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Statistical optimization of medium constituents and conditions for improved antimicrobial compound production by marine *Streptomyces* sp. JRG-04

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Abstract: A recently isolated *Streptomyces* sp. JRG-04 from a mangrove estuary was identified as a producer of a broad-spectrum antimicrobial compound against various pathogens, including multidrug resistant (MDR) pathogens, with no cytotoxic effect on H9C2 cells. In this study, the concentrations of various nutrient factors and culture conditions were optimized by both classical and statistical methods for an improved titer of the antimicrobial compound production. Among nutrient factors, carbon and nitrogen sources such as maltose and yeast extract stimulated the production of the antimicrobial compound with the highest titer. The production medium, with a pH 7.5 at 28°C, promoted increased antimicrobial compound production. All non-statistically optimized nutrients and environmental conditions were used for subsequent statistical optimization using a Plackett-Burman design (PBD) and response surface methodology (RSM). Maltose, yeast extract and the inorganic salt NH_4Cl were found to be significant components for antimicrobial compound production by the PBD method. Interactions between important variables were evaluated using central composite design (CCD) of response surface methodology. The final optimized medium (L^{-1}) contained: 10 g maltose, 2.9 g Na_2HPO_4, 2.3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $MgSO_4 \times 7H_2O$, 0.002 g $FeSO_4$, 0.5 g $CaCO_3$, 5.25 g yeast extract and trace elements in 5.0 mL salt solution (0.1 g $ZnSO_4 \times 7H_2O$, 0.3 g H_3BO_3, 0.2 g $COCl_2 \times 6H_2O$, 0.03 g $MnCl_2$ $4H_2O$, 0.03 g $Na_2MO_4 \times 2H_2O$, 0.02 g $NiCl_2 \times 6H_2O$, 0.01 g $CuCl_2 \times 2H_2O$). The medium provided an overall 42.8% increase in antibiotic activity when compared to the unoptimized medium, from 140.57±0.80 to 210.33±0.57 U/mL.

Key words: marine *Streptomyces*; medium optimization; central composite design; response surface methodology

INTRODUCTION

Streptomyces, the largest genus of the phylum Actinomycetes, are well-known filamentous bacteria endowed with a complex secondary metabolite biosynthesizing capability with diverse biological functions [1] Members of this genus are ubiquitous in different habitats, ranging from terrestrial regions, marshy areas, fresh and brackish waters, salty regions, etc. [2-5]. Many recent studies have reported the occurrence of novel *Streptomyces* species in marine environments rich in diverse chemicals used for the development of therapeutic materials [6]. During characterization of novel marine *Streptomyces* with a potential for bioactive compound production, we recently reported [7] a marine *Streptomyces* sp. JRG-04, which displayed bioactivity against various disease-causing infectious microbial pathogens, including MDR clinical pathogens, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. Although the antimicrobial metabolite exerted a broad-spectrum biological effect against different pathogenic microorganisms, it was not toxic to the H9C2 animal cell line. The bioactive metabolite was identified as a polyketide antibiotic compound with structural similarity to aromatic benzoisochromanequinone [7].

The marine origin of the *Streptomyces* sp. JRG-04 delimits the maximum antimicrobial compound production under laboratory conditions due to the requirement for optimized culture conditions and nutrient requirements. Sources of carbon, nitrogen and sodium chloride can influence the synthesis of pharmaceutically important secondary metabolites [8,9]. The stable, reproducible production of a specific bioactive compound can be achieved by optimizing nutritional

and physiological conditions for specific species. The optimization also provides the best selection choice for the most favorable ingredients of the medium to facilitate product formation [10-13]. Media optimization by classical approach does not show any significant interactions of selected variables in the medium [14]. In order to overcome this problem, a statistical medium optimization approach such as the Plackett-Burman design (PBD) method and response surface methodology (RSM) provide a significant effect on antimicrobial compound production. The medium optimization process that involves both classical and statistical approaches can provide a substantial improvement in bioactive compound production [5]. The PBD is a widely used statistical method to screen important factors required for increased quantities of product formation. RSM is also used to study the linear, square and interaction effect of ingredients crucial for the production of antibacterial compounds [15]. In the present study, the effect of different environmental conditions, such as temperature, pH and nutrient factors, such as carbon and nitrogen sources, was evaluated for its ability to improve the antimicrobial compound capability of *Streptomyces* sp. JRG-04. The improved antimicrobial compound was further evaluated by PBD and RSM based on statistical media optimization processes.

