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Optimization of culture medium for improved production of antimicrobial compounds by *Amycolatopsis sp.* -AS9 isolated from vermicasts

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ABSTRACT

Amycolatopsis sp.-AS9 (GenBank Accession no.: MG890287), isolated and characterized from vermicasts, has been identified as an antibiotic producing actinomycetes isolate with antimicrobial activity. In the present study, optimization of the culture medium for antibiotic production by the isolate *Amycolatopsis sp.*-AS9 was performed using a statistical approach based experimental designs. Response surface methodology (RSM) with central composite rotatable design (CCRD) was employed to optimize the influence of various parameters to improve the antibiotic production by *Amycolatopsis sp.*-AS9. In the RSM experiments, pH, temperature, inoculum volume, and agitation speed were found to have a significant effect on the antibiotic output which was directly proportional to the antimicrobial activity. The optimum conditions for the highest antimicrobial activity was found when the inoculum size (0.3%), pH (7.24), temperature (30 °C) and agitation speed (50.8 rpm) were employed.

1. Introduction

The gradual increase in the incidence of acquired infections caused by drug-resistant bacteria is currently a pressing issue creating a threatening impact on societies worldwide. Among all the discoveries made in the 20th century development and exploitation of microorganisms for the production of antibiotic was one of the significant and noted advances in the pharma industries. In the present day, due to the growing gap between drug resistance and emergence of super bugs, there is an increasing need for the development of more new potent and selective antimicrobial agents, through drug discovery. Research on antibiotics provides a wide range of structurally diverse and active agents for the treatment of several microbial infections (Durand et al., 2019).

Antibiotics are produced by different species of actinomycetes (Clardy et al., 2006; Grasso et al., 2016) including the well-known species of *Amycolatopsis* and *Streptomyces*, which are playing a vital role in the production of novel secondary metabolites with antibacterial properties (Hassan et al., 2011; Hopwood, 2007; Xiao et al., 2017). Biologically active molecules can act as an agents to block the antibiotic resistance ability of the microbes, in order to improve the host resistance to the human pathogens (Wright, 2016).

Culture media and physical parameters play a vital role which significantly affect the production of antimicrobials by actinomycetes (Djinni et al., 2014). The production of secondary microbial metabolites through fermentation has been found to be unstable, leading to inconsistent yield of the target active ingredients either through the physical or chemical parameters like pH, temperature, and time period that critically impact yield of such substances (Kiranmayi et al., 2011)). Important enzymes also play a critical role in antibiotic production, which can also be affected by pH of the medium (Gao et al., 2016). Several physical factors (incubation temperature, pH, agitation speed, aeration and phase variation of the bacteria) are known to affect the growth and production of antibiotics of *Xenorhabdus spp.* (Webster et al., 2002).

Statistical experimental approaches enhance the quality and quantity of the desired product to be produced by preventing inconsistency, saving time and cost. The application of statistical experimental design methodologies for physical parameters can improve the product yield and reduce time, when compared to one factor at a time (OFT) method (Ghasemi et al., 2014; Goupy, 1999). The single factor optimization has few drawbacks. It is unreliable, is highly laborious and time-consuming

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Table 1
Experiment design and results of optimization of Strain AS9 fermentation conditions by the central composite design observed value and predicted values of fermentation conditions.

