Optimizing the medium conditions for production of tetracycline by solid state fermentation of Streptomyces aureofaciens NCIM 2417 using statistical experimental methods

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Abstract

Tetracycline belongs to one of the extracellular produced polyketide antibiotics having hydrophilic properties. Statistical based optimization, Plackett–Burman design (PBD) and response surface methodology (RSM) were occupied to screen and optimize the medium conditions for the production of tetracycline from Streptomyces aureofaciens NCIM 2417, using solid state fermentation. pineapple peel waste was used as both the curious solid support and carbon and nitrogen sources and inorganic salts for the growth of Streptomyces aureofaciens NCIM 2417. Based on the positive influence of the half normal plot obtained from PBD on tetracycline production, three medium conditions – peanut meal, incubation period and soluble starch were screened. Central composite design (CCD) was occupied using these three medium conditions at five levels, for advance optimization, and the second order polynomial equation was derived, based on the experimental data. Response surface methodology showed that the conditions of peanut meal (0.4%), incubation period (day 2) and soluble starch (2%) were the optimal levels for maximal tetracycline production (17.98 mg/g substrate) which were validated through experiments.

Keywords

Streptomyces aureofaciens NCIM 2417, solid state fermentation, pineapple peel, Placket Burman design, central composite design

1 Introduction

Tetracycline is one of the important polyketide antibiotics widely used in pharmaceutical preparations for local applications. It is active against gram positive and gram negative bacteria. They are also of benefit in spirochaetal infections, such as syphilis, leptospirosis and Lyme disease. Certain rare or exotic infections, including anthrax, plague and brucellosis, are also susceptible to tetracycline [1]. A main disadvantage in the large-scale production of tetracycline by submerged fermentation is the cost of the downstream processing, contamination and energy input. An attractive preference could be the use of culture conditions is solid-state fermentation (SSF) which has been shown to boost antibiotic titres in a broad range of production strains [2-3]. SSF is formed as any fermentation process occurring in low concentration of bountiful water, employing a natural substrate or an inert support [4]. In the SSF process, the solid substrate not only provender the nutrients to the culture, but also serves as an harborage for the production strains[5]. Application of fruit waste residues in solid state fermentation provides an alternative

way to replace the enlightened and expensive raw materials and the aggregate use of such materials will help to elucidate environmental pollutions [6]. Solid substrates conventionally fermented in the solid state include a diversity of fruit waste residues such as apple pomace **[**7**],** grape pomace [8],orange begas [9] and banana fruit pulp [10]. SSF deals with the utilization of fruit waste residues as its substrates. Application of fruit waste residues as substrates for SSF is extremely inexpensive and reduces pollution.Pineapple (*Ananas cosmosus*) is a tropical fruit which grows in countries which are located in the tropical and sub-tropical regions. Total pineapple production around the world is about 16 to 18 million tons [11]. Pineapple waste is a by-product of the pineapple processing industry and it consists of residual pulp, peels and skin. These wastes can create environmental pollution problems if not utilized properly. Recently there are investigations carried out on how to process these wastes. Pineapple peel is abundant in cellulose, hemicellulose and other carbohydrates [12]. The utilization of starchy materials in the place of the cheap refined sugar is economical for tetracycline production [13].

The "one- process parameter-at-a-time" approach is the traditional methods of optimization for obtaining high yield of the desired secondary metabolites such as antibiotics. However, this advance is time consuming and other process parameters involved at an unspecified constant level does not describe the combined effect of all the process parameters involved. These limitations of a single process parameter optimization process can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM) and Placket-Burmen Design (PBD).The application of design of experimental techniques which includes Placket-Burmen Design (PBD) and response surface methodology (RSM) in solid state fermentation process development can result in improved antibiotic yields, cut back process variability, near confirmation of the output response to nominal and target requirements and cut back development time and overall costs, it also helps in seeking optimal conditions and in building models to adjudicate the interactions among process variables for production of antibiotics. RSM can be used to adjudicate the relative significance of several affecting factors even in the presence of complex interactions [14- 17].

