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**OPTIMIZATION OF FERMENTATION CONDITIONS FOR ENHANCED ANTIFUNGAL
METABOLITE PRODUCTION BY *STREPTOMYCES* SP. S-9 ISOLATED FROM PIGEON
PEA RHIZOSPHERE**

DAVE A AND INGLE S*

Department of Microbiology and Biotechnology Centre, The Maharaja Sayajirao University of
Baroda

Vadodara, Gujarat, India

*Corresponding Author: E Mail: ingle05@yahoo.co.in; Contact: 9978811838

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ABSTRACT

Rhizosphere soil of Pigeon pea crops of Gujarat region have been used to isolate actinomycetes as an elite source for the production of bioactive metabolites. The actinomycete strain S-9 was isolated during our systemic screening of rhizospheric actinomycetes and was identified as *Streptomyces* sp. Based on morphological, physiological and molecular approaches. Strain S-9 showed antifungal activity against *Fusarium udum* phytopathogenic fungi. Glucose, yeast extract, malt extract and starchas culture medium was selected for the production of *Streptomyces* sp. Plackett – Burman design (PBD) has been used to select yeast extract, malt extract and calcium carbonate (CaCO₃) as parameters with significant effects on antifungal activity and a Box – Behnken design has been applied to further optimize the production medium. The analysis showed the optimal concentrations for the anti-*F. udum* activities of the tested variables were yeast extract 6.03 g/L, malt extract 7.05 g/L and CaCO₃ 4.59 g/L.

Keywords: *Streptomyces* sp, Plackett – Burman design (PBD), Box – Behnken design
Fusarium udum, bioactive metabolites

INTRODUCTION

In agriculture, crop diseases need to be controlled to maintain the quality and abundance of food and feed [1, 2]. Moreover, the excessive use of synthetic pesticides for the management of plant pathogens has given rise to problems due to the development of resistance in phytopathogenic fungi against chemical fungicides [3]. Therefore, the biological management of pathogens has gained interest as an alternative approach that is sustainable and safe both economically and environmentally [4]. Consequently, there has been an increase in research aiming not only to find new effective and safe biocontrol agents, but also to limit the use of chemical pesticides. From the 22,500 biologically active compounds that have been obtained so far from microbes, 45% are produced by actinomycetes, with approximately 75% of metabolites being produced by species of the genus *Streptomyces* [5]. *Streptomyces* species provide a rich source of natural products that may have potential agricultural uses. Their activity has been evaluated against different plant pathogens, and found to exhibit great potential in suppressing plant diseases caused specifically by fungal pathogen.

To improve the production of antifungal secondary metabolites in actinomycetes, different approaches were used to optimize

culture media using statistical methods to reduce time and expense [6]. Response surface methodology (RSM) is a very beneficial tool to optimize numerous parameters of trails and to find relativeness among the factors, as well as the best combination of parameters and response prediction [7]. This method was extensively used for the optimization of *Streptomyces* antifungal production [1, 8]. Due to the non-linear efficiencies of these networks, they are considered good estimators providing very accurate results. Hence, RSM is taken into consideration as a classical technique for the optimization. As a part of our continuous screening for bioactive metabolites from actinobacterium resulted in the isolation of a promising stain S-9 with a good antifungal capacity was identified as *Streptomyces* sp. The strain was isolated from rhizosphere soil sample of Pigeon pea from Gujarat, India, and its 16S rDNA sequence was deposited to GenBank under accession number MK158952. Cultural, morphological, and physiological properties of the strain were recorded. Hence, the objectives of the existing study include the polyphasic taxonomic characterization of the strain, effect of optimization of process parameters using RSM for maximizing the yield of the bioactive metabolites It is in this context that the present

research work was conducted to resume the investigation about the *Streptomyces* sp. S-9 strain to increase current knowledge in terms of modeling-based optimization approaches.

MATERIALS AND METHODS

Sample collection and actinomycete strain isolation

Pigeon pea (*Cajanus cajan*) rhizosphere soil was collected in Gujarat region. The soil samples were collected in sterile bags. Each soil sample was air dried, thoroughly ground and sieved. The samples were enriched with calcium carbonate. Subsequently; 1 g of each soil sample was suspended in 10 mL of sterile distilled water and then incubated at 55°C for 20 min in a shaking incubator at 180 rpm to prepare a suspension. The resulting supernatants were diluted serially 10-fold to prepare and aliquot suspensions at 10^{-1} , 10^{-2} and 10^{-3} . Aliquots of 100 μ L from each dilution were evenly spread plated on actinomycete isolation agar media in triplicate and incubated at 28°C for 2–4 weeks. Different single colonies were selected and purified on actinomycete isolation agar medium (AIA).