Std	Run	Block	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	
			A: pH (X_1)	B: Temp (X_2)	C:Agitation (X_3)	D: Inoculum size (X_4)	Observed value (mm)	Predicted value (mm)
22	1	Block 1	7	30	150	0.3	6	7
21	2	Block 1	7	30	−50	0.3	7	7
17	3	Block 1	3	30	50	0.3	3	3
30	4	Block 1	7	30	50	0.3	9	9
10	5	Block 1	9	25	0	0.4	5	5
15	6	Block 1	5	35	100	0.4	8	7
20	7	Block 1	7	40	50	0.3	4	4
12	8	Block 1	9	35	0	0.4	5	5
23	9	Block 1	7	30	50	0.1	7	8
13	10	Block 1	5	25	100	0.4	6	6
27	11	Block 1	7	30	50	0.3	11	9
2	12	Block 1	9	25	0	0.2	5	5
6	13	Block 1	9	25	100	0.2	4	4
8	14	Block 1	9	35	100	0.2	4	4
19	15	Block 1	7	20	50	0.3	3	3
9	16	Block 1	5	25	0	0.4	5	5
5	17	Block 1	5	25	100	0.2	5	5
25	18	Block 1	7	30	50	0.3	11	9
26	19	Block 1	7	30	50	0.3	11	9
14	20	Block 1	9	25	100	0.4	5	5
7	21	Block 1	5	35	100	0.2	6	6
11	22	Block 1	5	35	0	0.4	6	6
4	23	Block 1	9	35	0	0.2	4	4
29	24	Block 1	7	30	50	0.3	11	9
24	25	Block 1	7	30	50	0.5	11	9
1	26	Block 1	5	25	0	0.2	5	5
16	27	Block 1	9	35	100	0.4	5	5
3	28	Block 1	5	35	0	0.2	5	5
28	29	Block 1	7	30	50	0.3	11	9
18	30	Block 1	11	30	50	0.3	1	1

Note: Observed value was the diameter of zones of inhibition. X_1 pH;X_2 –temperature; X_3 rotary speed; X_4 – inoculation volume.

than the statistical methods (Adinarayana et al., 2003; Elibol, 2004; Rao et al., 2000). In the present study, an experimental design based on a statistical approach has been employed for selecting the significant variables which play a critical role in enhancing the production of antibiotics by *Amycolatopsis sp.*-AS9. The significant physical parameters can be optimized using response surface methodology with either central composite design (CCD) (Wang et al., 2011) or Box-Behnken Design experiments (Kanmani et al., 2013). Therefore in this study RSM can be used to evaluate the relative significance of several contributing factors even in the presence of complex interactions (Dey et al., 2001; Elibol, 2004; Hamsaveni et al., 2001; Rao et al., 2000).

Novel antibiotic producing actinomycetes may possibly be present in Arctic and Antarctic regions, marine or soil-associated environments (De Mol et al., 2018; Palla et al., 2018; Shah et al., 2017). The earthworms are known to harbour functionally different groups of microorganisms and the excretory pellets –the vermicasts enhance the richness and diversity of bacterial, fungal and actinomycetes population in natural eco-systems and in vermicomposting systems (Ananthavalli et al., 2019; Hoeffner et al., 2018; Kale and Karmegam, 2010). Recent studies reveal that the actinomycetes isolated from vermicasts in hill tracts possess antimicrobial properties due to their ability to produce antimicrobial secondary metabolites (Balachandar et al., 2018, 2016). However, the culture conditions and media optimization for the vermicast-isolated actinomycetes has not been documented till date. Hence, the present study was carried out to give an insight into the optimum production of antimicrobial compounds by *Amycolatopsis sp.*-AS9 using statistical method based optimization technique.

2. Materials and methods

2.1. Sample collection and isolation of actinobacterial isolates with antimicrobial activity

Six vermicast samples were collected from southern Eastern Ghats, Tamil Nadu, India, and transported to the laboratory. The collected vermicast samples were serially diluted up to 10^{-5} (Hayakawa et al., 1988), and from this 100 µl of the sample was spread over the surface of two different culture media which included actinomycetes isolation agar (AIA) and starch casein agar (SCA) (Küster and Williams, 1964) and incubated at 30 °C for 7–8 days. After incubation, the actinomycetes were identified based on the phenotypic characteristics. The isolated actinomycetes samples were further purified by standard pure culture techniques. The pure strains were selected, maintained in SCA and stored at 4 °C for further use. Out of six actinobacterial isolates, the isolate designated as AS9 showed higher zone of inhibition with a diameter of 16.0 mm, 11.5 mm and 9.0 mm respectively against *Staphylococcus aureus, Bacillus circulans* and *Bacillus subtilis* respectively by Kirby Bauer Agar well diffusion method on Mueller Hinton medium uniformly inoculated with a lawn of bacterial strains on the agar surface (10^7 CFU/mL^{-1}). Hence the actinomycetes isolate AS9 identified as *Amycolatopsis sp.*-AS9 using 16S rRNA partial sequencing (GenBank Accession no.: MG890287) was used for further optimization studies.