The aim of this work was to optimize tetracycline production by a novel fruit waste as a solid substrate, pine apple peel. The strategy chosen for enhancement of tetracycline titres in solid state fermentation was the application of a Placket-Burmen Design (PBD) in combination with central composite design.

2. Materials and methods

2 .1 Growth and maintenance of the production strain

Streptomyces aureofaciens NCIM 2417 was used as the production strain. The actinomycetes, *Streptomyces aureofaciens* NCIM 2417, obtained from the National Collection of Industrial Microorganisms, Pune, India. *Streptomyces aureofaciens* NCIM 2417 was well-kept in agar slants contained the following components (in g I^{-1}): glucose, 4; yeast extract, 5; malt extract, 10; $CaCO_,$, 2; agar, 15. The strain was well-kept on agar medium and subcultured every month. Inoculation medium contained (in g I^{-1}): sucrose, 30; yeast extract, 5; $MgSO_4$. $7H_2O$, 0.5; $NH_4(NO)_3$, 3; NaCI, 4; KH_2PO_4, 1; $CaCO_3$ 0.2 (final pH = 7) and 1 ml of this mycelial suspension was used to inoculate the solid culture medium.

2.2 Pineapple peel

Pineapple grown in the south canara area, in south-western India, was used as the sole nutrient source for the fermentation studies and the production of tetracycline antibiotics. Samples of fresh pineapple peel were collected from a local fruit processing industries and stored at−24◦C. For any

given series of experiments, sub-samples (250 g) were taken and dried in an oven at 60◦C for 48 h. The solid was then milled in a materialistic mill and sieved. The mean diameter (D50, the diameter below which 50% of the particles fall) of the milled pineapple peel was 0.74 mm.

2.3 Solid-state fermentation

Solid substrate was preheated to discharge moisture before use. An amount of 10 g of pineapple peel powder was added in the 500 ml Earlenmeyer flask. The pineapple peel powder was supplemented with peanut meal (according experimental design), calcium carbonate (according experimental design) and soluble starch (according experimental design). Initial moisture was familiarized to according experimental design; each flask was concealed with hydrophobic cotton and autoclaved at 121◦C for 20 min. Preliminary studies showed that no changes in moisture content of the substrate after autoclaving were detected. After cooling, each flask was inoculated with inoculum suspension formerly prepared (according experimental design) and incubated period (according experimental design) with incubation temperature (according experimental design) and initial pH (according experimental design).

2.4 Determination of tetracycline by HPLC

At the end of the fermentation process, the solid fermented material was soaked in 100 ml sterile phosphate buffer (pH 8.0) by palpitation for 30 min at 30^{O}C and was stored for 6 h in the refrigerator. The consequent extract was centrifuged and clear supernatant was used for the termination of tetracycline content by HPLC at 280 nm according to the method described by Gontier et al. 1996[18]. Agilent chromatograph module (280 nm Equipped with a UV-DAD detection wavelength and auto sampler) was used. Condition for tetracycline determination by HPLC (Column: 100 ˇ 4 mm Hypersil BDS C18, 3 µm; Mobile phase: water with sulfuric acid and acetonitrile; Gradient:start with 15 % B at 10min 60% acetonitrile; Flow rate: 0.5 ml/min; Column compartment: 25 ºC; Detector:;UV-DAD detection wavelength 280 nm).

2.5 Experimental design

Carbon sources, nitrogen sources and physical parameters for the production of the tetracycline have been screened by conventional ‘one-variable-at-a-time’ approach [19].

2.5.1 Plackett–Burman design

For screening purpose, seven independent process factors were screened in twelve combinations (Table 1) integrated according to the Plackett–Burman design [20]. All trials were performed in triplicate and the average of tetracycline yield (mg/g substrate) observations were considered as responses. Seven process parameters, namely; initial moisture (X1), initial pH (X2), incubation temperature(X3), incubation period (X4), calcium carbonate (X5), peanut meal (X6) and soluble starch (X7)were included in the model. The main effect of each process parameters was measured as the difference between the average of measurements made at the high setting (+) and the average of measurements observed at the low setting (-) of that process parameter.

