Improved antimicrobial compound production by a new isolate Streptomyces hygroscopicus MTCC 4003 using Plackett-Burman design and response Surface methodology

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Abstract:
An active strain, isolated from soil of Chhattisgarh, India, showed broad-spectrum antimicrobial activity against various pathogenic bacteria and fungi in glucose soybean meal broth. Strain was characterized as Streptomyces hygroscopicus MTCC 4003 based on 16S rRNA sequencing from Microbial Type culture Collection (MTCC), IMTECH, Chandigarh, India. Identification of the purified antimicrobial compound was done by using Infra-red (IR), Mass, Ultraviolet (UV), 1H and 13C nuclear magnetic resonance (NMR) spectra. Plackett-Burman design (PBD) and response surface methodology (RSM) methods were used for the optimization of antibiotic production. Effects of the four medium components soybean meal, glucose, CaCO3 and MgSO4 showed positive effect on antibiotic production, were investigated with the help of PBD. The individual and interaction effects of the selected variables were determined by RSM using central composite design (CCD). Applying statistical design, antibiotic production was improved nearly ten times (412 mg/ L) compared with unoptimized production medium (37 mg/ L).

Key words: Streptomyces hygroscopicus, Plackett-Burman design (PBD), Central composite design, Response surface/ contour plots, and Antibiotic production

Background:
With the extensive use of antibiotics, the severe problem of antibiotic resistance has become far-reaching. Therefore, intensive search for new antibiotics is required on a global basis. Production of secondary metabolites by microorganisms differs qualitatively and quantitatively depending on the strains and species of microorganisms used as well as on their nutritional and cultural conditions [1] and as fermentation moves into lower-value, higher-volume substrates, it becomes necessary to maximize the efficiency and minimize costs by using waste by-products to complete effectively with traditional high-value, low-volume compounds [2]. To make the production of antibiotics feasible, it is necessary to optimize the antibiotic production conditions. This paper reports the influence of medium components on antibiotics production by Streptomyces hygroscopicus a new soil isolate using a statistical design.

Methodology:
Statistical optimization using Plackett-Burman design (PBD)
Streptomyces hygroscopicus, was maintained on ISP-2 slants containing (g/ L) yeast extract 4.0, malt extract 10, glucose 4.0, CaCO3 2.0 and agar powder 20. Submerged fermentation was carried out by cultivating the active isolate for 3 days at 28 °C, 180 rpm in 1 L Erlenmeyer flask with 200 ml production medium comprising of (g/ L) soybean meal 10, CaCO3 3, MgSO4.7H2O 0.5, (NH4)2HPO4 0.5, NaCl 3, K2HPO4 1, glucose 15, pH 6.9-7.0. To identify the most important medium
components for antibiotic production Plackett-Burman design (PBD) was used in the present study [3]. The Plackett-Burman design (PB) was based on the first-order model, with no interaction among the factors [4]. In this experiment, four independent and three dummy variables were selected for the screening in 8 trials. Each variable was represented at two levels high (H) and low (L) Table 1 (see supplementary material). Dummy variables were used in design to estimate error in experiment. The effect of each variable was determined by the following equation:

\[ E(x_i) = \frac{2(\sum M_{Hi} - \sum M_{Li})}{N} \rightarrow (1) \]

where \( E(x_i) \) is the concentration effect of tested variable, \( M_{Hi} \) is the antibiotic activity from the trials where variable was present at the high concentration, \( M_{Li} \) is the antimicrobial activity from trials where the variable present at the low concentration, and \( N \) is the total number of trials. Experimental error was estimated by calculating the variance among the dummy variables as given below

\[ V_{eff} = \sum (E_d)^2 / n \rightarrow (2) \]

where \( V_{eff} \) is the variance of the concentration effect, \( E_d \) is the concentration effect for the dummy variable, and \( n \) is the number of dummy variables. The standard error (SE) of the concentration effect was the square root of the variance of the effect, i.e., \( SE = \sqrt{V_{eff}} \). The significance level (p value) of each concentration effect was determined using Student’s t-test

\[ SE = \sqrt{V_{eff}} \rightarrow (3); b_{x(i)} = E(x_i) / SE \rightarrow (4) \]

Three variables were found to be most effectual components for the antibiotic production on the basis of PBD, were selected to identify the optimized conditions for the maximum production of antimicrobial components using central composite design (CCD) and response surface methodology (RSM) [5]. Optimization of the selected medium components by RSM using CCD response surface designs are used to explore non-linear relationships between independent (medium components) and dependent (antibiotic yield) variables. These relationships facilitate in selecting the optimum medium components concentrations for production of higher amount of product [5]. Total twenty experiments with eight cube points, six star points and six replicates of the central point were employed to fit the second order polynomial model. Design along with the range and levels of the selected variables are shown in Table 2 (see supplementary material).