2.2. Selection of culture medium for antibiotic production by Amycolatopsis sp.-AS9

The potent actinomycetes isolate, *Amycolatopsis sp.*-AS9 was examined in two different media (SCA and AIA), inoculated with spore-containing seed culture and incubated at 28 °C for 7 days. The experiments were conducted in triplicates, and the average antimicrobial

Table 2
Analysis of variance of response surface model to predict zone of inibition efficiency.

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F-value	p-value Prob > F	
Model	223.8833	14	15.99166667	37.38312	< 0.0001	Significant
A-pH	7.041667	1	7.041666667	16.46104	0.0010	
B-Temperature	1.041667	1	1.041666667	2.435065	0.1395	
C-Agitation	0.041667	1	0.041666667	0.097403	0.7593	
D-Inoculum size	9.375	1	9.375	21.91558	0.0003	
AB	1.5625	1	1.5625	3.652597	0.0753	
AC	1.5625	1	1.5625	3.652597	0.0753	
AD	0.0625	1	0.0625	0.146104	0.7076	
BC	0.5625	1	0.5625	1.314935	0.2695	
BD	0.5625	1	0.5625	1.314935	0.2695	
CD	0.5625	1	0.5625	1.314935	0.2695	
A^2	130.003	1	130.0029762	303.9031	< 0.0001	
B^2	89.0744	1	89.07440476	208.2259	< 0.0001	
C^2	30.36012	1	30.36011905	70.97171	< 0.0001	
D^2	5.002976	1	5.00297619	11.69527	0.0038	
Residual	6.416667	15	0.427777778			
Lack of Fit	3.083333	10	0.308333333	0.4625	0.8598	Not significant
Pure Error	3.333333	5	0.666666667			
Cor Total	230.3	29				

Table 3
Analyses of variance (ANOVA) for the quadratic model for optimization of the culture condition.

Parameter	Values	Parameter	Values
Std. Dev.	0.654047229	R-Squared	0.972138
Mean	6.3	Adj R-Squared	0.966133
C.V. %	10.38170205	Pred R-Squared	0.940816
PRESS	22.56	Adeq Precision	21.17203

Note: Coefficient of determination $R^2 = 0.972138$; Coefficient of determination adjusted R^2 = D 0.966133.

activity was assessed based on the zone of inhibition.

2.3. Experimental design and optimization of physical parameters

A four-factor three levels of central composite design was used in this study, requiring 30 experiments. The fractional factorial design consisted of nine factorial points, fourteen center points, and 9 axial points with four parameters. The level of various physical parameters used for the production of secondary metabolites were: X_1: *pH* (3–11), X_2: temp (25–40), X_3: Agitation (50–150), X_4: Inoculation volume (0.1%–0.5%) (Table 1). Contour plots were generated to illustrate the main and interactive effects of the independent variables on the dependent ones. The optimum combination of parameters can be determined on the basis of the maximum ridge analysis and the canonical analysis using the optimization function of the MINITAB 14 software. The optimum value of any variable for maximum antimicrobial activity was determined by the response optimizer tool in the software.

2.4. Optimization of significant physical parameters by response surface methodology (RSM)

Response surface regression procedure was used for the fitted experimental results of response surface methodology (RSM). The variables were given coded values according to the equation:

$$X_i = \frac{(X_i - X_i)}{X_i} = 1,2,3, \ldots\ldots K, \quad (1)$$

Where, *Xi* is an independent variable coded value, *X*i is the independent variable's real value, *X* is the independent variable's real mean, and *X*i is the step change value. The second-order polynomial model was fitted to a response curve fitting the equation:

$$\hat{\mathscr{Y}} = \hat{\beta}_0 + \sum_{i=1}^{k} \hat{\beta}_i x_i + \sum_{i=1}^{k} \hat{\beta}_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{i-1} \hat{\beta}_{ij} x_i x_j \quad (2)$$

Where, $\hat{\mathscr{Y}}$ is the measured response; $\hat{\beta}_0$ is the intercept term; $\hat{\beta}_i$, $\hat{\beta}_{ii}$, and $\hat{\beta}_{ij}$ are measures of the effects of variables x_i, x_i^2, and $x_i x_j$, respectively. The variable $x_i x_j$ represents the first-order interaction between x_i and x_j ($i < j$).