Pathogenic filamentous fungi

The pathogenic filamentous fungi used in this study *Fusarium udum* ITCC 3241 (ITCC, IARI, New Delhi) the fungi were maintained on Petri plates containing potato dextrose agar

(PDA) at 4°C and were subculture every 2 months.

Antagonistic strains screening

Fusarium udum (ITCC, IARI, New Delhi) used in antifungal study. The antifungal activities of actinomycetes were tested by dual culture method. The isolated strains were seeded over the entire surface of AIA+PDA medium Petri dishes. As soon as the organism developed, agar discs were cut out by the cork borer (6 mm diameter) and transferred to the surface of AIA+PDA agar plates seeded with the test organisms i.e. *F. udum*. The Petri dishes were then kept in incubator at 27°C to allow the growth of test organisms. If the antibiotic produced by the organism inhibits the growth of the test organism, a dead zone is formed round the discs. The isolates that showed positive result in preliminary screening were taken for shake flask fermentation. Spores from a mature slant culture of the actinomycetes that showed promising activity in preliminary screening were inoculated aseptically into 250 ml Erlenmeyer flask containing ISP-1(Hi-media, Mumbai) Casein enzymic hydrolysate 5.000 Yeast extract 3.000 Final pH (at 25°C) 7.0 ± 0.2 in a 250 ml flask and incubated at 30°C for 4 days under agitation at 200 rpm. After incubation, the fermented broth was centrifuged at 10,000 rpm for 10 min. and

filtered with Whatmann No.1 filter paper. The mycelium free culture filtrate was assayed for antifungal activity. Wells of 6 mm diameter was made in the centre of the plates with the help of cork borer. 100 µl of the test samples were transferred into the wells and plates were incubated at 27°C for 48 hrs. The plates were then observed for zone of inhibition. Percent inhibition was calculated using the following formula

$PI = (C - T)/C \times 100$. where, PI is the (%) inhibition, C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture.

Polyphasic taxonomy

The morphological, cultural, physiological, and characterization of the isolate was carried out as described in the international Streptomyces project. The cultural traits of the strain were recorded on different media which include tryptone-yeast extract agar (ISP-1), YMD agar (ISP-2), oatmeal agar (ISP -3), starch inorganic salts agar (ISP-4), glycerol asparagine agar (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7), starch-casein agar [9]. The utilization of carbohydrates by the strain was carried out in minimal medium containing different carbon sources at 1% concentration according to the method described by [10].

Molecular identification via 16S rDNA sequencing and phylogenetic analysis

S-9 was inoculated into International Streptomyces project-1 broth kept overnight for shaking, genomic DNA was extracted using SDS-lysozyme extraction method [11] and PCR targeting the 16S rDNA gene amplified using universal primer gene corresponding to positions 8-27 for the forward primer and 1492-1510 for the reverse primer (Forward primer (27F): 5'-AGAGTTTGATCMTGGCTCAG-3' Reverse primer (1492 R): 5'-TACGGYTACCTTGTTACGACTT -3'). The 16S rRNA gene sequence was further elucidated by a database search using the Ezbiocloud (<https://www.ezbiocloud.net>). Phylogenetic relatedness of isolate was determined by constructing the dendrogram.

Selection of basic medium

Antifungal activity against *Fusarium udum* was determined upon the growing of the Streptomyces sp. S-9 strain on five different basal production media as follows: (BM1) Bennett's medium: glucose 10 g/L, pancreatic digest of casein 2 g/L, yeast extract 1 g/L and beef extract 1 g/L, pH 7.0; (BM2) Czapek medium: sucrose 30 g/L, NaNO₃ 3 g/L, K₂HPO₄ 1 g/L, KCl 0.5 g/L, MgSO₄·7H₂O 0.5 g/L and FeSO₄·7H₂O 0.01 g/L pH 7.3; (BM3) GYM + S medium: starch 20 g/L, malt