Following regression equation was developed by the application of RSM showing a relationship between the coded units of the medium components and the logarithmic values of antibiotic yield.

\[ Y = b_0 + b_{x1}x_1 + b_{x2}x_2 + b_{x3}x_3 + b_{x1x2}x_1x_2 + b_{x1x3}x_1x_3 + b_{x2x3}x_2x_3 + b_{x1x2x3}x_1x_2x_3 \rightarrow (5) \]

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. ‘Statistica 7.0’ was used for the graphical and statistical analysis of the data obtained from CCD. The optimum values of the selected variables were obtained by analyzing the response surface/contour plots and also by analyzing the regression equation [6].

**Discussion:**

Active purified compound from Streptomyces hygroscopicus showed activity against Gram positive and Gram negative bacterial as well as fungal pathogens (Table 1) and chemically characterized as hygromycin. Absorbance at 203 nm in the UV-vis spectra, suggested the presence of amide group in the structure. Presence of M+H peak at 527.47 confirmed that the molecular weight was corresponding to the other reported hygromycin b. Structure elucidation has been done with the detailed analysis of IR, 1H, 13C NMR data and elemental analysis which is being communicated elsewhere (data not shown). Chemical structure of hygromycin is represented in (Figure 1).
significant [7-9]. Higher significance of linear, quadratic and interactive effects of soybean meal, glucose, and CaCO₃ (p x₁ = 0.000013, p x²_1 = 0.000001, p x x₁ = 0.000000, p x x₁ x₂ = 0.000003, p x₁ x₁ x₁ = 0.000114, p x₁ x₁ x₁ x₁ = 0.002580, p x₁ x₁ x₁ x₁ = 0.002735) suggested that they have a direct relationship with the antibiotic production. Interaction effect of x₁ x₂ and x₁ x₃ has low significant value, however linear effect of p x₃ had no significant effect on antibiotic production (p x₃ = 0.241140). Analysis of variances (ANOVA), to validate the regression coefficient was performed which suggested the adequacy of the second order response surface model Table 5 (see supplementary material). The Fisher F-test with a very low probability value (F = 55.67784, p = 0.000000) shows the high statistical significance of the regression model [6]. High value of correlation coefficient (R = 0.990169) explains an excellent correlation between the independent variables i.e. medium components [7].

The goodness of the fit of the model is explained by the determination coefficient (R² = 0.9804343) which indicates that the second order polynomial model eq. 6 could explain 98% of the total variation and only 2% of the total variations were not explained by the model. Higher value of the adjusted determination coefficient (adj. R² = 0.962825) further point outs the high significance of the model [3, 12]. The application of RSM yielded following regression equation showing positive linear and negative quadratic effect. This equation expressed a relationship between the logarithmic values of antibiotic yield and concentration of the medium components. Since the overall regression equation is significant hence the terms which are individually non-significant are also considered in the equation [1, 6, 11].

Prediction equation for antibiotic yield:

\[ Y = 0.012746 + 0.039455x₁ - 0.001300x₁² + 0.031763x₂ - 0.000807x₂² - 0.004645x₃ - 0.002750x₃² - 0.001312 x₁x₂ + 0.001186x₁x₃ + 0.001174 x₂x₃ \rightarrow (6) \]

3D graphs assist understanding of the main as well as the interaction effects of two factors. 3D graphs were created for the pair-wise combination of the three factors while keeping the other one at its optimum levels for antibiotic production (Figure 2). It is obvious from the plots that the higher concentration of glucose, middle concentration of the soybean meal and CaCO₃ are responsible for the higher antibiotic production where the production of crude was predicted 405 mg/ L. The predicted yield was verified by performing an experiment with the optimized concentrations in basal medium and the antibiotic production was around 412 mg/ L which was found to be close to the predicted value and ten times more than with the normal unoptimized production medium (37 mg/ L).

