BIOINFORMATION
Discovery at the interface of physical and biological sciences

open access www.bioinformation.net Hypothesis

Volume 8(21)

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

Neha Singh* & Vibhuti Rai

Biochemistry and Microbiology laboratory, School of Studies in Life Sciences, Pt. Ravishsankar Shukla University, Raipur, 492010, India; Neha Singh – Email: nevi0007@gmail.com; *Corresponding author

Received October 09, 2012; Accepted October 15, 2012; Published October 31, 2012

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), ^{1}H and ^{13}C 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, $CaCO_3$ and $MgSO_4$ 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, $CaCO_3$ 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, $CaCO_3$ 3, $MgSO_4.7H_2O$ 0.5, $(NH_4)_2HPO_4$ 0.5, NaCl 3, K_2HPO_4 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:

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

where E(xi) is the concentration effect of tested variable; M_{IH} is the antibiotic activity from the trials where variable was present at the high concentration, M_{IL} 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 = $\sqrt{Vef}$. The significance level (*p* value) of each concentration effect was determined using Student's t-test

$$SE = \sqrt{V_{eff}} \rightarrow (3); t_{(X1)} = E_{(X1)} / SE \rightarrow (4)$$

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 replicas of the central point were employed to fit the second order polynomial model. Design along with the range and levels of the three 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.

$$Y = b_0 + b_1x_i + b_2x_{ii} + b_3x_{iii} + b_4x^2_i + b_5x^2_{ii} + b_6x^2_{iii} + b_7x_i.x_{ii} + b_8x_i.x_{iii} + b_9x_{ii}.x_{iii} \rightarrow (5)$$

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, ^{1}H, ^{13}C NMR data and elemental analysis which is being communicated elsewhere (data not shown). Chemical structure of hygromycin is represented in (**Figure 1**).

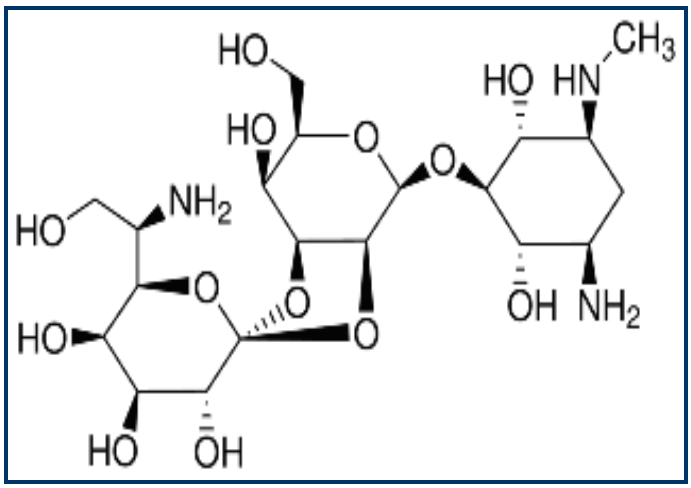

Figure 1: Chemical structure of hygromycin b

Screening of important components for the antibiotic production

Antibiotic production with normal unoptimized medium was found to be 37 mg/L. For medium optimization the effects of various medium components at different concentrations were investigated with the help of PBD, in which probability values less than 0.05 ($p < 0.05$) indicated significance of the model term whereas *p*-values greater than 0.1 ($p > 0.1$) indicated model terms were not significant. Therefore the medium components, soybean meal (t = 4.02, $p = 0.001$), $CaCO_3$ (t = 7.14, $p = 0.0001$) and glucose (t = 3.21, $p = 0.001$) with higher t-value and lower *p* values were considered as significant components for optimum antibiotic production **(Table 2)**.

