 Preliminary screening of nutritional factors for statistical optimization

The production strain is unique in their molecular, biochemical, metabolic, and antibiotics production properties. Hence, an in depth appreciation of antibiotics production from any new strain is an essential for the assessment of its biotechnological possibility [22]. The nutritional factors were optimized to boost clavulanic acid secretion. The effect of inorganic salts was shown in Fig 1. In this article, among all the supplementary inorganic salts (1% [w/w]), ferric chloride has been found to be the better source for clavulanic acid production. These results were in conformity with reported clavulanic acid production by submerged fermentation in the presence of diverse inorganic salts [14]. When different concentrations of ferric chloride (1% [w/w]), supported the maximum clavulanic acid production (11.56 mg/gds) at 8^{th} day of incubation. It was shown in actinobacteria that the production of clavulanic acid was induced by ferric chloride in the liquid culture medium [5].The other inorganic salts such as potassium dihydrogen phosphate and manganese chloride supported clavulanic acid production. Among nitrogen sources, the accessory of yeast extract supported maximum clavulanic acid production (Fig 2) at 8^{th} day of incubation. When deviating concentrations of yeast extract were supplemented, yeast extract 1% [w/w] supported the maximum clavulanic acid production (12.89 mg/gds). The supplemented yeast extract stirred clavulanic acid production. These results are in accordance with the observation made with *Streptomyces clavuligerus* NRRL 3585 in liquid fermentation [24].The sources such as beef extract, soy peptone, malt extract and bacteriological peptone also enhanced clavulanic acid production. In SSF, the addition of carbon sources enhanced clavulanic acid production except fructose and maltose. Among the carbon sources, glycerol enhanced the highest clavulanic acid production (10.78 mg/gds) at 8^{th} day of incubation. These results were in

accordance with the surveillance made with *Streptomyces clavuligerus* in liquid fermentation [24]. Fructose (1% [w/w]) and maltose (1% [w/w]) inhibited clavulanic acid production (Fig 3).

3.3 Identification of important nutritional factors using Plackett-Burman design

The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. Ten nutritional factors were screened by twelve experimental runs. The Pareto chart displays the magnitude of each factor estimate and it is a convenient way to view the results of a Plackett–Burman design [25]. Variation reflects the influence of medium optimization to accomplish higher productivity of clavulanic acid. The pareto chart (Fig 4) was used to appear the effect of all the nutritional factors on clavulanic acid production. A p-value less than 0.05 for the five variables viz., yeast extract(X7), beef extract(X8), sucrose(X2) , malt extract (X10) and ferric chloride (X4) indicates that they are significant. In addition, the coefficient of determination (R^2) of the model was 0.9999 which explains the 99.99% variability of the data. In the Pareto chart (Fig 4), the maximal effect was presented in the upper part and then progressed down to the minimal effect. Yeast extract (X7), beef extract(X8), sucrose(X2) and malt extract (X10) had a confidence level of above 95% in comparison to ferric chloride (X4) and thus reasoned to be most significant for clavulanic acid production. The coefficient estimate was positive to yeast extract, sucrose and ferric chloride. This states that the higher levels of yeast extract, sucrose and ferric chloride concentrations would benefit clavulanic acid production. The coefficient estimate was negative to beef extract and malt extract; these indicated that the enhance of beef extract and malt extract concentration in the jackfruit seed powder medium will approve for antibiotic production. The five significant nutritional factors such as yeast extract, beef extract, sucrose, malt extract and ferric chloride were further optimized with central composite design (CCD).

3.4 Response surface methodology

The effect of the five variables (yeast extract, beef extract, sucrose, malt extract and ferric chloride) on clavulanic acid production was evaluated by CCD and RSM. The CCD model helps to study the interactions between the different variables, and RSM helps to investigate the optimum concentrations of each of the variables. The highest production of the clavulanic acid was ascertained at run 3 (Table 4).The results obtained from CCD were analyzed using ANOVA (Table 6). The model F-value of 6.43 implies that the model is significant (Table 5).There is only a 0.3% eventuality that a “Model F-value” this ample could appear due to noise. Values of “Prob >F” less than 0.05 denote that model terms are significant. In this case,X7, X8, X2, $X7^2$, $X2^2$, $X10^2$, X7 x X8, X7 x X2, X7 x X4, X8 x X10 and X2 x X4 are significant model terms. The second-order polynomial model was used to associate the independent variables with clavulanic acid production. In Fig.5A, the normal probability plot, since the points exhaustively from an near straight line, it implies the data follow near normal distribution.. The odd points not divergent much from the straight line, are less in significance to affect the model. Hence the normal distribution provides an exemplary model for the data. It shows an agreeable correlation between measured and predicted values of the model. The coefficient of determination (R^2) was calculated to be 0.9212, indicating that the model could clarify 92.12% of the variability. The nearer the value of R squared is to the unity, the perfect the empirical model fits the actual data. The smaller the value of R squared is, the less applicable the dependent variables in the model have to explain