MATERIALS AND METHODS

Strain and culture conditions

The bacterial isolate *Streptomyces* sp. JRG-04 was grown on a modified ISP-4 medium containing per liter: soluble starch, 20 g; NaCl, 0.5 g; KNO_3, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \times 7H_2O$, 0.5 g; $FeSO_4$, 0.002 g, agar, 18 g. The initial inoculum was incubated at 30°C for 4 days. The isolate was maintained at 4°C on modified ISP-4 agar plates for further studies. The 16S rRNA gene sequence of the isolate was deposited in the GenBank database under accession number JX486678.

Media and culture condition optimization by a one-factor-at-a-time (OFAT) approach

The effects of pH, temperature, carbon and nitrogen source on growth and antimicrobial compound produced by the *Streptomyces* sp. JRG-04 we examined. To carry out this study, the media components were supplemented with the basal medium containing: 2.9 g Na_2HPO_4, 2.3 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4 \times 7H_2O$, 0.002 g $FeSO_4$, 0.5 g $CaCO_3$ and a 5.0-mL trace element salt solution from a stock solution (0.1 g $ZnSO_4 \times 7H_2O$, 0.3 g H_3BO_3, 0.2 g $COCl_2 \times 6H_2O$, 0.03 g $MnCl_2 \times 4H_2O$, 0.03 g $Na_2MO_4 \times 2H_2O$, 0.02 g $NiCl_2 \times 6H_2O$, 0.01 g $CuCl_2 \times 2H_2O$ in 1 L water). Non-statistical media optimization was performed according to the OFAT approach in which one factor is changed and other factors are kept constant. For carbon source optimization, different carbon sources (glucose, sucrose, maltose, fructose, starch and glycerol) were used independently at the same concentration (1%), supplemented with basal medium. The effect of nitrogen source on the basal medium used for antimicrobial compound production was examined using peptone, tryptone, yeast extract, beef extract and urea. The effectiveness of suitable parameters was assessed based on their antimicrobial activity against the test organism, *Staphylococcus aureus*. All experiments were performed in triplicate. The outcome of the OFAT method was used for statistical media optimization.

Statistical optimization

The Plackett-Burman design method was used to identify the significant factor for improving the antimicrobial compound production. Optimized factors such as maltose, yeast extract, Na_2HPO_4, KH_2PO_4, NH_4Cl, $MgSO_4 \times 7H_2O$, $FeSO_4$, $CaCO_3$, asparagine, vitamin B12 and trace element salt solutions were used. The selected variables were applied at both high (+1) and low (-1) levels. The experimental design is shown in Table 1. Twelve runs were examined using the PBD, and significant factors were selected on the basis of antimicrobial activity and biomass. The experiments were carried out in 500-mL conical flasks containing 100 mL of production medium, incubated at 28°C, with shaking at 120 rpm for seven days. The response surface methodology was implemented to examine independent and dependent variables. Interactions of selected medium components provide maximum product formation, and study of the interaction effect of the components is a widely accepted method [16]. The central composite design (CCD) of RSM was used to optimize three important factors, NH_4Cl, maltose and yeast extract. The optimum concentrations of three independent variables

Table 1. Media components for optimization according to the PBD.