Plackett–Burman experimental design is based on the first order model

$$Z = bo + \sum bixi \Rightarrow (1)$$

Where Z is the tetracycline yield (mg/g substrate), *bo* is the model intercept and *bi* is the linear coefficient, and *xi* is the level of the independent process parameters. This model does not

designate interaction among process parameters and it is used to screen and assess the crucial process parameters that alter the response.

2.5.2 Central composite design

Process factors touching tetracycline production were optimized using the central composite design which is a RSM [21]. Three process parameters, namely; peanut meal (X6), incubation period (X4) and soluble starch (X7) were included in the model. Each process parameter was examined at five different levels (relatively low, low, basal, high, relatively high) coded (a, -, 0, +, A) as given in Table 4. According to the practical design, 20 combinations were done and their observations were fitted to the following second order polynomial model:

$$Z = b_o + b_1X6 + b_2X4 + b_3X7 + b_{12}\ X6X4 + b_{13}X6X7 + b_{23}\ X4X7 + b_{11}X6^2 + b_{22}X4^2 + b_{33}X7^2 \Rightarrow (2)$$

Where Z is the tetracycline yield (mg/g substrate); X61,X4, and X7 are the independent process parameters as mentioned above; b_0 is the regression coefficient at centre point; b1, b_2 and b_3 are linear coefficients;b_{12}, b_{13} and b_{23} are second order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients.

The values of the coefficients, the optimum levels as well as R^2 were definite as mentioned above. The experiments were carried out using stalactical software, JMP10 (Trial version)

3. Results

3.1 Optimization by Plackett-Burman design (PBD)

The consideration of the seven process parameters, the initial moisture, initial pH, incubation temperature, incubation period, calcium carbonate, peanut meal and soluble starch for tetracycline production was investigated by PBD. The results showed the effects of these process parameters on the response and significant levels in Tables 2 and 3. The values chosen were based on a number of factors, including preliminary results (not shown), previous work on single-stage solid state fermentation of initial moisture, initial pH, incubation temperature, incubation period, calcium carbonate, peanut meal and soluble starch [13,19, 22- 25].

According to statistical analysis of the data by JMP (Trial version 10) software, the results showed that only peanut meal, incubation period and soluble starch had confidence levels above 95% ($p < 0.05$) and were well-advised to influence tetracycline production significantly. The others had confidence levels below 95% and hence were well-advised insignificant. In these results, R^2= 0.9465 indicated that 94.65% of the changeability in the response could be explained in the model.

PBD results indicated that the absolute contrast of peanut meal, incubation period were positive. Decreasing the two process parameters levels might result in higher production of antibiotic tetracycline. The absolute contrast of soluble starch was negative. Increasing the one process parameters levels might result in higher production of antibiotic tetracycline. From the Half normal (Fig.1) the process parameters viz., peanut meal (X6), incubation period (X4) and soluble starch (X7) were preferred and their optimal levels were identified further using response surface methodology.

3.2 Response surface methodology and the range of values chosen

In design of experiments the treatment combinations are at the centre, axial and the midpoints of edges of the process space, giving a geometry which suggests a sphere within the process space.

Here this method as used to determine the optimal values of three important process parameters in maximizing tetracycline yields. Thus, tetracycline yield was related to accompanying changes in peanut meal concentration (0.25, 0.5, 0.75, 1.0 and1.25%), incubation period (1.36, 2, 3, 4 and 4.63 in days) and the soluble starch (2.5, 3.0, 3.5, 4.0 and 4.5%) of the solid fermentation medium.