Statistical analysis of the model was performed in the form of analysis of variance (ANOVA), including the Fisher's F-test, associated probability P (F), determination coefficient R^2, and correlation coefficient R that measures the goodness of fit regression model. The analysis also included Student's t-value for the estimated coefficients and associated probabilities, P (t). For each variable, the quadratic models were represented as contour plots.

2.5. Extraction of the fermentation broth

The *Amycolatopsis sp.*-AS9 was inoculated in a flask containing starch casein nitrate broth and incubated at 37 °C for 7–10 days. After incubation the fermentation broth was centrifuged at 15000 rpm for 5 min. Then, the supernatant was collected and mixed with an equal volume (1:1 v/v) of ethyl acetate. The ethyl acetate layer containing active substance was concentrated by evaporating to dryness at 55 °C, and crude extract was obtained.

2.6. Partial purification and identification of bioactive compounds

The crude extract was purified by silica gel column chromatography. Fractions of bioactive compounds were collected by using 10% methanol as a solvent. The bioactive compounds present in the fractions were identified by using GC-MS. GC-MS analysis of a crude extract of isolates was performed in JEOL GC MATE II model equipped with quadrupole with double focusing mass analyzer on HP5 column. The initial oven temperature was 50 °C for 2 min, increased at 10 °C/min to 25 °C and held for 5 min. Helium gas as the carrier gas was at a constant flow rate of 1 ml/min. The mass transfer and source temperature were both set at 25 °C.