Factors	Code	Unit	Low Level	High Level
Maltose	A	g L^{-1}	5	20
Yeast extract	B	g L^{-1}	2.5	10
Potassium dihydrogen phosphate	C	g L^{-1}	1	5
Vitamin B12	D	g L^{-1}	0.005	0.02
Magnesium sulfate	E	g L^{-1}	0.01	0.1
Ferrous sulfate	F	g L^{-1}	0.002	0.2
Calcium carbonate	G	g L^{-1}	0.005	0.02
Ammonium chloride	H	g L^{-1}	0.5	5
Asparagine	J	g L^{-1}	0.005	0.02
Disodium hydrogen phosphate	K	g L^{-1}	1	5
Trace elements	L	ml L^{-1}	2	10

Table 2. Levels of factors tested in the central composite design (CCD).

Variables (Factors)	Code	Unit	α	+	0	-	-α
Maltose	X_1	g L^{-1}	25	22	10	5	2.5
Yeast extract	X_2	g L^{-1}	15	12	5	3	1
Ammonium chloride	X_3	g L^{-1}	2	1.5	1	0.5	0.1

were determined using 20 runs with five different levels (-1.682, -1, 0, 1, and 1.682) (Table 2).

The factors were implied according to the following equation:

$$dxi = \Sigma(Xi\text{-}X0)$$
$$\Delta X\ ,\ i = 1, 2, 3, \ldots, k \quad (1)$$

where *xi* was the coded independent factor, *Xi* was the real independent factor, X_0 was the value of *Xi* at the center point and ΔX was the step change value.

Antimicrobial compound production (response variable) was explained by the second-order polynomial equation:

$$Y = \beta 0 + \Sigma\, \beta i\, xi + \Sigma\, \beta iixi$$
$$2 + \Sigma\, \beta ijxixj,\ i = 1, 2, 3, \ldots, k \quad (2)$$

where *Y* was the predicted response, β_0 was the intercept, *x*i and *x*j were the coded independent factors, βi was the linear coefficient, βii was the quadratic coefficient and βij was the interaction coefficient.

Statistical analysis and method validation

Design Expert version 8.0.2 (STAT-EASE Inc., Minneapolis, MN, USA) was used to analyze the experimental model and for regression analysis of the results. The accuracy of the second-order polynomial model was evaluated with coefficient determination (R^2). The statistical significance of the above model was determined with $p \leq 0.05$. The response graph was drawn using the same software. The classical and statistically optimized media were compared with unoptimized media for biomass and antimicrobial compound production of *Streptomyces* sp. JRG-04 using a laboratory-scale batch bioreactor.

Production, extraction and bioassay of antimicrobial compound

For the comparative experimental study, the laboratory-scale fermenter (New Brunswick, scientific model number Bioflo/Celligen 1150 from Eppendorf, N.J, USA) with optimized and controlled parameters (pH and temperature) and medium was used for the production of the antimicrobial compound. The antimicrobial compound was also obtained using unoptimized medium. The 7-day-old culture broth was harvested using a harvesting tube and centrifugation. The cell-free supernatant was collected and acidified to pH 3.0 with 1N HCl [17]. The bioactive compound was extracted twice with an equal volume of chloroform and concentrated using a rotational vacuum concentrator, RVC 2-18 CD (CHRIST, Germany). The extracted bioactive compound was examined for antimicrobial activity by disk diffusion [18]. The test organism, *S. aureus* ATCC 6518 (1×106 CFU/mL), was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and used for evaluation of antimicrobial activity.

An equal volume of chloroform-extracted bioactive compound was loaded on a sterile disk and air dried. A sterile disk soaked with chloroform served as a control. The dried disks were placed on Mueller-Hinton agar plates containing test organisms. The plates were incubated at 37°C for 24 h. The zone of growth inhibition around the bioactive compound loaded disk was measured using a measuring tool. The size of the zones of inhibition can be considered as a measure of antibiotic titer [19-21]. Antibiotic activity was expressed as units of activity per mL of crude substance of the cultures, where 1 U was defined as a 1.0 mm annular clearing around the antibiotic disk.