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### Table 1: High (H) and low (L) values of the independent variables in the PBD analysis

| Independent variables | H | L |
|-----------------------|---|---|
| X1 Soybean meal (g/L) | 20| 1 |
| X2 CaCO₃ (g/L)       | 6 | 1 |
| X3 MgSO₄ (g/L)       | 1.5 | 0.5 |
| X4 Glucose (g/L)     | 25| 5 |

### Table 2: Plackett-Burman design and result

| Runs | X1 (Soybean meal) | X2 (CaCO₃) | X3 (MgSO₄) | X4 (Glucose) | D1 (NH₄H₂PO₄) | D2 (NaCl) | D3 (K₂HPO₄) | Yield (g/L) |
|------|------------------|------------|------------|--------------|---------------|-----------|------------|-------------|
| 1    | H                | H          | H          | H            | L             | L         | L          | 0.030       |
| 2    | L                | H          | H          | H            | L             | L         | L          | 0.027       |
| 3    | L                | L          | H          | H            | L             | H         | L          | 0.031       |
| 4    | H                | L          | L          | H            | H             | H         | L          | 0.037       |
| 5    | L                | H          | L          | H            | H             | H         | L          | 0.025       |
| 6    | H                | L          | H          | L            | H             | H         | H          | 0.021       |
| 7    | H                | H          | L          | H            | L             | L         | H          | 0.033       |
| 8    | L                | L          | L          | L            | L             | L         | L          | 0.019       |

### Table 3: CCD (coded and uncoded test variables) with observed and predicted yield of antibiotic

| Run No | Soya g/L | Glucose g/L | CaCO₃ g/L | Yield (g/L) |
|--------|----------|-------------|-----------|-------------|
|        | Coded    | Coded       | Coded     | Observed    |
| 1      | 1        | 15          | 1         | 0.278000    |
| 2      | 1        | 15          | -1        | 0.352300    |
| 3      | -1       | 5           | 1         | 0.390898    |
| 4      | 1        | 15          | 1         | 0.287607    |
| 5      | 1        | 15          | -1        | 0.302140    |
| 6      | -1       | 5           | 1         | 0.340000    |
| 7      | -1       | 5           | -1        | 0.215464    |
| 8      | -1       | 5           | -1        | 0.320110    |
| 9      | -2       | 0.001       | 0         | 0.254665    |
| 10     | 0        | 10          | -2        | 0.210000    |
| 11     | 0        | 10          | 0         | 0.319595    |
| 12     | 2        | 20          | 0         | 0.302323    |
| 13     | 0        | 10          | 2         | 0.320000    |
| 14     | 0        | 10          | 0         | 0.205359    |
| 15     | 0        | 10          | 0         | 0.412200    |
| 16     | 0        | 10          | 0         | 0.404243    |
| 17     | 0        | 10          | 0         | 0.404243    |
| 18     | 0        | 10          | 0         | 0.404243    |
| 19     | 0        | 10          | 0         | 0.404243    |
| 20     | 0        | 10          | 0         | 0.404243    |

### Table 4: Estimated regression coefficients for yield (antibiotic production)

| Coefficient | SE  | t-value | p-value |
|-------------|-----|---------|---------|
| Intercept   | -0.149831 | 0.064303 | -2.3301 | 0.042044 |
| x₁          | 0.037031  | 0.004681  | 7.9109  | 0.000013 |
| x₂          | -0.001269 | 0.000103  | -12.2863 | 0.000000 |
| x₃          | 0.051065  | 0.004953  | 11.1188  | 0.000001 |
| x₄          | -0.003104 | 0.000103  | -13.5927 | 0.000000 |
The p-values less than 0.05 are significant

**Table 5:** Analysis of variance (ANOVA) for the quadratic model

| Source      | SS     | DF | MS      | F-value | p-value |
|-------------|--------|----|---------|---------|---------|
| Regression  | 0.083461 | 9  | 0.009273 | 55.67784 | 0.000000 |
| Residual    | 0.001666 | 10 | 0.000167 |         |         |

SS: sum of squares; DF: degree of freedom; MS: mean square; R = 0.990169; R² = 0.980434; R² (adj) = 0.962825