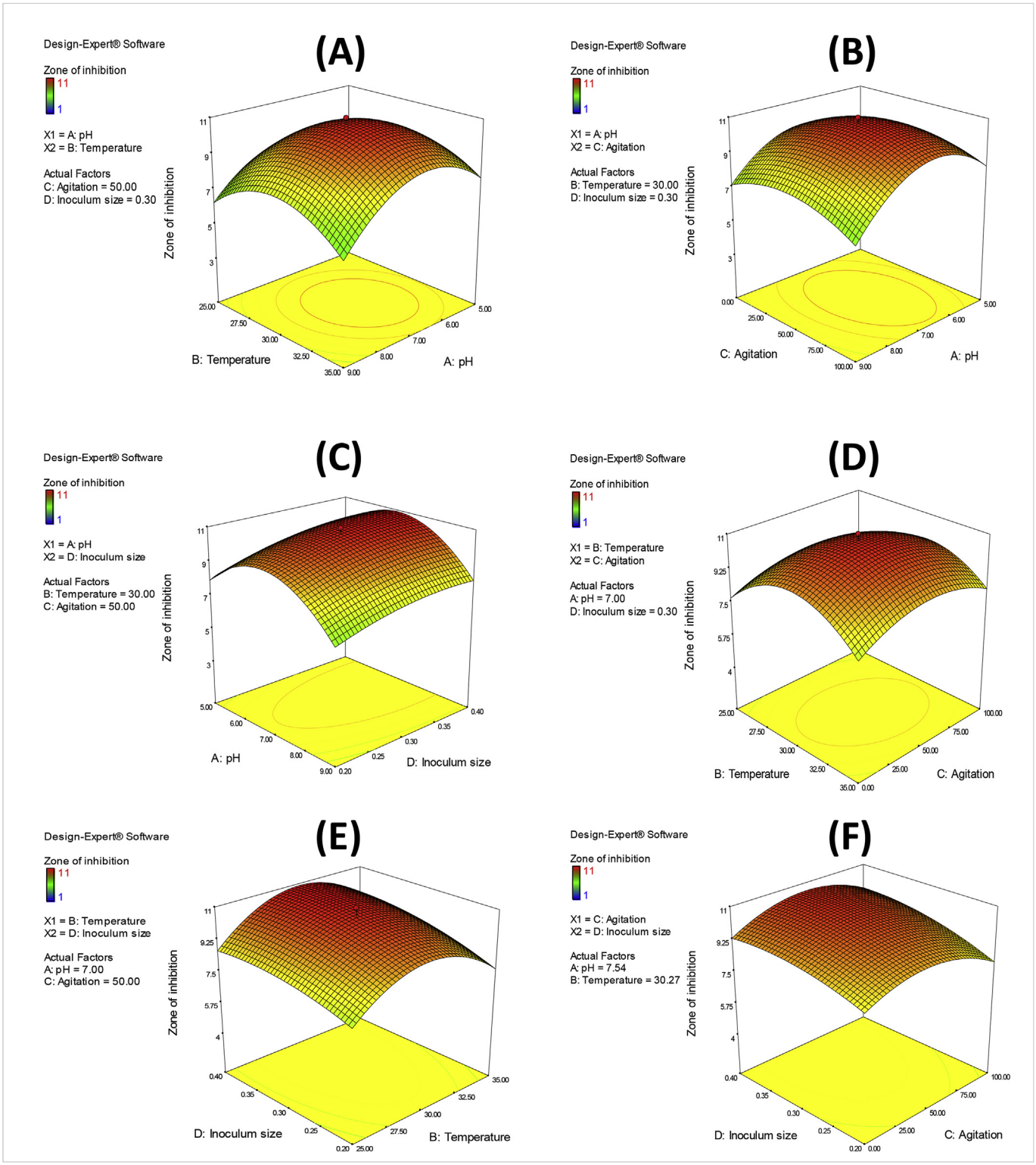

Fig. 1. Response surface plots and contour plots showing individual and interactive effect of variables on zone of inhibition efficiency with respect to (A) pH and temperature, (B) Agitation speed and pH, (C) pH and inoculum size, (D) Agitation speed and temperature, (E) Temperature and inoculum size, and (F) Inoculum size and agitation speed.

3. Results and discussion

3.1. Optimization of fermentation conditions

The effect of various physical parameters on secondary metabolite production was investigated using a central composite experimental design. Thirty experiments with a different range of parameter conditions were performed to improve the production of antimicrobial compounds (Table 1). Temperature, pH, inoculum volume and agitation speed had a significant effect on antimicrobial compounds production. In most aerobic fermentation dissolved oxygen plays a crucial role in the cell growth and production of secondary metabolites (Dou

Table 4
The antimicrobial compounds in ethyl acetate extract of the actinomycetes isolate-AS9.

Compound name	Molecular formula	Molecular wt. (g/mol)	Activity reported	Reference
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.457	Antibacterial and antifungal activity	Hsouna et al. (2011)
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	Antioxidant, antibacterial, antitumour, cancer preventive and immune stimulant activity	Selvin et al. (2009)
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.4721	Anticancer, anti-inflammatory activity	Yu et al. (2005)
9-Octadeconic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	296.495	Antifungal, antitumour and antibacterial activity	Abou-Elela et al. (2009)
Methyl 9-cis,11-trans-octadecadienoic acid	$C_{19}H_{34}O_2$	294.479	Antibacterial and antifungal activity	Pinto et al. (2017)
Methyl stearate	$CH_3(CH_2)_{16}$ CO_2CH_3	295.50	Antibacterial and antifungal activity	Pinto et al. (2017)

et al., 2013; X. X. Wang et al., 2010; Y.-H. Y.-H. Wang et al., 2010). Uniform distribution of oxygen in the fermentation medium can be achieved by agitation.

By applying multiple regression analysis to the experimental data, the following second-order polynomial equation was found to give an adequate description describing the antibiotic activity.

$$\text{Zone of Inhibition} = 10.67 - 0.54167A + 0.2083B 0.04167C + 0.625D - 0.3125AB - 0.3125AC - 0.0625AD + 0.1875BC + 0.1875BD + 0.1875CD - 2.17708A^2 - 1.80208B^2 - 1.05208C^2 - 0.42708D^2.$$

Where, Y is antibiotic activity response; and X_1, X_2, X_3 and X_4 are the coded values of the independent factors, *viz.,* pH, temperature, agitation speed and inoculum volume respectively.