In order to examine for the optimum combination of major process factors, using central composite experimental design, a total of 20 experiments with different combination of peanut meal, incubation period and soluble starch were performed according to the CCD experimental plan. The actual values and coded values of the sovereign process parameters are given in Table 4. The results of experimental for studying the effects of three sovereign process parameters, viz., peanut meal, incubation period and soluble starch on tetracycline production are presented in Table 5 along with the observed and predicted response. The results were analyzed using the analysis of variance (ANOVA) as competent to the experimental design used. The calculated regression equation for the optimization of process parameters showed tetracycline production units were functions of the levels of peanut meal (X6, %), incubation period (X4, in day) and the soluble starch (X7, %).

By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the tetracycline yield (mg/g substrate):

$$Z = 6.3201\text{-}X6\ 2.2844 + X4\ 0.7874 - X7\ 3.2463 + X6*X4\ 1.3762 + X6*X7\ 3.4012 + X4*X7\ 2.3162 + X6*X6\ 1.9598 - X4*X4\ 1.0589 + X7*X7\ 1.2923 \Rightarrow (3)$$

Where Z is the response, that is, the tetracycline production units and X6,X4 and X7 are the coded values of the sovereign process parameters, viz., peanut meal, incubation period and soluble starch, respectively.

The results of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 6. The goodness of fit of the model based on RSM can be checkered by the coefficient of determination (R^2), which provides a measure of how much changeability in the observed response values can be explained by the experimental process parameters and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the better the model is and the better it predicts the response [26]. In this case, the value of the determination coefficient (R^2= 0.9299) implies that the sample changeability of 92.99% for tetracycline production is attributed to the given independent processes parameters. The R^2 also indicates that only 7% of the total changeability are not explained by the model. The value of the adjusted determination coefficient (adjusted R^2= 0. 0.8335) is also high to uphold a high significance of the model (Table 7). These measures indicate that the nearness and general ability of the polynomial model are good and that analysis of the response trends using the model is admissible. The ANOVA of the quadratic regression model demonstrates that the model is highly significant, as is assured from the low P-value of the Fisher's F-test (F model, mean square regression/mean square residual = 9.65) [(Pmodel>F) = 0.0018]. Moreover, the computed F-value is much superior than the tabulated F-value($F_{0.01(9,5)}$= 10.15), indicating that the treatment differences are highly significant. This proves that the model equation as verbalized in Eq. (3) provides a appropriate model to designate the response of the experiment pertaining to tetracycline production. The model also showed a statistically insignificant lack of fit, as is assured from the lower calculated F-value (0.1984) than the tabulated F-value ($F_{0.01(9,5)}$= 10.15) even at the 99% confidence level. The plot of predicted versus experimentally measured tetracycline yield in Fig. 2. also approves the sufficiency of the model since all the points are

placed around the solid line representing x=y. The model was found to be effective for prediction within the range of process parameters employed.

The regression coefficients, along with the corresponding P-values, for the model of the tetracycline yield by *Streptomyces aureofaciens* NCIM 2417, are presented in Table 6. The student's t-test and Prob-values were used as a tool to ascertain the significance of each coefficient, which also indicated the interaction strength between each dominant process factors. The larger the breadth of the t-value and smaller the Prob-value, the more significant is the corresponding coefficient [27]. It can be observed from the degree of significance (Table 8) that the linear (peanut meal and soluble starch), quadratic (peanut meal) and interaction (between peanut meal and soluble starch and between incubation period and soluble starch) ($P< 0.01$) are more significant than the other process parameters. These suggest that the levels of peanut meal, incubation period and soluble starch have a direct association with the tetracycline production in this particular complex solid medium. Since the concentration of soluble starch is also very significant in the quadratic level ($P< 0.0104$), meaning that it can act as limiting nutrients and a little deviation in their concentrations will alter the tetracycline production. The carbon and nitrogen have a significant effect on tetracycline production at a second-order level. This means that the mineral stimulates the production of tetracycline. The interactions between the preferred process parameters have insignificant effects on tetracycline production.