extract 10 g/L, CaCO₃ 4 g/L, glucose 4 g/L, and yeast extract 4 g/L, pH 7.2; (BM4) ISP4 medium: starch 10 g/L, CaCO₃ 2 g/L, (NH₄)₂SO₄ 2 g/L, K₂HPO₄ 1 g/L, MgSO₄·7H₂O 1 g/L, NaCl 1 g/L, FeSO₄·7H₂O 1 mg, MnCl₂·7H₂O 1 mg and ZnSO₄·7H₂O 1 mg, pH 7.2 and (BM5) Waksman's Glucose medium: glucose 10 g/L, peptone 5 g/L, beef extract 5 g/L and NaCl 5 g/L, pH 7.5 [2]. All the media (100 mL) were inoculated with 10 mL of the seed culture and incubated at 30 °C under agitation (250 rpm) for 4 days. After incubation, the crude extract from the culture broth was assayed against *Fusarium udum*. The medium that showed high antifungal activity was selected for subsequent statistical optimization.

RSM based modeling

Screening of essential medium components using the Plackett–Burman design

The Plackett–Burman design was used to analyze important factors. Twelve experiments were conducted in triplicate to evaluate five factors. Five components: A: starch; B: malt extract; C: CaCO₃; D: glucose; E: yeast extract, were selected for the study, with each variable being represented at two levels, high (+) and low (–), as well as two dummy variables in twelve assays. The effect of each variable on the anti-*F. udum* activity was calculated and their significance was

determined via Student's t-test using Minitab 15.0 version (Minitab Inc. PA, USA). The variables with confidence levels above 95% were considered to have a significant effect on antifungal compound production, and thus chosen for further optimization.

Box–Behnken design and optimization by RSM

The significant variables were optimized for enhanced antifungal activity by employing a Box–Behnken design [12]. Three variables: B: malt extract; C: CaCO₃ and E: yeast extract were selected for studying the effect and significance on anti-*F. udum* activity. These selected variables were analyzed at three levels low, medium, and high coded as -1, 0, and +1, respectively, in twelve runs. The dummy variables were used to calculate the standard error. Each run was carried out with three replicates. The behavior of the system was explained by a second-order polynomial equation [Eq (1)].

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 + \dots \quad (1)$$

Where Y is the predicted response, b₀ is offset term b_i is linear effect, b_{ii} is squared effect, b_{ij} is interaction effect, and X_i is coded value of independent variables under study. This design was used to evaluate the main effects, interaction effects and quadratic effects. It is also used to optimize the levels of parameters for enhancing antifungal activity. The

statistical software (Minitab 15.0 version) was used for the experimental design and data analysis. Three-dimensional response surface plots were drawn to illustrate the relationship between the responses and the experimental levels of each independent variable. An optimum level of the variables for maximum anti- *F. udum* activity was determined by the response optimizer tool of the software.

Statistical analysis

A one-way analysis of variance (ANOVA) and Tukey's post-hoc test was performed to determine significant differences between the responses using SPSS 19 statistical package (SPSS Ltd. Woking, UK). Means and standard errors were calculated. Differences among the mean values of the various responses were determined by the least significant difference test. A probability level of $P < 0.05$ was used in testing the statistical significance of all experimental data.

RESULTS AND DISCUSSION

Isolation of actinomycete strains and antagonistic strain screening

From the rhizosphere soil of Pigeon pea samples, 165 morphologically distinct actinomycete strains were isolated. Screening found 25 strains exhibited antifungal activity against *F. udum*. Screening was performed to ascertain the broad-spectrum antifungal properties of these 25 actinomycetes strains.

Strain S-9 exhibited maximum antifungal activity against *F. udum* showed in (Table 1). Thus, strain S-9 was selected for further studies.

Morphological, cultural and physiological characterization

ISP media used to characterize actinomycetes on the basis of colony morphology, (1) the mass color of mature, sporulating aerial surface growth, (2) for the color of the substrate mycelium as viewed from the reverse side, and (3) for diffusible soluble pigments (eg. Melanins). The morphology of different isolates was noted. The colour of the substrate mycelium was determined by observing the plates after 7 to 10 days. It was done only after seeing the heavy spore mass surface. The most important factors considered for classification of actinomycetes include colonial characterization especially ability to produce vegetative and aerial hyphae, their colour and organization on defined media under standard conditions of cultivation shown in (Table 2). Thus all cultures were grown on 7 (ISP-1 to ISP-7) specifically designed International Streptomyces Project media as recommended by ISP-1 (Tryptone yeast extract) and ISP-2 (Yeast malt agar) were used to classify vegetative growth; ISP-3 (Oat meal agar) and ISP-4 (Inorganic salt starch agar) promoted sporulation; ISP-5 (Glycerol asparagine agar),

ISP-6 (Peptone yeast extract iron agar) and ISP-7 (Tyrosine agar) [Hi-media, Mumbai, India]. Promoted miscellaneous features like secretion of reverse and diffusible pigment and other potent antimicrobial compounds. Actinomycetes colonies with various colours (aerial hyphae and spores), consistency (dry to mucoid), shape (irregular to concentric), secretions (colourless to golden) and pigmentation were observed as shown in (Table 3).