RESULTS

Effect of pH and temperature on antimicrobial activity

A suitable pH for increasing the biomass and antimicrobial activity (183 U/mL±5.7) was observed at pH 7.5 (Fig. 1A). Maximum antimicrobial activity was found at neutral pH compared. Optimum growth and a higher level of antimicrobial activity (193 U/mL±5.7) was observed at 28°C (Fig. 1B).

Effect of carbon and nitrogen source on antimicrobial activity

Among the tested carbon sources, maltose showed maximum antimicrobial activity (170 U/mL±10.00) against *S. aureus*, followed by lactose (143 U/mL±5.7). Lower antimicrobial activities were observed for other carbon sources (glucose, starch, fructose, and sucrose; Fig. 2A). Examination of the effect of nitrogen showed that yeast extract-supplemented medium provided maximum growth and antimicrobial activity (176 U/mL±5.77) (Fig. 2B). Other organic nitrogen sources such as tryptone and beef extract exhibited moderate antimicrobial activities (126 U/mL±0.57 and 130 U/mL±0.00, respectively). Urea and peptone showed moderate growth and less antibiotic activity compared to other nitrogen sources. Maltose and yeast extract were selected as the best carbon and nitrogen sources for growth, respectively, and were used for subsequent medium optimization experiments.

Plackett-Burman Design for selection of important components

The PBD was adapted to identify the interaction of the important factors for enhancing the production of the antimicrobial compound by *Streptomyces* sp. JRG-04. Eleven important ingredients were selected and experiments were carried out with twelve different combinations (Table 3). Factors A, B and H (A – maltose, B – yeast extract, H – ammonium chloride) influenced the growth and maximum antimicrobial activity and the result is shown in Table 4. The experimental results were subjected to regression analysis and analysis of variance (ANOVA). Maltose, yeast extract and ammonium chloride produced statistically

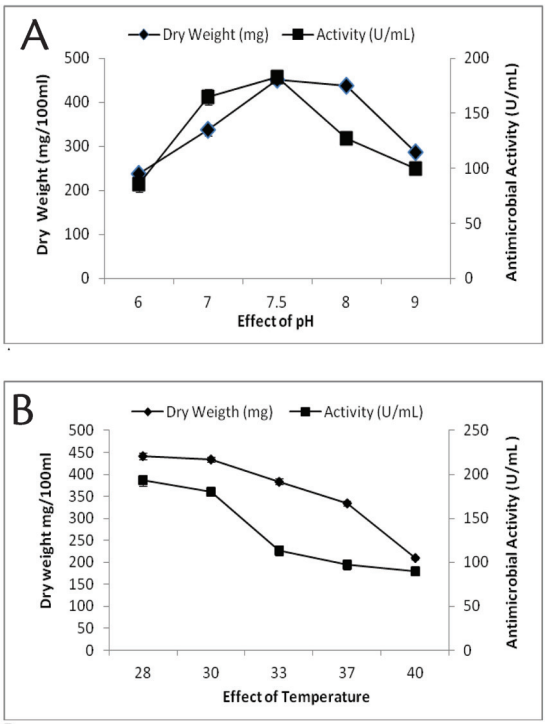

Fig. 1. Effect of pH (**A**) and temperature (**B**) on biomass and antimicrobial compound production by *Streptomyces* sp. JRG-04