The three-dimensional response surface plots described by the regression model are drawn to illustrate the effects of the sovereign process parameters, and interactive responding to the combined effects of soluble starch–incubation period, soluble starch–peanut meal, and incubation period – peanut meal, respectively, from which the tetracycline production for different levels of the process parameters can be predicted.

The three-dimensional response surfaces plot at various peanut meal and incubation period are plotted in Fig. 3. From the graph it was ascertained that the content of tetracycline decreased sharply when the peanut meal below 0.5% concentration. However the content of tetracycline increased with the increase in incubation period when keeping the optimum peanut meal concentration, where soluble starch was kept at centre point values. The response surface plot for the effect of peanut meal and incubation period interaction for tetracycline production established the computed optimum values for the sovereign process parameters.

Fig.4 depicted the three dimensional surface plot showing the response surface from the interaction between peanut meal and soluble starch while keeping incubation period at its zero level. It can be seen from Figure, when concentration of soluble starch was at a fixed level, the tetracycline production increased with the increasing concentration of peanut meal but decreased beyond the range. On the contrary, when concentration of peanut meal was at a fixed level, the effect of concentration of soluble starch on the tetracycline production response was similar to that of concentration of peanut meal. These indicated that maximum tetracycline could be obtained in the middle concentration of both peanut meal and soluble starch.

Fig.5 showed the effect of incubation period and soluble starch on tetracycline production while the peanut meal was fixed at its middle level. It was obvious that higher level of incubation period and higher concentration of soluble starch increase the yield of tetracycline. This study proves that the production of tetracycline production as secondary metabolites is thoroughly influenced by the kind and quality of nutritional elements available and environmental processes parameters.

3. 3 Validation of the model

On the basis of production medium optimization, the model predicted the highest production of tetracycline as 18.78 mg/g substrate, in the presence of 0.4% peanut meal, day 2 incubation period and 2% soluble starch. To verify the predicted results, a validation experiment was performed in triplicate tests. Under the optimized condition, the observed experimental yield of mean tetracycline was 17.98 mg/g substrate, which is a 2.87-fold increase as compared to the yield in non-optimized production media, suggesting that experimental and predicted values of tetracycline yield were in good agreement. This result therefore verified the predicted values and the effectiveness of the model, indicating that the optimized production medium favors the production of tetracycline.

4. Discussions

The gain of solid-state fermentation is that the process requires low equipment and economical, enough fruit waste as solid substrate [28]. Pineapple peel, an inexpensive and easily available fruit-residue as solid substrate, was the most suitable carbon source for tetracycline production by solid state fermentation and showed higher significant effect. The optimal environment for solid-state fermentation includes many processes parameters such as incubation period, initial pH, initial moisture content, concentration of inoculums. incubation period. nitrogen and carbon sources for fermentation [29].

Production strain *Streptomyces aureofaciens* is a well-known tetracycline production strain [13,19, 23, 30] and therefore, an enough of nutrients and processes conditions are required for cell growth and metabolism. The influences of initial pH, initial moisture, incubation temperature and calcium carbonate were not significant in this screening experiment, because this test was carried out close to the optimal conditions of these four processes parameters [13,19,23,30]. Some authors studied the effects of less expensive organic nitrogen source such as peanut meal. Agenes et al., 2005 [19]reported that peanut meal affects the tetracycline yield. A considerable increase in tetracycline production by the addition of soluble starch was already reported in the case of *S. aureofaciens* [13,19,23]. Agenes et al., 2005 [19]; Yang and Swei 1996[23]; Yang and Yuan 1990[30];Yang and Ling 1989 [13] were observed that increase in tetracycline yield from day 2 incubation period. The optimization of the fermentation conditions of *Streptomyces aureofaciens* NCIM 2417 not only increased the tetracycline production, but also significantly altered the metabolite profile. However, the association between the increase of tetracycline production and up regulation of the metabolites of *Streptomyces aureofaciens* and whether the optimization of fermentation conditions could break previously enigmatic metabolites have not been discovered yet. In order to clarify these questions, further work will be guided on characterization of the metabolites of *Streptomyces aureofaciens* NCIM 2417 under optimized condition.