Identification of actinomycetes by 16srrna amplification

Four isolates were identified up to genus level on the basis of 16S rRNA gene sequences. The sequence was deposited in GenBank (NCBI) with the accession number of MK158952. After sequencing results all the isolates were found to be the *Streptomyces* sp. To identify actinomycete isolates up to genus species level, molecular approach of amplifying and sequencing 16S rRNA gene was carried out. Genomic DNA isolated from pure cultures was amplified to obtained 1.1 kb PCR product. Purified products were sequenced, aligned, checked for chimera, cleaned, identified (BLAST) and deposited (~700 bp) in GeneBank (NCBI) with a unique NCBI Accession number for each actinomycete sequence (Table 4). Thus, correlation of classical features to molecular

phylogeny was the criteria used for identification of actinomycetes. The phylogenetic analysis of actinomycete isolate from *C.cajan* rhizosphere was done in MEGA7.0 to infer the taxonomic affiliations. The phylogenetic tree was constructed for *C.cajan* rhizosphere of South Gujarat and Saurashtra region. The bootstrap consensus tree constructed from 1000 replicates was taken to represent the evolutionary history of taxa analyzed. Evolutionary analyses were conducted in MEGA 7.

Selection of basal medium

The antifungal activity of crude extract of *Streptomyces* sp. S-9 strain using five different basal production media was evaluate against *Fusarium udum*. As compiled in (Table 5), the crude extract showed varying degrees of antifungal activity. The inhibition zones were in the range from 0 to 19.85±0.51 mm. Among the five different growth media, the highest ($P < 0.05$) anti- *F.udum* activity (19.85±0.51 mm) was observed in S-9 BM3 shown in Table 5. Therefore, BM3 was selected for further optimization study using statistical approaches.

Selection of significant media components by the Plackett– Burman design

Five variables supposed to affect anti-*F. udum* activity were evaluated under twelve experiments for the Plackett– Burman design

(PBD). **Table 6A** shows the responses obtained in terms of anti-*F. udum* activity. The model was highly significant ($P < 0.05$), $R^2 = 0.8404$ for anti-*F. udum* activity, suggesting that 84.04% of the total variability in the response could be explained by this model (**Table 6 A**). As shown in **Table 6C**, the adequacy of the model was calculated, and the variables proving statistically significant effects were screened via Student's t-test for the Analysis of Variance (ANOVA). Factors evidencing P-values of less than 0.05 were considered to have significant effects on the response, and were therefore selected for further optimization studies. The P-values of the important variables in the PBD as given below were the most significant variables affecting antifungal compounds produced by *Streptomyces* sp. S-9 strain (**Table 6 B**): yeast extract ($P = 0.010$), malt extract ($P = 0.021$) and CaCO_3 ($P = 0.030$). From the experimental data, these three variables could clearly affect the anti-*F. udum* activity. Moreover, yeast extract ($P = 0.010$), malt extract ($P = 0.021$) and CaCO_3 ($P = 0.030$) affect positively the anti-*F. udum* activity. Considering the results displayed in **Table 2B** and after the exclusion of the insignificant model terms ($P > 0.05$) the reduced polynomial Eq. (2) may be written as follows:

$$\text{Anti-Fusarium sp: activity} = 18:333 + 1:833 \times B + 1:666 \times C + 2:166 \times E \dots\dots\dots(2)$$

Where B: malt extract; C: CaCO_3 ; E: yeast extract

Based on the results of the Plackett–Burman design, it is obvious that this method was useful in the detection of most significant ingredients. In this study, for further optimization of anti-*F. udum* activity, the levels of the most significant factors were optimized by Box–Behnken design, while insignificant ones were used in all assays at their optimum level.