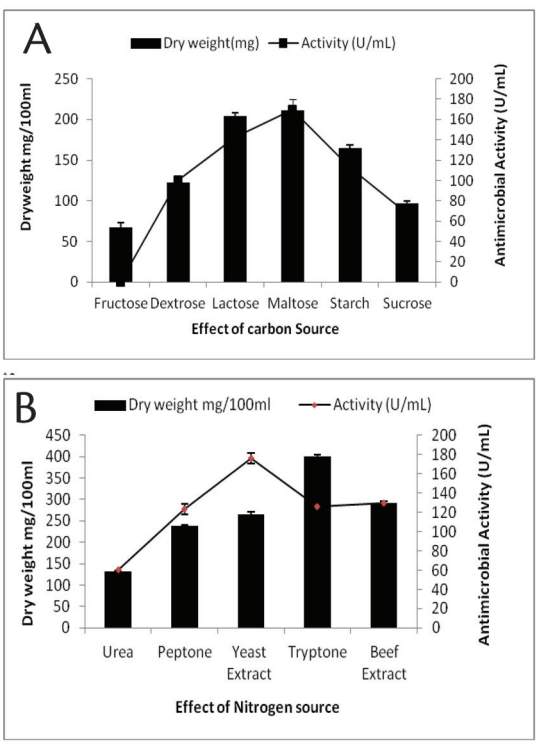

Fig. 2. Effect of carbon (**A**) and nitrogen source (**B**) on biomass and antimicrobial compound production by *Streptomyces* sp. JRG-04.

Table 3. PBD for 11 factors in 12 combinations (runs) at coded levels with zone of inhibition as response.

Run	A	B	C	D	E	F	G	H	J	K	L	RESPONSE Antimicrobial activity (U/mL) (SEM)
1	+	-	+	-	-	-	+	+	+	-	+	160.00±5.00
2	+	+	-	+	-	-	-	+	+	+	-	60.66±4.04
3	-	+	+	-	+	-	-	-	+	+	+	110.66±1.15
4	+	-	+	+	-	+	-	-	-	+	+	52.00±2.51
5	+	+	-	+	+	-	+	-	-	-	+	51.00±1.00
6	+	+	-	+	+	-	+	-	-	-	+	50.00±0.00
7	+	+	+	-	+	+	-	+	-	-	-	101.66±2.88
8	-	+	+	+	-	+	+	-	+	-	-	121.66±2.88
9	-	-	+	+	+	-	+	+	-	+	-	170.00±5.0
10	-	-	-	+	+	+	-	+	+	-	+	51.66±2.88
11	+	-	-	-	+	+	+	-	+	+	-	101.66±2.88
12	-	-	-	-	-	-	-	-	-	-	-	60.00±0.00

A – maltose (g); B – yeast extract (g); C – KH_2PO_4 (g); D – vitamin B12 (g); E – $MgSO_4 \times 7H_2O$ (g); F – $FeSO_4$ (g); G – $CaCO_3$ (g); H – NH_4Cl (g); J – asparagine (g); K – Na_2HPO_4 (g); L – trace element salt solution (mL).

Table 4. Central composite design with observed and predicted responses.

			Coded level Antimicrobial Activity (U/mL) SEM		
Run	X_1	X_2	X_3	Actual Value	Predicted Value
1	-1	-1	-1	70.00±0.00	70.32
2	-1	-1	1	140.00±5.00	140.96
3	-1	1	-1	50.33±0.57	60.42
4	-1	1	1	150.00±0.00	140.31
5	1	-1	-1	60.00±0.57	70.01
6	1	-1	1	130.66±1.15	110.90
7	1	1	-1	50.33±0.57	40.86
8	1	1	1	100.00±0.00	100.01
9	-1.682(-α)	0	0	190.66±1.15	190.68
10	1.682(+α)	0	0	190.33±0.57	190.68
11	0	-1.682(-α)	0	201.66±2.88	190.68
12	0	1.682(+α)	0	211.66±2.88	190.68
13	0	0	-1.682(-α)	60.00±5.00	40.90
14	0	0	1.682(+α)	150.00±5.00	150.64
15	0	0	0	116.66±5.77	110.45
16	0	0	0	90.00±0.00	90.10
17	0	0	0	171.66±2.88	160.46
18	0	0	0	121.66±2.88	120.58
19	0	0	0	201.66±7.63	210.19
20	0	0	0	210.33±0.57	210.19