In the present study, three processes parameters (peanut meal, incubation period and soluble starch) were identified to be the most influencing condition for decorative tetracycline production by using Placket Burmen design, and then their optimal condition were obtained by using response surface methodology such as central composite design. The production of tetracycline increased to 17.98 mg/g substrate under the optimum conditions, with 2.87-fold increase compared to the nonoptimized medium. On comparison with appropriate references [13,19,23,30], a significant higher yield tetracycline was obtained in our studies. Response surface designs are commonly used to explore nonlinear relationships between independent processes parameters (medium condition) and the dependent (tetracycline yield) variables [31,32]. It was evident that the response surface methodology had the benefit of identifying the most significant medium composites and their optimal levels, and thus was useful for operating

the fermentation towards the concentration of precious secondary metabolites. To our knowledge, this is the first report of decorative tetracycline production using response surface methodology. However, this paper is only an attempt to demonstrate the applicability of statistical theories to the study of tetracycline production by solid state fermentation processes, and the next step for decorative tetracycline yield should be the production in a bioreactor under optimized conditions.

This study proves that the production of tetracycline as secondary metabolites is thoroughly influenced by the kind and quality of processes conditions.

5. Conclusions

Production medium of tetracycline fermentation by *Streptomyces aureofaciens* NCIM 2417 was optimized by using the Plackett–Burman design and CCD, and the maximum yield of tetracycline in the Earlenmeyer flask could reach 17.98 mg/g substrate. The great improvement of the yield may be attributed to both the optimum medium and the overproducing strain.

Statistical experimental designs appear to be a powerful tool for optimization of process parameters to improve the tetracycline production. Considering that few reports centered on optimization of process factors for tetracycline production using statistical experimental methodologies, this study could also serve as a guide for optimization of tetracycline production.

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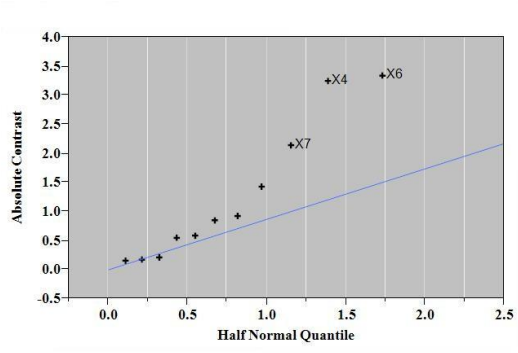

Figure1. Half normal plot showing the absolute contrast of process parameters on tetracycline production.

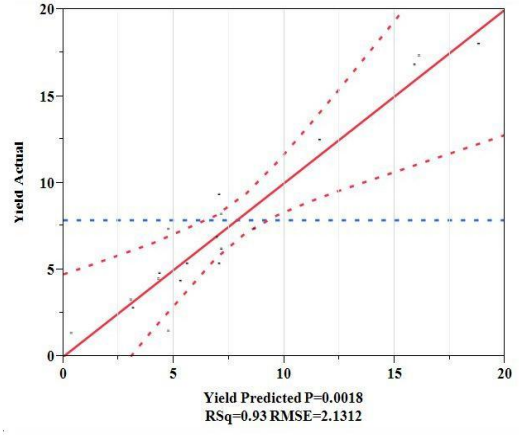

Figure 2. Correlation between observed and predicted values of tetracycline yield.

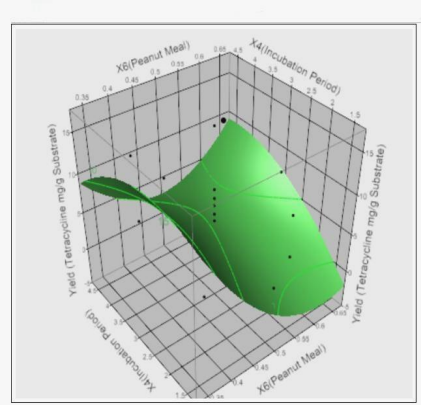

Figure 3. Response surface plot of tetracycline by *Streptomyces aureofaciens* NCIM 2417 : peanut meal vs. incubation period.