the behavior variation [26-27]. Residual Vs Fit graph shows (Fig 5B) a pattern of randomness in scatter which indicates un-biased variance. A histogram of the residuals (Fig 5C) also confirms and the residuals being normally distributed around the mean residual of 0.00. It also highlighted the outliers located in the results. A value of >0.75 indicates advantageous for the model. Analyses of residuals vs. experimental run of clavulanic acid production (Fig 5D) showed no significant drift with time and no discernible pattern which reinforces that all runs were conducted independently from one another. The "Lack-of-Fit F-value" of 0.16 implies that the lack of fit is not significant relative to the pure error. There is only a 9.77% chance that a "Lack-of-Fit F-value" this ample could appear due to noise. The p values are used to account the significance of each coefficient, which also denote the interaction effectiveness between each impendent variable (Table 6). The smaller the p value, the larger the significant of the corresponding coefficient [28].The response (Y) was good fitted with a quadratic second-order polynomial equation.

Clavulanic acid yield (Y) = 6.41- 1.56 X7 + 2.24 X8 - 1.70 X2 -0.57 X10 -0.75 X4 + 1.57 $X7^2$ - 0.06 $X8^2$ +1.02 $X2^2$ +1.21 $X10^2$- 0.0044 $X4^2$-2.37 X7 x X8 -1.51 X7 x X2+ 0.87 X7 x X10-1.57 X7 x X4-0.18 X8 x X2+2.20 X8 x X10- 0.79 X8 x X4+1.02 X2 x X10+1.92 X2 x X4 + 0.34 X10 x X4 $\Rightarrow$ (3)

Where X7 is yeast extract (%), X8 is beef extract(%), X2 is sucrose (%), X10 is malt extract (%) and X4 is ferric chloride (%)

3.5 Response surface plots

Response surface three dimensional and two dimensional contours plots graphs help to appreciate the association between the response and experimental levels of each variable. These plots also show the type of interaction between test variables and help to achieve the optimum conditions [29]. A total of three response surfaces were shown by considering all the possible combinations. These plots show the type of interaction between the tried variables and hence allow us obtain the optimum conditions [30]. A circular contour plot represents a insignificant interaction between the independent variables, while perfect interactions were indicated by the elliptical contours. The maximum predicted value is represented by the surface restricted in the smallest ellipse in the contour diagram [31]. The optimum value of each variable was identified based on the hump in the three-dimensional plot, or from the central point of the equivalent contour plot. Each contour curve represents a countless number of combination of the two tested variables, with the other three maintained at zero levels.

The effect of yeast extract (X7) and beef extract (X8) on the clavulanic acid production was shown in Fig 6 A and B. There was the optimum in the clavulanic acid production (more than 30 mg/gds) when low concentration of yeast extract (X7) (2.5-3.0 %) and beef extract (X8) (0.7-1.0 %) were used. The interaction effect of yeast extract (X7) and sucrose (X2) on the clavulanic acid production was shown in Figure. 6 A and B An increased in the sucrose (X2) concentration to 3.2 % at the high concentration solution of yeast extract (X7) (3.2 %) increased the clavulanic acid production to the maximum level (more than 20mg/gds).

The effect of sucrose(X2) concentration on the clavulanic acid production was observed for the

yeast extract (X7) concentration (Fig 7 C and D). An increased in the sucrose(X2) concentration with yeast extract (X7) added at the high concentration (3.2 % and 3.2 %) increased the clavulanic acid production to more than 20 mg/gds. However, the trend was reversed at low yeast extract (X7) (2.4-2.9%) and low sucrose(X2) (less than 2.3%) concentrations.

The interaction effect of yeast extract (X7) and malt extract (X10) concentrations on the clavulanic acid production in Fig 8 (E and F) did not clearly indicate a proper combination of the clavulanic acid production. In Fig 9 (G and H), an increased in the ferric chloride (X4) concentration to 3.2 at high yeast extract (X7) concentration (3.0-3.2 %) increased the clavulanic acid production to the maximum level (more than 20 mg/gds).

The effect of beef extract (X8) and sucrose (X2) concentrations on the clavulanic acid production was shown in Fig 10 (I and J). An increased in the sucrose (X2) concentration to 3.2 % at high beef extract (X8) concentration (0.6%) increased the clavulanic acid production to the maximum value.

Fig 11 (K and L), shows the maximum clavulanic acid production (more than 15 mg/gds) at the concentrations of beef extract (X8) less than 0.2% and malt extract (X10) less than 0.2%. However, the trend was decreased at high beef extract (X8) concentration (more than 0.6%).