X_1 – maltose, X_2 – yeast extract, X_3 – ammonium chloride
SEM – standard error mean

significant interactions (based on their *p* values). If the *p* value is less than 0.05, it indicates significance of the model [22]. According to this model, the *p* value of factors A, B and H was less than 0.05 when compared to other factors that are statistically significant. The "Pred R-Squared" of 0.9862 is in reasonable agreement with the "Adj R-Squared" of 0.9989, and the

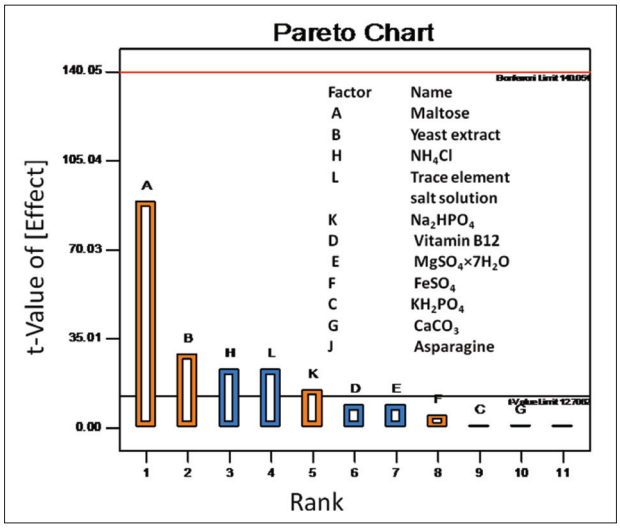

Fig. 3. Pareto chart (PBD) showing the ranking of the medium components on the basis of t value limit: A – maltose, B – yeast extract, H – NH_4Cl, L – trace element salt solution, K – Na_2HPO_4, D – vitamin B12, E – $MgSO_4 \times 7H_2O$, F – $FeSO_4$, C – KH_2PO_4, G – $CaCO_3$, J – asparagine.

"R-Squared" of 0.9999 is in reasonable agreement with the "Adj R-Squared" of 0.9989 for the antimicrobial activity, respectively. The ranking of the media components is also presented in the Pareto chart based on their coefficient estimates (Fig. 3).

Response surface methodology

A central composite experimental design of three important components such as maltose, yeast extract and ammonium chloride was used for antimicrobial compound production in the fermentation medium. The design matrix and their responses showed an improved antimicrobial compound from PDB-selected factors. The model was then subjected to statistical analysis ($p>0.05$) to exclude the insignificant factor. A second-order polynomial equation showed important factors as follows: response antimicrobial activity (U/mL): $Y=204.84+32.27\ X_1 - 12.97\ X_2 - 11.28\ X_3 + 1.17\ X_1X_2 - 6.25\ X_1X_3 - 3.75\ X_2X_3 - 40.26\ X_1^2 - 32.13\ X_2^2 - 25.53\ X_3^2$, where Y indicated the predicted response of X_1 (maltose), X_2 (yeast extract) and X_3 (ammonium chloride), respectively.

The statistical significance of the model was checked by the F-test and ANOVA of the response (Table 5). In this experimental model, the F-value 25.06 implied that the model is significant. There is

Table 5. ANOVA of the regression model for the response.

Source	Sum of squares	df	Mean squares	F-value	p-Value	prop>F
Block	1498.06	1	1498.06			
Model	58786.50	9	6531.83	25.06	<0.0001	Significant
X_1 – maltose	14221.26	1	14221.26	54.56	<0.0001	
X_2 – yeast extract	2298.06	1	2298.06	8.82	0.0157	
X_3 – ammonium chloride	1738.59	1	1738.59	6.67	0.0296	
$X_1 X_2$	10.90	1	10.90	0.042	0.8425	
$X_1 X_3$	312.50	1	312.50	1.20	0.3020	
$X_2 X_3$	112.50	1	112.50	0.43	0.5277	
X_1^2	23339.58	1	23339.58	89.54	<0.0001	
X_2^2	14863.32	1	14863.32	57.02	<0.0001	
X_3^2	9385.77	1	9385.77	36.01	0.0002	
Residual	2346.03	9	260.67			
Lack of fit	1997.08	5		4.58	0.0828	Not significant
Pure Error	348.95	4	399.42			
Core Total	62630.60	19	87.24			