Optimization of the selected medium components by statistical experimental design

CCD is an important tool to determining the optimal level of medium constituents and their interaction. Based on the PBD soybean meal, glucose and $CaCO_3$ were selected for their remarkable effect on the antibiotic production at CCD was used for further optimization. The experimental responses of the RSM for studying the effect of soybean meal, glucose and $CaCO_3$ on the antibiotic production are summarized in **Table 3 (see supplementary material)**. The regression coefficient of each variable in terms of linear, quadratic and interaction along with t and *p*-values are shown in **Table 4 (see supplementary material)**. Coefficients with lower p-values ($p < 0.05$) are more

significant **[7-9]**. Higher significance of linear, quadratic and interactive effects of soybean meal, glucose and $CaCO_3$ (p x_i = 0.000013, p x^2= 0.000001, p x^2_i = 0.00000, p x^2_{ii} = 0.00000, p x^2_{iii} =0.000003, p $x_i.x_{ii}$ = 0.000114, p $x_i.x_{iii}$ = 0.002580, p $x_{ii}.x_{iii}$ = 0.002735) suggested that they have a direct relationship with the antibiotic production. Interaction effect of $x_i.x_{iii}$ and $x_{ii}.x_{iii}$ has low significant value, however linear effect of p x_{iii} had no significant effect on antibiotic production (p x_{iii} = 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 = 0.00000) 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]**.

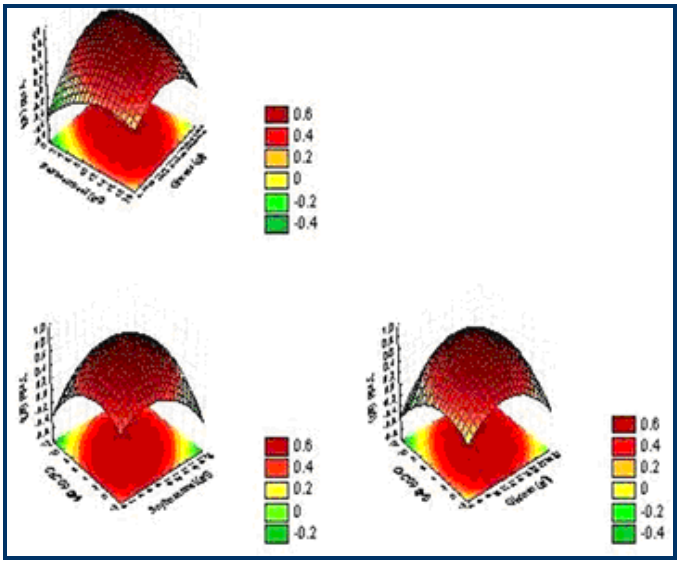

Figure 2: Response surface plots showing effect of independent variables soybean meal, glucose and calcium carbonate concentrations on antibiotic production

The goodness of the fit of the model is explained by the determination coefficient (R^2 = 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. R^2 = 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:

$$Y = 0.012746+0.039455x_i -0.001300x^2_i +0.031763x_{ii} -0.000807x^2_{ii} -0.004645x_{iii} -0.002750x^2_{iii} -0.001312\ x_i.x_{ii} +0.001186x_i.x_{iii} +0.001174\ x_{ii}.x_{iii} \rightarrow (6)$$

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 $CaCO_3$ 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).

References:

[1] Mao *et al*. *Proc Biochem*. 2007 **42**: 878
[2] Khan *et al*. *Ind J Exp Biol*. 2006 **44:** 151
[3] Devillers *et al*. *Chemosphere*. 1989 **19:** 1693
[4] Yee L & Blanch HW, *Biotechnol Bioeng*. 1993 **41:** 221 [PMID: 18609541]
[5] Adinarayana K & Ellaiah P, *J Pharm Pharm Sci*. 2002 **5:** 272 [PMID: 12553896]
[6] Lam *et al*. *J Ind Microbiol*. 1989 **4:** 105
[7] Bauer AW *et al*. *AMA Arch Intern Med*. 1959 **104:** 208 [PMID: 13669774]
[8] Hamedi *et al*. *Biotechnol Lett*. 2002 **24:** 697
[9] John RP *et al*. *Appl Microbiol Biotechnol*. 2007 **74:** 524 [PMID: 17225102]
[10] http://www.stat.ufl.edu/~ufakhuri/pdf/Publish08.pdf
[11] Chen *et al*. *J Climate* 2002 **15:** 3698
[12] Elibol M, *Process Biochem*. 2004 **39:** 1057