The effect of beef extract (X8) and ferric chloride (X4) concentrations on the clavulanic acid production was shown in Fig 12 (M and N). There was the optimum in clavulanic acid production (more than 14 mg/gds) at high concentration of beef extract (X8) (0.4-0.6%) and ferric chloride (X4) concentration (2.8-3.2%).

The interaction effect of sucrose(X2) and malt extract (X10) concentrations on the clavulanic acid production (Fig 13 O and P) indicated that the increasing of the concentration of sucrose (X2) lower than 2.3% showed the direct effect on the increasing of clavulanic acid production. The 0.4-0.1% malt extract (X10) concentration could increase the clavulanic acid concentration to more than 20 mg/gds.

The effect of the sucrose(X2) and ferric chloride (X4) concentrations on the clavulanic acid production was shown in Fig 14 (Q and R). The clavulanic acid production reached the maximum level at 20 mg/gds in below 2.3% sucrose(X2) and below 2.3% ferric chloride (X4) concentration.

An interactive effect of malt extract (X10) and ferric chloride (X4) can be seen (Fig. 15 S and T). The response is expected to increase with increase in the malt extract (X10) concentration. At low malt extract (X10) concentrations, supplementation with ferric chloride (X4) can enhance the production of clavulanic acid. However, these results would be applicable in the evaluated range of variables and in order to evaluate the activity out of the range further experimentation is required. An increase in malt extract (X10) concentration is favorable for clavulanic acid production and supplementation of the medium with ferric chloride (X4) in low levels can further enhance it.

3.6 Verification of predictive model

The suitability of the model equations for predicting optimum response values was tested under the conditions: yeast extract 2.5%, beef extract 0.5%, sucrose 2.5%, malt extract 0.25% and ferric chloride 2.5%. This set of conditions was determined to be optimum by the RSM optimization approach and was also used to validate experimentally and predict the values of the responses using the model equation. A mean value of 18.90 (N= 3), obtained from real experiments, demonstrated the validation of the RSM model, indicating that the model was adequate for the extraction process (Table 4).

3. CONCLUSIONS

The Plackett–Burman design was successfully employed to screen various process variables and response surface methodology was applied to optimize the screened variables involved in the production of clavulanic acid by *Streptomyces clavuligerus* MTCC 1142 under solid state fermentation. The results indicated that the nutritional factors selected in this study had a significant effect on the antibiotics production, and acted as supplement effects along with jackfruit seed powder. Model summary statistics showed that the predicted model was in close agreement with the experimental data. From the ANOVA results, the second-order polynomial regression model developed has a high correlation R^2 value. The best conditions were found to be, yeast extract 2.5%, beef extract 0.5%, sucrose 2.5%, malt extract 0.25% and ferric chloride 2.5% and the predicted maximum clavulanic acid production was 19.37 mg/gds. Under these optimized conditions, the experimental values of enzyme production closely agreed with the predicted values.

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Table1- Nutrients supplements to jackfruit seed powder for screening using Plackett–Burman design.

S No	Variables with designate	Lower level	Higher level
1	X1 Glycerol	1%	2%
2	X2 Sucrose	1%	2%
3	X3 Glucose	1%	2%
4	X4 Ferric chloride	1%	2%
5	X5 Calcium chloride	1%	2%
6	X6 Sodium chloride	1%	2%
7	X7 Yeast extract	1%	2%
8	X8 Beef extract	1%	2%
9	X9 Soy peptone	1%	2%
10	X10 Malt extract	1%	2%

Table 2- Experimental design using Plackett–Burman method for screening of nutrients supplemented to jackfruit seed powder.

Run Order	Blocks	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	Clavulanic acid (mg/gds)
1	1	2	2	1	2	1	1	1	2	2	2	2.89
2	1	2	2	1	2	2	2	2	1	1	1	17.34
3	1	2	1	1	1	2	1	2	1	2	2	8.34
4	1	1	2	2	1	2	1	1	1	2	2	7.45
5	1	1	2	2	2	1	2	2	1	2	1	18.34
6	1	1	2	1	1	1	2	2	2	1	2	7.23
7	1	1	1	1	2	2	2	1	2	2	1	1.34
8	1	1	1	1	1	1	1	1	1	1	1	4.89
9	1	1	1	2	2	2	1	2	2	1	2	5.23
10	1	2	1	2	2	1	2	1	1	1	2	4.89
11	1	2	1	2	1	1	1	2	2	2	1	7.56
12	1	2	2	2	1	2	2	1	2	1	1	5.45

X1 Glycerol (% w/w), X2 Sucrose (% w/w), X3 Glucose (% w/w), X4 Ferric chloride (% w/w), X5 Calcium chloride (% w/w), X6 Sodium chloride (% w/w), X7 Yeast extract (% w/w), X8 Beef extract (% w/w), X9 Soy peptone (% w/w), X10 Malt extract (% w/w) * Each data point represents the mean of triplicate determinations ± SD.