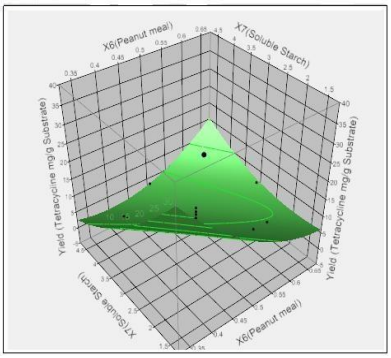

Figure 4. Response surface plot of tetracycline by *Streptomyces aureofaciens* NCIM 2417 : peanut meal vs. soluble starch.

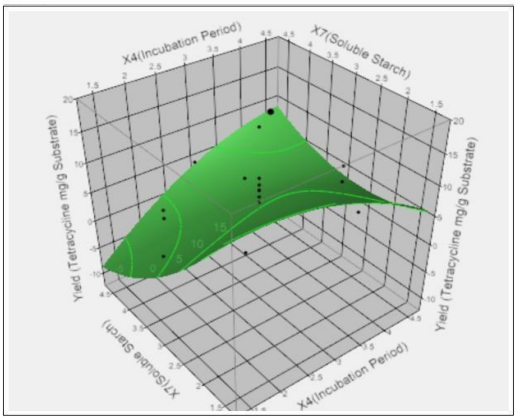

Figure 5. Response surface plot of tetracycline by *Streptomyces aureofaciens* NCIM 2417 : incubation period vs. soluble starch.

Table 1. The Plackett-Burman design for screening process parameters (range and levels) in tetracycline production.

S No	Variables with designate	Lower level	Higher level
1	X1 Initial Moisture	40%	60%
2	X2 Initial pH	6.0	8.0
3	X3 Incubation Temperature	30°C	35°C
4	X4 Incubation Period	Day 5	Day 10
5	X5 Calcium Carbonate	1%	3%
6	X6 Peanut Meal	1%	3%
7	X7 Soluble Starch	1%	3%

Table 2. The Plackett-Burman design matrix for screening of important process parameters (in coded and uncoded levels) with tetracycline production as response. (X1-initial moisture ,X2-initial pH , X3-incubation temperature, X4-incubation period , X5-calcium carbonate , X6-peanut meal and X7- soluble starch).

Pattern	X1	X2	X3	X4	X5	X6	X7	Tetracycline (mg/g Substrate)
−−+−−+	40%	6	30°C	Day 10	1%	1%	3%	11.76
+++++++	60%	8	35°C	Day 10	3%	3%	3%	17.34
+−−−+−−	60%	6	30°C	Day 5	3%	1%	1%	4.78
+−+++−−	60%	6	35°C	Day 10	3%	1%	1%	11.89
−+−−+−+	40%	8	30°C	Day 5	3%	1%	3%	1.43
+−−+−++	60%	6	30°C	Day 10	1%	3%	3%	14.45
−+++−−−	40%	8	35°C	Day 10	1%	1%	1%	16.89
−−+−−+−	40%	6	35°C	Day 5	1%	3%	1%	15.23
++−−−+−	60%	8	30°C	Day 5	1%	3%	1%	16.98
+++−−−+	60%	8	35°C	Day 5	1%	1%	3%	3.54
−−+−+++	40%	6	35°C	Day 5	3%	3%	3%	8.89
−+−+++−	40%	8	30°C	Day 10	3%	3%	1%	17.43

Table 3. Absolute Contrast, t-values and p-Value obtained from the yields of tetracycline in the Screening experiments (Placket Burman Design) in solid state fermentation.