The ANOVA was performed to inspect the second-order response surface model and also to test the significant and insignificant effects of the equations, and the same has been reported for a zone of inhibition after the treatment period (Table 2). Relatively high coefficient of determination value ($R^2 = 0.9020$) showed that the model was significant and the developed models for maximizing zone of inhibition are statistically accurate for the system under the given experimental conditions (i.e., at a 99% confidence interval). A high value (0.9461) of adjusted R-Square (R_{adj}^2) indicates the significance of the models. The value of the coefficient of variation (CV) was found to be 1.03%, and these values are < 10%, which indicated that the results from experiments were reliable and accurate (Reed et al., 2002). The insignificance of "lack of fit" (> 0.05) indicates that the model was statistically appropriate for further use. It can be observed from Table 3, where the coefficients for linear terms, interaction terms, and the square terms are quite significant. The present study results fall in line with a highly significant coefficient of determination (R^2) value of 0.9935 and determination coefficient (R^2 adjusted) value of 0.9819 reported while optimizing the media for *Streptomyces sp.* (Souagui et al., 2015).

3.2. Response surface and contour plots

The RSM provides a clear understanding of the interaction between the optimum levels of each parameter (Silva and Roberto, 2001). In this study, RSM was used to optimize the effective parameters with the aim of maximizing zone of inhibition. It was investigated by three-dimensional (3D) surface and two-dimensional (2D) contour plots. The 3D surface is a graphical representation of a 3D view that shows the individual and cumulative effects of the variables and interaction between variables (Xin-hui et al., 2014). The interactive effect of two selected parameters on the zone of inhibition was assessed by plotting as 3D surface curves against the two parameters as the independent variables, by keeping the other two variables at its central level. The geometric nature of the response surface obtained may be maximized or minimized to determine the optimum level. The contour plot is the projection of the response surface as a 2D plane by plotting constant *z*-slices.

Fig. 1 shows the 3D response surface plots of the relationship between differential parameters for the zone of inhibition after incubation time. The interactive effect of two variables (pH value (A) and temperature (B) was in the range of 4–8 and 25–45 °C, respectively) on zone of inhibition [Fig. 1 (A)]. The predicted optimum temperature level and pH value were 30 °C and 7.3, respectively, for the maximized zone of inhibition (11 mm). In Fig. 1(B), a 3D surface plot shows the interactive effect of agitation speed (*C*) and pH (*A*) on zone of inhibition at fixed inoculum size (0.3) and temperature (30 °C). As per RSM, maximized inhibition occurred (11 mm) with the optimum speed of 53 rpm and a pH value of 7.0. The elliptical nature of the contour plot confirms that the interaction between these two independent variables was significant. Fig. 1(B), The interactive effect of the two variables pH value (*A*) and inoculum size (*D*) was found in the range of 4–8 and 0.1–0.9,