Table 3- Levels of solid medium components applied for the optimization studies.

Factors Code	Name of factors	Units			levels		
			Axial (-2)	Low (-1)	Center (0)	High (+1)	Axial (+2)
X7	Yeast extract	% w/w	2.25	2.50	2.75	3.0	3.25
X8	Beef extract	% w/w	0.125	0.25	0.375	0.50	0.625
X2	Sucrose	% w/w	2.25	2.50	2.75	3.0	3.25
X10	Malt extract	% w/w	0.125	0.25	0.375	0.50	0.625
X4	Ferric chloride	% w/w	2.25	2.50	2.75	3.0	3.25

Table 4- Central composite design with actual and predicted values

SO	RO	PT	Blocks	X7	X8	X2	X10	X4	Actual Yield	Predicted Yield
11	1	1	1	2.50	0.500	2.50	0.500	3.00	13.89	14.90
18	2	-1	1	3.25	0.375	2.75	0.375	2.75	9.56	9.65
19	3	-1	1	2.75	0.125	2.75	0.375	2.75	1.34	1.73
21	4	-1	1	2.75	0.375	2.25	0.375	2.75	15.34	13.98
22	5	-1	1	2.75	0.375	3.25	0.375	2.75	6.56	7.16
5	6	1	1	2.50	0.250	3.00	0.250	2.50	6.96	6.55
17	7	-1	1	2.25	0.375	2.75	0.375	2.75	16.76	15.91
20	8	-1	1	2.75	0.625	2.75	0.375	2.75	11.87	10.72
2	9	1	1	3.00	0.250	2.50	0.250	2.50	18.76	18.61
9	10	1	1	2.50	0.250	2.50	0.500	2.50	2.36	2.49
8	11	1	1	3.00	0.500	3.00	0.250	2.50	3.34	2.26
15	12	1	1	2.50	0.500	3.00	0.500	2.50	15.45	15.47
27	13	0	1	2.75	0.375	2.75	0.375	2.75	4.87	6.47
24	14	-1	1	2.75	0.375	2.75	0.625	2.75	10.67	10.19
31	15	0	1	2.75	0.375	2.75	0.375	2.75	12.78	6.47
7	16	1	1	2.50	0.500	3.00	0.250	3.00	15.45	15.92
10	17	1	1	3.00	0.250	2.50	0.500	3.00	5.34	5.73
13	18	1	1	2.50	0.250	3.00	0.500	3.00	8.34	8.48
6	19	1	1	3.00	0.250	3.00	0.250	3.00	6.89	6.74
26	20	-1	1	2.75	0.375	2.75	0.375	3.25	6.32	4.95
28	21	0	1	2.75	0.375	2.75	0.375	2.75	5.32	6.47
12	22	1	1	3.00	0.500	2.50	0.500	2.50	17.89	18.17
25	23	-1	1	2.75	0.375	2.75	0.375	2.25	7.34	7.95
32	24	0	1	2.75	0.375	2.75	0.375	2.75	6.34	6.47
1	25	1	1	2.50	0.250	2.50	0.250	3.00	10.56	11.14
29	26	0	1	2.75	0.375	2.75	0.375	2.75	5.32	6.47
3	27	1	1	2.50	0.500	2.50	0.250	2.50	18.90	19.37
4	28	1	1	3.00	0.500	2.50	0.250	3.00	4.30	5.03
23	29	-1	1	2.75	0.375	2.75	0.125	2.75	12.78	12.50
16	30	1	1	3.00	0.500	3.00	0.500	3.00	7.57	7.86
14	31	1	1	3.00	0.250	3.00	0.500	2.50	4.67	4.08
30	32	0	1	2.75	0.375	2.75	0.375	2.75	3.45	6.47

X7 Yeast extract (% w/w), X8 Beef extract (% w/w), X2 Sucrose(% w/w), X10 Malt extract(% w/w), X4 Ferric chloride (% w/w)Each data point represents the mean of triplicate determinations ± SD.Value of α is 2. SO-Standard Order, RO-Run order, PT-Point Type

Table 5- Sequential model fitting for the clavulanic acid production .