Term	Absolute Contrast	Lenth t-Ratio	Individual p-Value
X6	3.33583	3.84	0.0123
X4	3.24250	3.73	0.0135
X7	-2.14917	-2.47	0.0370
X5	-1.42417	-1.64	0.1095
X3	0.57917	0.67	0.5002
X2	0.55083	0.63	0.5840
X1	-0.22083	-0.25	0.8272
X6*X4	-0.92250	-1.06	0.2655
X6*X7	0.17083	0.20	0.8663
X4*X7	0.85417	0.98	0.2982
X6*X5	0.16417	0.19	0.8711

* p value less then 0.05indicate model terms are significant.

Table 4. Experimental range and levels of the independent processes parameters (-α - negative axial level,-1-low level, 0-center level,+1-high level and +α- positive axial level).

Independent process parameters	Code	Range and levels				
		$-\alpha$	-1	0	+1	$+\alpha$
Peanut Meal (%)	X6	0.25	0.5	0.75	1.0	1.25
Incubation Period (in days)	X4	1.36	2	3	4	4.63
Soluble Starch (%)	X7	2.5	3.0	3.5	4.0	4.5

Table 5. Central composite design with three independent process parameters .

Pattern	Block	X6	X4	X7	Actual Yield	Predicted Yield
−−−	1	0.4	2	2	17.98	18.78
+−+	1	0.6	2	4	1.32	0.34
000	1	0.5	3	3	1.45	4.75
000	1	0.5	3	3	7.34	4.75
++−	1	0.6	4	2	4.78	4.35
−++	1	0.4	4	4	4.45	4.31
000	2	0.5	3	3	5.35	7.05
−−+	2	0.4	2	4	2.78	3.16
−+−	2	0.4	4	2	14.34	15.27
+++	2	0.6	4	4	12.45	11.60
000	2	0.5	3	3	9.34	7.05
+−−	2	0.6	2	2	6.87	6.96
000	3	0.5	3	3	6.23	7.14
0A0	3	0.5	4.63	3	5.34	5.60
00a	3	0.5	3	1.36	16.78	15.89
000	3	0.5	3	3	8.23	7.14
A00	3	0.66	3	3	7.34	8.63
0a0	3	0.5	1.36	3	3.24	3.03
a00	3	0.33	3	3	17.34	16.09
00A	3	0.5	3	4.63	4.34	5.28

Table 6. Analysis of variance and regression for tetracycline production (DF-degree of freedom, SS-sum square, MS-mean square and F-Fishure`s ratio).

Source	DF	SS	MS	F ratio	Prob>F
Model	11	482.24	43.84	9.65	0.0018
Error	8	36.33	4.54		
C. Total	19	518.58			
Lack of Fit	5	9.03	1.80	0.1984	0.9431
Pure Error	3	27.30	9.10		
Total Error	8	36.33			

a Value of "p>F" less than 0.05 indicate model terms are significant.

Table 7. Fit statistics for Z .

	Master model	Predictive model
R Square	0.9299	0.9299
R Square Adj	0.8335	0.8335
Root Mean Square Error	2.1312	2.1312
Mean of Response	7.8645	7.8645

Table 8. Estimated linear, squared and interactive effects ranked by magnitude from analysis of central composite design (t-student t test).

Term	Estimate	t Ratio	Prob>|t|
Intercept	6.3201	7.2717	<.0001
X6(0.4,0.6)	-2.2844	-3.9141	0.0045
X4(2,4)	0.7874	1.3492	0.2142
X7(2,4)	-3.2463	-5.5621	0.0005
X6*X4	1.3762	1.8265	0.1052
X6*X7	3.4012	4.5139	0.0020
X4*X7	2.3162	3.0740	0.0153
X6*X6	1.9598	3.3418	0.0104
X4*X4	-1.0589	-1.8057	0.0846
X7*X7	1.2923	2.2036	0.0587
Block[1]	-1.5622	-2.2464	0.0812
Block[2]	0.7394	1.0633	0.3187