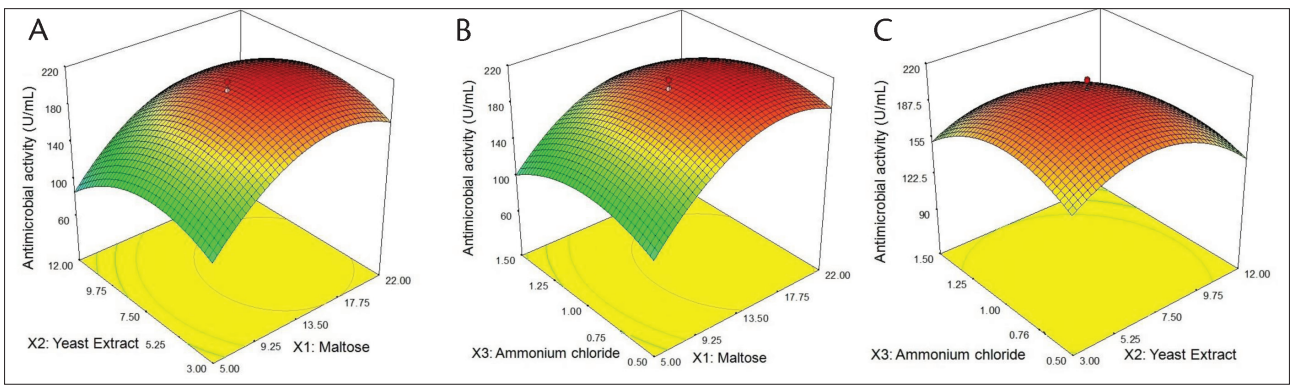

Fig. 4. Three-dimensional response surface curve for antimicrobial compound production by *Streptomyces* sp. JRG-04. **A** – interaction of maltose and yeast extract with different concentrations. Ammonium chloride was kept constant at 1 g L^{-1}. **B** – the interaction of ammonium chloride and maltose with different concentration. Yeast extract was kept constant at 5.25 g L^{-1}. **C** – the interaction of ammonium chloride and yeast extract with different concentrations. Maltose was kept constant at 10 g L^{-}.

only a 0.01% chance that a model F-value could occur due to noise. The predicted R squared of 0.6819 is in reasonable agreement with the adjusted R squared of 0.9232, which indicates that the model is in good agreement with the observed and predicted response.

Fig. 4A-C shows the 3D response surface curves of the effects of maltose, yeast extract and ammonium chloride on the antimicrobial activity. These results show that the highest antimicrobial activity was achieved with maltose, 10.0 g L^{-1}, followed by yeast extract, 5.25 g L^{-1} and ammonium chloride, 1.0 g L^{-1}. The 3D response surface curves provided the interaction between the significant factors and the optimal concentration was identified for further experimental conditions.

Experimental validation

Based on the media optimization, the quadratic model predicted that the higher antimicrobial activity was at 210.33 U/ml±0.57 and overall it represented a 42.8% increase. The optimum concentration of the test factors with coded levels were X_1=0, X_2=1.682 and X_3=0 for maltose 10 g L^{-1}, yeast extract 5.25 g L^{-1} and ammonium chloride 1.0 g L^{-1}, respectively. To determine the predicted values, the validation experiments were performed in triplicate. Under the optimized medium, the antimicrobial activity was at 210.33 U/ml±0.57, and the predicted value was 210.9 (U/ml). These results indicate that the observed and predicted values were in reasonable agreement with each other.