Term	Coefficient	SE Coefficient	T	P
Constant	6.47193	0.9655	6.703	0.000
X7	-1.56458	0.4941	-3.167	0.009
X8	2.24875	0.4941	4.551	0.001
X2	-1.70375	0.4941	-3.448	0.005
X10	-0.57792	0.4941	-1.170	0.267
X4	-0.75125	0.4941	-1.520	0.157
X7 x X7	1.57807	0.4469	3.531	0.005
X8 x X8	-0.06068	0.4469	-0.136	0.894
X2 x X2	1.02557	0.4469	2.295	0.042
X10 x X10	1.21932	0.4469	2.728	0.020
X4 X X4	-0.00443	0.4469	-0.010	0.992
X7 x X8	-2.37688	0.6051	-3.928	0.002
X7 x X2	-1.51938	0.6051	-2.511	0.029
X7 x X10	0.87562	0.6051	1.447	0.176
X7 x X4	-1.57063	0.6051	-2.595	0.025
X8 x X2	-0.18812	0.6051	-0.311	0.762
X8 x X10	2.20438	0.6051	3.643	0.004
X8 x X4	-0.79687	0.6051	-1.317	0.215
X2 x X10	1.02687	0.6051	1.697	0.118
X2 x X4	1.97812	0.6051	3.269	0.007
X10 x X4	0.34563	0.6051	0.571	0.579
R-Sq = 92.12%				

Table 6- ANOVA of the response surface model for the clavulanic acid production .

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Regression	20	753.071	753.071	37.654	6.43	0.001
Linear	5	271.342	271.342	54.268	9.26	0.001
X7	1	58.750	58.750	58.750	10.03	0.009
X8	1	121.365	121.365	121.365	20.71	0.001
X2	1	69.666	69.666	69.666	11.89	0.005
X10	1	8.016	8.016	8.016	1.37	0.267
X4	1	13.545	13.545	13.545	2.31	0.157
Square	5	132.798	132.798	26.560	4.53	0.017
X7 x X7	1	61.583	73.049	73.049	12.47	0.005
X8 x X8	1	1.455	0.108	0.108	0.02	0.894
X2 x X2	1	25.817	30.853	30.853	5.27	0.042
X10 x X10	1	43.943	43.611	43.611	7.44	0.020
X4 x X4	1	0.001	0.001	0.001	0.00	0.992
Interaction	10	348.931	348.931	34.893	5.96	0.003
X7 x X8	1	90.393	90.393	90.393	15.43	0.002
X7 x X2	1	36.936	36.936	36.936	6.30	0.029
X7 x X10	1	12.268	12.268	12.268	2.09	0.176
X7 x X4	1	39.470	39.470	39.470	6.74	0.025
X8 x X2	1	0.566	0.566	0.566	0.10	0.762
X8 x X10	1	77.748	77.748	77.748	13.27	0.004
X8 x X4	1	10.160	10.160	10.160	1.73	0.215
X2 x X10	1	16.872	16.872	16.872	2.88	0.118
X2 x X4	1	62.608	62.608	62.608	10.69	0.007
X10 x X4	1	1.911	1.911	1.911	0.33	0.579
Residual Error	11	64.452	64.452	5.859		
Lack-of-Fit	6	10.385	10.385	1.731	0.16	0.977
Pure Error	5	54.067	54.067	10.813		

Total	31	817.524				

Adj SS -Adjusted Sum Square, Seq SS-Sequential Sum Square, Adj MS-Adjusted Mean Square

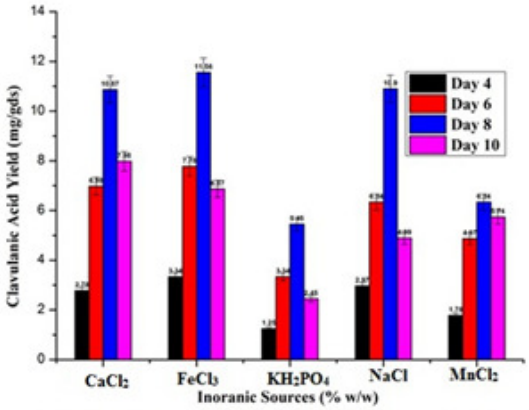
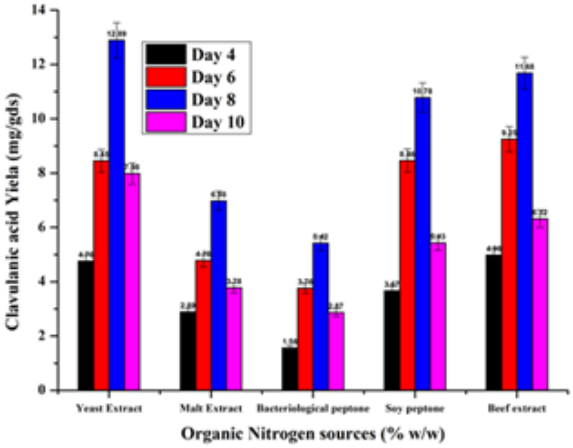


Figure 1- Effect of minerals (1% w/w) on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142 using jackfruit seed powder under solid-state fermentation.