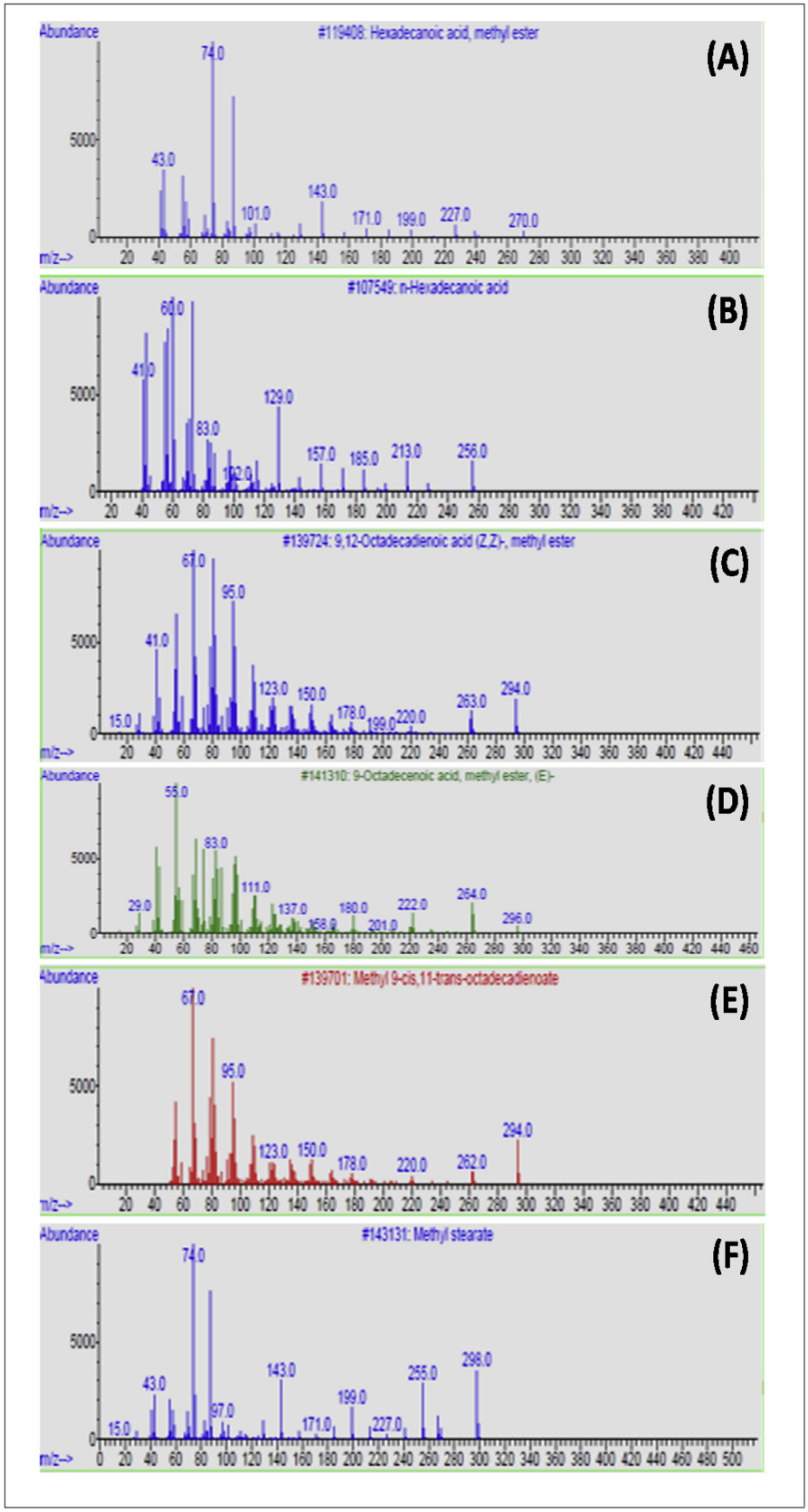

Fig. 2. GC-MS chromatogram showing major antimicrobial components in ethyl acetate extract of the actinomycetes isolate-AS9 (A) Hexadecanoic acid, methyl ester, (B) n-Hexadecanoic acid, (C) 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, (D) 9-Octadeconic acid, methyl ester, (E)-, (E) Methyl 9-cis, 11-trans-octadecadienoic acid, and (F) Methyl stearate.

respectively. It is clear from the figure that there is a combined effect of pH and inoculum size at a constant agitation speed (50 rpm) and temperature (30 °C). The maximum of 11 mm zone of inhibition occurred at an initial pH of 7.4 and an inoculum size of 0.35%. The elliptical nature of the corresponding contour plot showed a significant interaction between these two independent variables. It means that antibiotic production was dependent on the pH value and inoculum size. The maximum inhibition at optimum pH indicates the sensitiveness of the process to pH value.