Regression analysis and model fitting

The data obtained from the experiments were used to estimate the coefficients by regression analysis. Regression analysis data such as estimated coefficients and P-value were shown in (**Tables 7A and 7B**). The response of activity can be expressed in the following regression equation [Eq. (3)]:

$$\text{Anti_F:udum activity} = 23:50 + 3:62 \times E + 4:4 \times B - 6 \times C - 0:281 \times E^2 - 0:185 \times B^2 + 1:22 \times C^2 + 0:025 \times E \times B - 0:187 \times E \times C - 0:125 \times B \times C$$

The fitness and adequacy of the models were analyzed by ANOVA. P-values were used to evaluate the significance of different coefficients, which provides the information required to understand the interaction patterns among the experimental variables [13]. The P-values less than 0.05 are always accepted as the values for statistical significance with confidence level greater than 95% and smaller

P-values refer to larger significance of the respective coefficient [13]. Based on P-values represented in **Tables 7A and 7B**, the linear effect of malt extract and yeast extract ($P < 0.05$) and square effect of malt extract, CaCO_3 and yeast extract ($P < 0.05$) were found to be more significant than other variables in anti-*F. udum* activity. The linear and square effects of the variables were found to be more significant than the interaction effects between the variables. The model fitness was expressed by the coefficient of determination (R^2) and adjusted coefficient of determination R^2 . It should be noted that R^2 is a tool to check the accuracy of fit between the experimental and predicted results [13]. In our case, the R^2 value of 0.9463 showed that the model was higher than 94% accurate to express the variability of the data (**Table 7A**). It revealed that there was a good relation between the experimental and predicted values for anti-*F. udum* activity. The adjusted coefficient of determination (R^2_{adj}) plays the role of

correcting the R^2 value as a function of the sample size and number of parameters in the model. Its values (0.8496) were also high enough to express the high significance of the model (**Table 7 A**).

Validation of the optimized condition

Based on numerical optimization, the predicted models with the maximum anti-*F. udum* activity was 32.29 mm, when the optimal values of test factors were (malt extract 7.05 g/L, $\text{CaCO}_3 = 4.59$ g/L and yeast extract = 6.03 g/L). To verify the predicted results, the growth inhibition experiment was performed in triplicate. An increase of 64.15% was shown between the original medium and the optimized medium whose values were 19.67 ± 0.51 mm (original medium) and 32.50 ± 2.75 mm (optimized medium), respectively. Validated and predicted values are basically the same, indicating that the model is reasonable and effective.

Table 1: Antifungal activity of S-9 isolate against *F. udum*

Sr.No	Strain no	Phytopathogenic fungi	Percentage inhibition of Phytopathogenic fungus
1	S-9	<i>F. udum</i>	85

Table 2: Cultural and morphological characteristics of S-9

Strain	Colony appearance	Aerial mycelia	Aerial spore	Pigmentation	DAP Isomer	Gram staining
S-9	white, dry, powdery	White	Grey	None	L-DAP	Positive, filamentous, branched

Strain no	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-6	ISP-7
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S-9	Chocolate brown (chalky)	White chalk	Dark brown	White chalk	Dark brown	Chocolate brown (chalky)	Dark grey
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Table 3: Cultural characteristics of the isolate S-9 on different ISP Medium

Table 4: Identification of antifungal actinomycetes

Strains	Scientific name	NCBI Accession number
S-9	<i>Streptomyces</i> sp	MK158952

Table 5: Effect of five different production media on antifungal activity production of *Streptomyces* sp.S-9 strain extracts

Basal medium	Anti- <i>F.udum</i> activity (mm)
BM1	11.27 ± 1.1
BM2	15.13 ± 1.15
BM3	19.76 ± 0.51
BM4	10.00 ± 1.00
BM5	0.00 ± 0.00

Table 6A: Plackett–Burman design and the experimental response obtained for *Streptomyces* sp. S-9 strain

Trials	Variables					Anti- <i>F. udum</i> activity (mm)
	A	B	C	D	E	
1	+(30)	+(15)	-(2)	+(6)	+(6)	20.12±2.02
2	-(10)	-(5)	+(6)	+(6)	+(6)	19.42±2.62
3	-(10)	+(15)	-(2)	-(2)	-(2)	17.44±0.55
4	-(10)	-(5)	-(2)	+(6)	+(6)	17.30±0.37
5	+(30)	-(5)	-(2)	-(2)	+(6)	19.62±2.12
6	+(30)	-(5)	+(6)	+(6)	-(2)	18.12±1.25
7	-(10)	+(15)	+(6)	+(6)	-(2)	20.96±1.32
8	-(10)	+(15)	+(6)	-(2)	+(6)	23.65±1.58
9	+(30)	+(15)	+(6)	-(2)	+(6)	23.06±2.41
10	+(30)	+(15)	-(2)	+(6)	-(2)	16.00±2.00
11	-(10)	-(5)	-(2)	-(2)	-(2)	10.16±1.04
12	+(30)	-(5)	+(6)	-(2)	-(2)	14.85±2.02