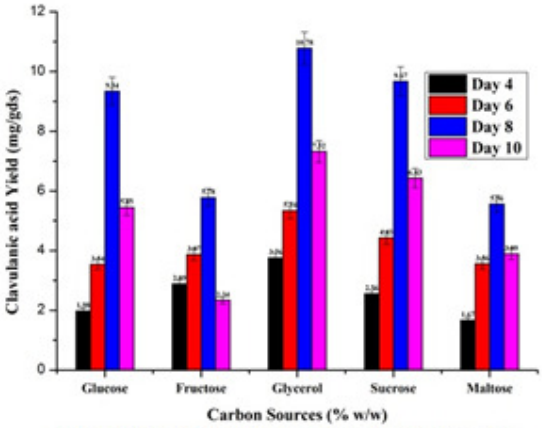


Figure 3- Effect of minerals (1% w/w) on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142 using jackfruit seed powder under solid-state fermentation.

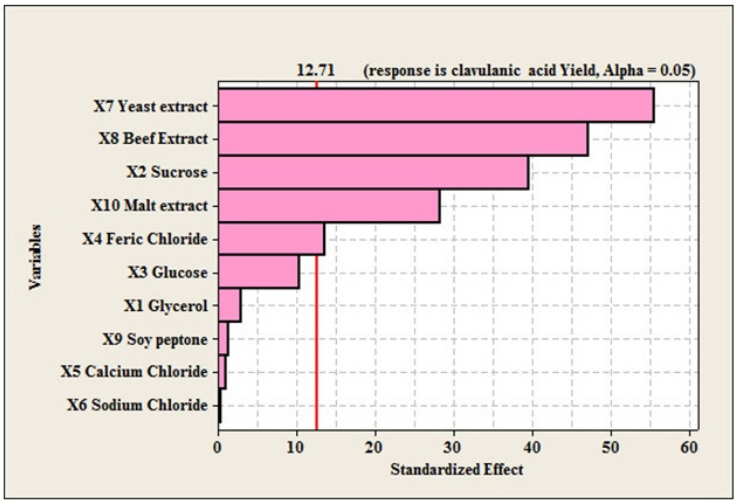


Figure 4- Pareto chart of ten nutational factors standardized effects on clavulanic acid production. The important terms were yeast extract,beef extract,sucrose, malt extract and ferric chloride.

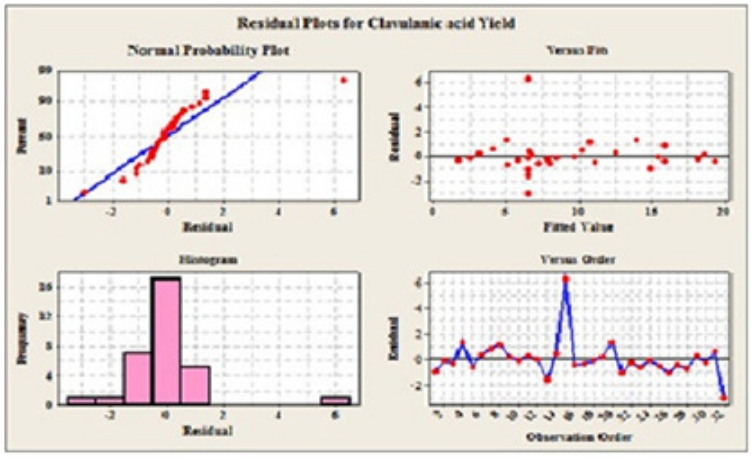


Figure 5-Residuals analyses plots.

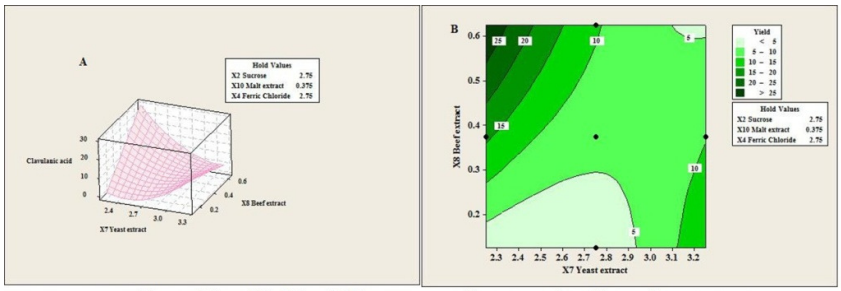


Figure 6 A and B- 3D and 2D response surface curve for effects of yeast extract X7,beef extract X8 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