The interactive relationship between the parameters, temperature (*B*), and agitation (*C*) on the zone of inhibition at fixed inoculum size (0.3%) and pH value (7.0) as shown in Fig. 1(D). The elliptical nature of the contour plot indicates that the interaction between the two independent variables was significant in inhibition. The predicted optimum levels for temperature and agitation were 33.1 °C and 55 rpm, respectively, for maximized zone of inhibition (11 mm). An increasing trend with rise in temperature from 25 to 30 °C was observed. In contrast, further increase in temperature beyond 30 °C did not show any higher efficiency, which might be due to the adverse effect of high temperature. Temperature had great impact on production, at low temperature environmental pressure affected the secondary metabolites and resulted in amplified production (Holder and Boyce, 1994).

The synergy between the parameters, temperature (*B*), and Inoculum size (*A*) on zone of inhibition at fixed agitation speed (50 rpm) and initial pH value (7.0) is shown in Fig. 1(E). The predicted optimum levels for temperature and Inoculum size were 31.3 °C and 0.35%, respectively, for maximized zone of inhibition (11 mm). The effect of two variables, agitation speed (*C*) and inoculum size (*D*) was in the range of 50–250 rpm and 0.1–0.9% for zone of inhibition [Fig. 1(F)]. According to this plot, the zone of inhibition was increased by increasing the agitation speed and inoculum size until reaching the optimum level. The maximized zone of inhibition (11 mm) was observed at the optimum agitation speed of 52.5 rpm and an inoculum size of 0.3. Beyond this, a decrease in the inhibitory level was found. This might be due to decrease in effective reactivity by high shear stress among inoculum size while processing above the optimum agitation speed. The contour plot points show that interaction between these two independent variables (inoculum size and agitation speed) was highly significant. It is also apparent that the inhibition was highly influenced by these parameters.

The optimum predicted cultural conditions were as follows: initial pH = 7, temperature = 30 °C, agitation = 50 rpm and inoculum volume = 0.3%. These values predict a 11.9 mm diameter inhibition zone. The optimized cultural parameter was verified by carrying shake flask cultures. The maximum antimicrobial activity obtained experimentally was 11.0 ± 1.5 mm diameter of zone of inhibition. As a result, the model developed was considered to be accurate and reliable for predicting the production of antibiotic by the actinomycetes-AS9 isolated from vermicasts. Antimicrobial activity was improved by 20% after optimization of cultural physical parameters. RSM was found to be a suitable optimization technique while optimizing antifungal production by an alkalophilic and halotolerant actinomycete, *Streptomyces sp.* SY-BS5 (Souagui et al., 2015), production of vanillin by *Escherichia coli* (Chakraborty et al., 2016) and antibacterial thiopeptide nocathiacin I production by *Amycolatopsis fastidiosa* LCB1001 (YANG et al., 2017). These results suggest that RSM is a suitable technique for optimization processes which were similar to that of the present study.

3.3. Extraction and purification

Fermentation broth was extracted with ethyl acetate (1:1 *v/v*). After drying 1 g of crude extract was obtained. Bioactive compound were purified from the crude extract by using silica gel column chromatography. Five fractions were collected and subjected to reveal the bioactive compounds present in the fractions by GC-MS analysis. Six different compounds, hexadecanoic acid, methyl ester; n-hexadecanoic acid; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; 9-octadeconic acid, methyl ester, (E)-; Methyl 9-cis, 11-trans-octadecadienoic acid and methyl stearate were identified as a bioactive compounds against pathogens (Table 4; Fig. 2). The GS-MS derived bioactive compounds along with chemical formula and molecular weight, and activities reported are provided in Table 4.

Conclusions

In this present study, *Amycolatopsis sp.*-AS9 with antimicrobial activity was isolated from vermicasts, and subjected to culture optimization studies using CCD and RSM methods. The optimum predicted cultural conditions were as follows: initial pH = 7, temperature = 30 °C, agitation = 50 rpm and inoculum volume = 0.3%. These values predict a 11.9 mm diameter inhibition zone. Furthermore, the optimum cultural conditions obtained is useful for the development of *Amycolatopsis sp.*-AS9 cultivation process for the efficient production of antibiotics in large fermentation in the fermenter.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101186.

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