A: starch; B: malt extract; C: CaCO₃; D: glucose; E: yeast extract; for each parameter, are statistically different (P < 0.05).Table 6B: Effect estimates for anti-*F.udum* activity from the result of the Plackett–Burman design.A: starch; B: malt extract; C: CaCO₃; D: glucose; E: yeast extract

Anti <i>F.udum</i> activity					
	Effect	t	p	Coefficient	SE coefficient
Constant		30.11	0.000	18.325	0.5893
A	0.666	0.54	0.594	0.333	0.6554
B	3.666	3.11	0.023	1.833	0.5836
C	3.333	2.80	0.031	1.664	0.5237
D	0.333	0.28	0.784	0.166	0.5325
E	4.333	3.68	0.010	2.163	0.5569

R-Sq = 84.04%; R-Sq(adj) = 70.74%.

Table 6 C: ANOVA for the anti-*F. udum* activity based on the Plackett–Burman design.A: starch; B: malt extract; C: CaCO₃; D: glucose; E: yeast extract

Anti <i>F.udum</i> activity						
Source	DF	Seq ss	Adj ss	Adj ms	F value	P value
Main effects	5	13.167	13.16	26.3333	6.30	0.020
A	1	1.333	1.333	1.33	0.30	0.590
B	1	40.333	40.333	40.334	9.64	0.020
C	1	33.333	33.333	33.325	8.00	0.032
D	1	0.333	0.333	0.333	0.05	0.78
E	1	56.333	56.333	56.333	12.54	0.010
Residual error	6	25.000	25.000	4.1667		
Total	11	156.667				

Table 7A: Regression coefficients and their significance for response surface model of anti- *F. udum* activity

Anti- <i>F. udum</i> activity					
	P value	t	Coefficient	SE Coefficient	
Constant	0.041 0.6892	1.29	23.50		0.6892
E	0.04	1.46	3.625		0.689
B	0.007	4.44	4.4		0.6892
C	0.06	-2.42	-6		0.6892
E ²	0.01	-1.11	-0.281		1.0145
B ²	0.006	-4.559	-0.185		1.0145
C ²	0.005	4.81	1.218		1.0145
E×B	0.808	0.26	0.025		0.9747
E×C	0.476	-0.77	-0.187		0.9747
B×C	0.256	-1.28	-0.125		0.9747
R ²		R-Sq = 94.63%		R-Sq(adj) = 84.96	

Table 7 B: ANOVA for the anti-*F. udum* activity based on the Box-Behnken design

Anti- <i>F. udum</i> activity						
Source p value	DF	Seq SS	Adj SS	Adj MS	F-value	p value
Regression	9	334.733	334.733	37.1926	9.79	0.011
Linear	31	140.500	140.500	46.8333	12.32	0.010
B	1	18.000	18.000	18.000	4.74	0.007
C	1	98.000	98.000	98.000	25.79	0.06
E	1	24.500	24.500	24.500	6.45	0.040
Square	3	185.483	185.483	61.8278	16.27	0.005
B×B	1	92.857	78.981	78.9808	20.78	0.006
C×C	1	87.750	87.750	87.7500	23.09	0.005
E×E	1	4.876	4.673	4.6731	1.23	0.01
Interaction	3	8.750	8.750	2.9167	0.77	0.559
B×C	1	6.250	6.250	6.2500	1.64	0.25
E×B	1	0.250	0.250	0.250	0.07	0.808
E×C	1	2.250	2.250	2.250	0.59	0.476
Residual Error	5	19.000	19.000	3.8000		
Lack-of-Fit	3	19.000	19.000	6.333		
Pure Error	2	0.000	0.000	0.000		
Total	14	353.733				

CONCLUSION

The experimental isolate was identified as *Streptomyces* sp S-9 on the basis of cultural, morphological, physiological characteristics and 16S rRNA analysis. Factors that affect the antifungal activities were investigated and assessed using the statistical experimental design. To achieve maximum metabolite production, fermentation of *Streptomyces* sp. S-9 was optimized through response surface methodology. The optimal conditions for the production of bioactive compounds by S-9 and its inhibitory effect against the responses using

the RSM statistical design were determined.

Further research concerning the purification and characterization of bioactive compounds produced by the strain is in progress.

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