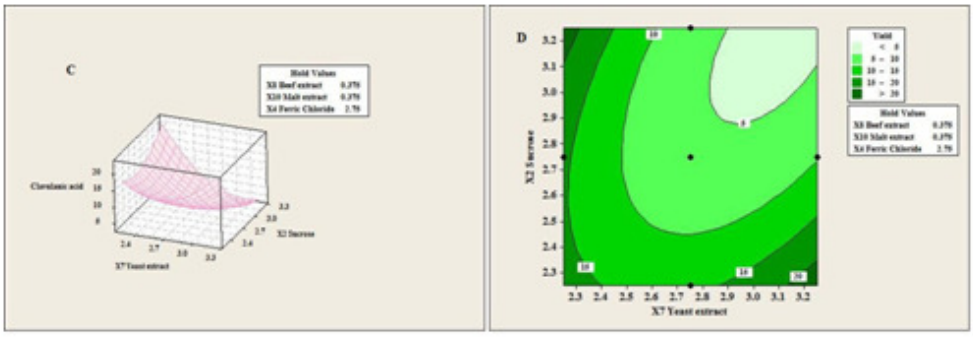


Figure 7 C and D-3D and 2D response surface curve for effects of yeast extract X7, sucrose X2 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

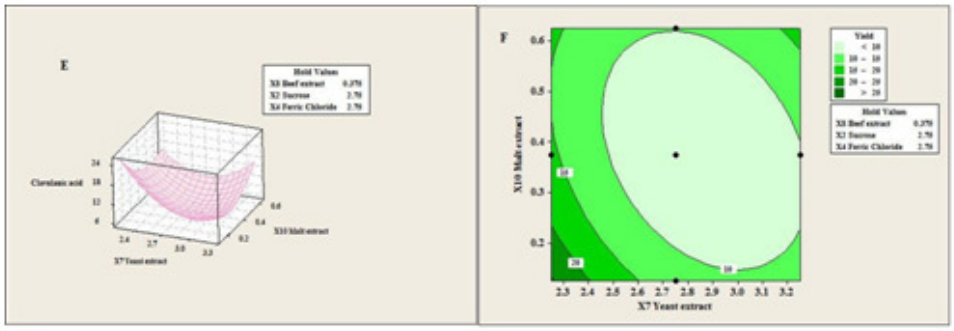


Figure 8 E and E- 3D and 2D response surface curve for effects of yeast extract X7, malt extract X10 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

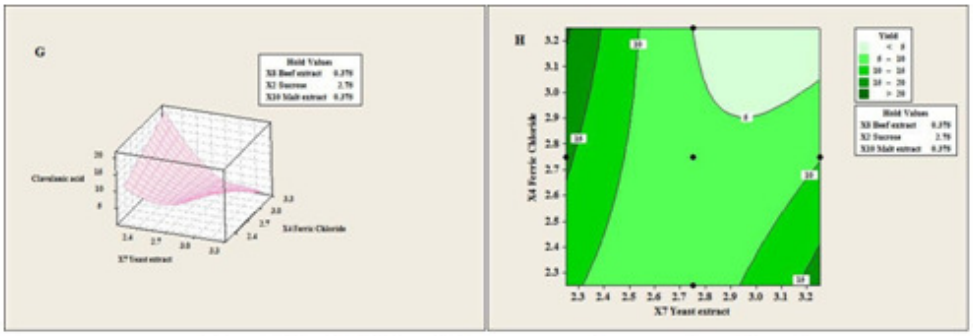


Figure 9 G and H- 3D and 2D response surface curve for effects of yeast extract X7, ferric chloride X4 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

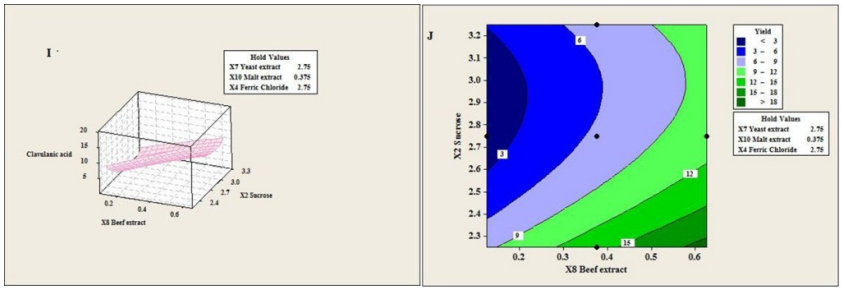


Figure 10 I and J- 3D and 2D response surface curve for effects of beef extract X8, sucrose X2 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

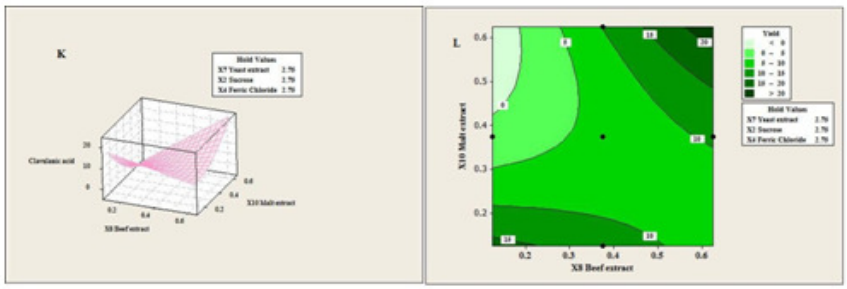


Figure 11 K and L- 3D and 2D response surface curve for effects of beef extract X8, malt extract X10 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