DISCUSSION

The genera *Streptomyces* is known to produce diverse secondary metabolites possessing different biological activities, such as antifungal, antibacterial, antitumor and plant growth promoting [1]. *Streptomyces* sp. JRG-04 was previously reported from sediments taken from the root zone of mangroves at 0.7-10-cm depth from the Karangadu mangrove forest, Tamil Nadu, India. The strain *Streptomyces* sp. JRG-04 had the ability to inhibit the growth of many pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* with a significant level of MIC. The broad-spectrum bioactive property and non-cytotoxicity against normal H9C2 cell lines are due to the aromatic polyketide nature of the compound. This feature is an important criterion for developing antimicrobials against pathogens [7]. However, its bioactive production potential titer is significantly affected by incubation conditions and components of the unoptimized production medium. The combination of classical and statistical optimization process provides maximum production of secondary metabolites [23]. Here, we developed suitable culture conditions and identified media components for optimal growth and antimicrobial compound production by the *Streptomyces* sp. JRG-04 isolate. Similar to this study, production media optimization was also reported for a newly isolated species of *Streptomyces* identified to the genus level [24,25].

The pH of the production medium is a crucial factor in defining growth, which in turn significantly influences product formation in secondary metabolite producers [20,26]. Neutral pH has a significant role in the growth of *Streptomyces* sp. JRG-04 as well as in the resulting bioactive compound production potential. Similar to this report, a study conducted on *Streptomyces* sp. KEH23 also yielded optimum antimicrobial substance production at pH 7.5 [27].

Examination of the effect of temperature showed optimum growth and a higher level of antimicrobial activity at 28°C. Extreme temperatures are generally unfavorable for bioactive metabolite production due to the harmful effect imparted on the enzymes involved in the biosynthetic pathway [5,28]. Carbon and nitrogen sources play an important role in growth and secondary metabolite production in various industrially important microorganisms. The present study revealed that *Streptomyces* sp. JRG-04 grown in a medium containing maltose as the sole carbon source produced maximum biomass and antimicrobial substance as compared to lactose, which only moderately supported the growth and antimicrobial activity, and other carbon sources. Similar to this study, maltose was reported as the best carbon source for growth and antimicrobial compound production in *Streptomyces albidoflavus* [29]. Nitrogen assimilation is the key factor in the regulation of antibiotic production in microorganisms [30]. In this study, yeast extract provided maximum growth and antimicrobial activity against the test organism. These results are in accordance with avilamycin production by *S. viridochromogenes* Tu 57-1 [31]. Other organic nitrogen sources provided for a moderate and lower antimicrobial activity.

It is important to identify components of the medium used in fermentation processes for antimicrobial compound production. In this study, we adapted the PBD method to select the factor suitable for enhancing the antibiotic production. A Pareto chart can be applied to screen significant factors in the t-value limit [10]. Based on the Pareto chart, the three important components, maltose, yeast extract and ammonium chloride, were selected, and their optimum concentrations were further identified by RSM.

Application of RSM shows the experimental relationship between the logarithmic values of antimicrobial compound production and the coded units of the test variables [15]. In general, a regression model having an *R* squared-value higher than 0.9 was considered as a high correlation [32]. The coefficient variation value CV=11.89% indicates that the experimental data were accurate and reliable [16]. The lack of fit of the p-value 0.0869 implied a nonsignificant lack of fit, indicating that the model is reliable. The value of "Prob>*F*" lower than 0.05 indicated that the model terms are significant [33]. The interaction of maltose (X_1), yeast extract (X_2) and ammonium chloride (X_3) indicated that the model is significant due to the p value <0.0001 in all the cases.

In the unoptimized medium, the antimicrobial activity was 140.57 U/ml±0.80. Overall, we obtained a 42.8% increase in activity. This result clearly indicates that the optimized medium favors enhanced antimicrobial compound production by *Streptomyces* sp. JRG-04.

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