Edited by P Kangueane
Citation: Singh & Rai, Bioinformation 8(21): 1021-1025 (2012)

BIOINFORMATION

open access

Supplementary material:

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	$CaCO_3$ (g/L)	6	1
X3	$MgSO_4$ (g/L)	1.5	0.5
X4	Glucose (g/L)	25	5

Table 2: Plackett-Burman design and result

Runs	X1 (Soybean meal)	X2 ($CaCO_3$)	X3 ($MgSO_4$)	X4 (Glucose)	D1 $(NH_4)_2HPO_4$)	D2 (NaCl)	D3 (K_2HPO_4)	Yield (g/L)
1	H	H	H	L	H	L	L	0.030
2	L	H	H	H	L	H	L	0.027
3	L	L	H	H	H	L	H	0.031
4	H	L	L	H	H	H	L	0.037
5	L	H	L	L	H	H	H	0.025
6	H	L	H	L	L	H	H	0.021
7	H	H	L	H	L	L	H	0.033
8	L	L	L	L	L	L	L	0.019
Difference	19	9	1	20	0.5	4	1	
Effect	4.75	2.25	0.25	5	0.125	1	0.25	
Mean square	2.82	0.632	0.007	3.125	0.0019	0.125	0.007	
SE	0.077	0.077	0.077	0.077	0.077	0.077	0.077	
t-test	4.02	3.21	0.35	7.14	0.178	1.42	0.35	
p-value	0.001	0.001	0.759	0.0001	0.149	0.18	0.759	

H = high concentration of the components; L = low concentration of the components, D = dummy variable, p value less than 0.05 are significant.

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

Run No	Soya		Glucose		$CaCO_3$		Yield (g/L)		
	Coded	g/L	Coded	g/L	Coded	g/L	Observed	Predicted	Residual
1	1	15	1	20	1	3	0.278000	0.294206	0.016206
2	1	15	-1	10	-1	3	0.352300	0.351816	0.000484
3	-1	5	1	20	-1	3	0.390898	0.386878	0.004020
4	1	15	1	20	-1	3	0.287607	0.294206	-0.00659
5	1	15	-1	10	1	9	0.302140	0.305645	-0.00350
6	-1	5	1	20	1	9	0.340000	0.339969	0.000031
7	-1	5	-1	10	1	9	0.215464	0.192144	0.023320
8	-1	5	-1	10	-1	3	0.320110	0.319595	0.000515
9	-2	0.001	0	15	0	6	0.254665	0.268352	-0.01368
10	0	10	-2	5	0	6	0.210000	0.220150	-0.01015
11	0	10	0	15	-2	0.001	0.365480	0.356327	0.009153
12	2	20	0	15	0	6	0.302323	0.289153	0.013170
13	0	10	2	25	0	6	0.320000	0.310365	0.009635
14	0	10	0	15	2	12	0.253559	0.263223	-0.00966
15	0	10	0	15	0	6	0.412200	0.405655	0.006545
16	0	10	0	15	0	6	0.404243	0.405655	-0.00141
17	0	10	0	15	0	6	0.404243	0.405655	-0.00141
18	0	10	0	15	0	6	0.404243	0.405655	-0.00141
19	0	10	0	15	0	6	0.404243	0.405655	-0.00141
20	0	10	0	15	0	6	0.404243	0.405655	-0.00141

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

	Coefficient	SE	*t*-value	*p*-value
Intercept	-0.149831	0.064303	-2.3301	0.042044
x_i	0.037031	0.004681	7.9109	0.000013
x^2_i	-0.001269	0.000103	-12.2863	0.000000
x_{ii}	0.051065	0.004593	11.1188	0.000001
x^2_{ii}	-0.001404	0.000103	-13.5927	0.000000

x_{iii}	-0.009475	0.007604	-1.2461	0.241140
x^2_{iii}	-0.002664	0.000287	-9.2825	0.000003
$x_i.x_{ii}$	-0.001249	0.000204	-6.1121	0.000114
$x_i.x_{iii}$	0.001355	0.000340	3.9850	0.002580
$x_{ii}.x_{iii}$	0.001342	0.000340	3.9489	0.002735

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