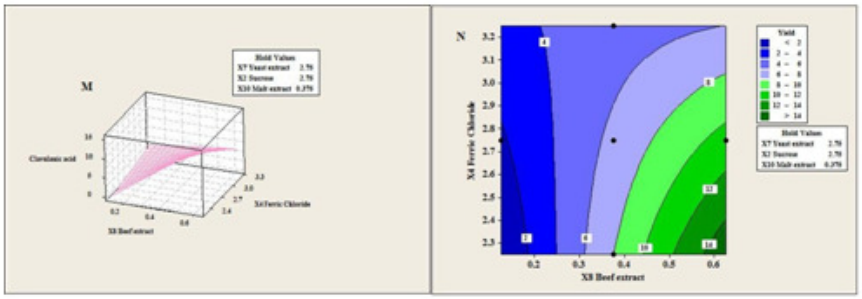


Figure M and N 12- 3D and 2D response surface curve for effects of beef extract X8, ferric chloride X4 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

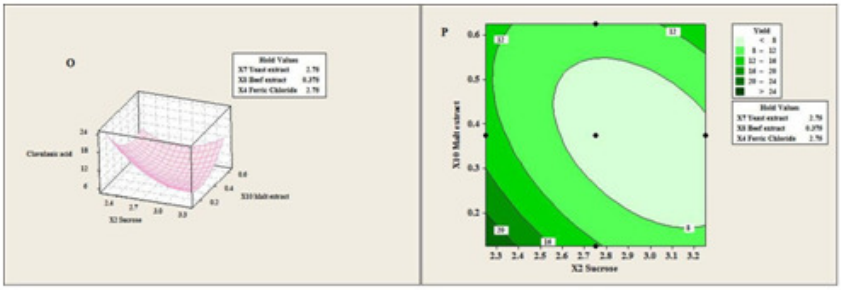


Figure O and P 13- 3D and 2D response surface curve for effects of sucrose X2, malt extract X10 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

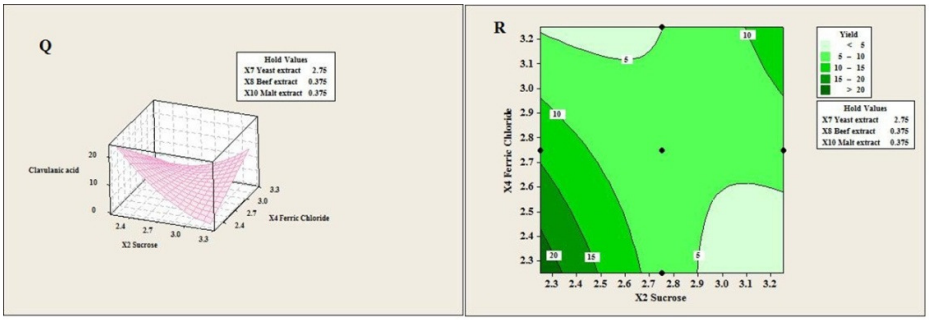


Figure 14 Q and R- 3D and 2D response surface curve for effects of sucrose X2, ferric chloride X4 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.

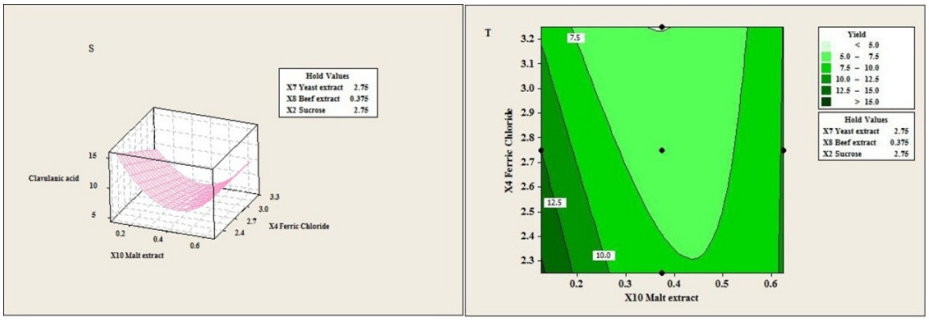


Figure 15 S and T- 3D and 2D response surface curve for effects of malt extract X10, ferric chloride X4 and their mutual interaction on clavulanic acid production by *Streptomyces clavuligerus* MTCC 1142. Other variables held at their zero level.