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Original Article

Improved Bioactive Metabolite Production by *Saccharopolyspora halotolerans* VSM-2 using Response Surface Methodology and Unstructured Kinetic Modelling

Ushakiranmayi Managamuri[1], Muvva Vijayalakshmi[1*], Mani Deepa Indupalli[1], Venkat Siva Rama Krishna Ganduri[2], Satish Babu Rajulapati[3], Sudhakar Poda[4]

Ushakiranmayi Managamuri[1], Muvva Vijayalakshmi[1*], Mani Deepa Indupalli1, Venkat Siva Rama Krishna Ganduri[2], Satish Babu Rajulapati[3], Sudhakar Poda[4]

[1]Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna nagar, Guntur-52510, Andhra Pradesh, INDIA.
[2]Department of Biotechnology, K L University, Vaddeswaram, Guntur, Andhra Pradesh, INDIA.
[3]Department of Biotechnology, National Institute of Technology, Warangal, Telangana, INDIA.
[4]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna nagar, Guntur-52510, Andhra Pradesh, INDIA.

Correspondence

Prof. Muvva Vijayalakshmi

Acharya Nagarjuna University, Nagarjuna nagar, Guntur-522510, Andhra Pradesh, INDIA.

Phone no : +91-9440870026

E-mail: profmvl08@gmail.com

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ABSTRACT

Background: This study targets to optimize and analyse the interactive effects of process variables for improved bioactive metabolite production using RSM and unstructured kinetic modelling by *S. halotolerans* VSM 2. **Materials and Methods:** RSM was applied to optimize the interactive effects of five variables, viz., time of incubation, pH, temperature, concentration of maltose and meat extract on bioactive metabolite production and its effect against the five responses viz., *S. flexneri*, *S. marcescens*, *P. vulgaris*, *P. aeruginosa* and *E. coli.* Models of Logistic and Luedeking-Piret were used to simulate the cellular increase and bioactive metabolite production. **Results:** RSM optimal conditions for the bioactive metabolite production recorded were incubation time (12days), pH (8), and temperature (25^{0}C), concentrations of maltose and meat extract (1 % w/v) (each). The effect of the bioactive metabolite produced (zone of inhibition) against the responses were found to be 17 mm for *S. flexneri*, 17 mm for *S. marcescens*, 16 mm for *P. vulgaris*, 17 mm for *P. aeruginosa* and 18 mm for *E coli*. The data obtained from experimental values are in close agreement with the predicted values of RSM. Model adequacy was evaluated using ANOVA variance where the quadratic effect of $p<0.0001$ which imply the significance of the model. The unstructured-, mathematical- kinetic models provided a better approximation of profiles of *S. halotolerans* VSM 2 growth, optimized media utilization and bioactive metabolite production. **Conclusion:** Optimization of the independent variables for the production of the bioactive metabolite using RSM by *S. halotolerans* VSM 2 and its effect against the five responses were documented. The predicted values are in good agreement with the experimental values. Unstructured models provided a better approximation of kinetic profiles for bioactive metabolite production by *S. halotolerans* VSM 2.

Key words: *Saccharopolyspora halotolerans*, Response surface methodology, Optimization, Bioactive metabolites, Kinetic modelling.

INTRODUCTION

Nature's extreme environments are untapped immense potential resources for discovery and isolation of novel microbes that are taxonomically significant. These microbes are an essential treasure for novel bioactive lead compounds which might be a consequence of their evolution and adaptation to metabolic biochemistry such as enzymes and antibiotics.[1] Hence marine microbes offer novel biocatalysis and value added molecules.[2] Marine microbial diversity enacts limitless pool of novel metabolite chemistry that contributed an important source to the innovative biotechnology.[3] The search for novel antibiotics from the terrestrial microbes have diminished since this resource has been extensively explored and precious compounds have been already derived from these microbes.[4] Hence the researchers and scientists switched over to explore new environments for the discovery of pharmaceutical compounds to combat human pathogens.

Actinomycetes are an important group of microbes that dwell in diverse ecological extreme environments[5] and are assuring sources for the unconventional anti-microbial compounds that are exclusive and carry unexplored metabolic pathways even at the species variants.[6] The production of the bioactive compounds by the actinomycetes is influenced by the potency of the strain, nutritional and physical conditions, since the metabolism of the strain influenced by the medium constituents for the production of the bioactive compounds. Several environmental factors including temperature, pH and incubation period greatly influence the metabolite production in addition to carbon and nitrogen source.[7]

Response Surface methodology (RSM) is a competent statistical method for model development and optimization of the complex process variables for

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the bioactive metabolite production due to its efficiency and experimental interpretation.[8] The key features of the RSM application are the reduced number of the experiments accompanied with the process variables interaction effects.[9] Hence RSM has been widely applied to design the model for the optimization of bioactive metabolite production which accomplishes it, as an expedient and acceptable method to that of classical optimization method.[10]

Mathematical models provide the complete knowledge of dynamic behavior that allows us to operate, optimize and control most of the fermentation processes. When unstructured models are applied for bioactive metabolite production, they would explain the kinetic relationships between substrate, product and biomass. These models obtained from stoichiometry and kinetic expressions describe each operation unit as reactor. Batch bioprocesses are hard to model, owing to the time-varying characteristics of cell systems, which often results in nonlinearities. For this purpose, model equations are solved, and the values of output variables are obtained as a function of time. Further, the evaluation of assumed unstructured model with experimental data for comparison is carried out, to find the best model that could describe the whole microbial system. Parameters estimation is an essential step in the model verification and subsequent use of a mathematical model. In general, unstructured models consider the cell mass to explain the biological system and are more effective in elucidating the fermentation profiles of microbial process for bio products.[11-13]

As the statistical methods viz., Full-factorial design cannot investigate the second order effects of process parameters and Taguchi design does not evaluate the interaction effects of parameters, the present study has been conducted with the following objectives: (i) to optimize the independent process variables using Central composite design of RSM which determines the optimal values and the interactive effects of the independent variables for the bioactive metabolite production by *Saccharopolyspora halotolerans* VSM 2 and its effect against the five responses. (ii) to assess the kinetic parameters (after verification of mathematical model) in the bioactive metabolite production by *Saccharopolyspora halotolerans* VSM 2.

MATERIALS AND METHODS

Isolation

Marine sediment samples were collected at different depths of the Bay of Bengal of north coastal Andhra Pradesh, India. Samples collected were transported to the laboratory in sterile bags and air dried at room temperature and then subjected to pre-treatment with dry heat at 100°C/one h[14] to increase the actinobacterial population in the sample and to restrain the unwanted contaminants like fungi and bacteria. Pre-treated sediment sample (1g) was suspended in 100 ml sterile distilled water, homogenized by vortexing. Serial dilutions were prepared and 100 µl of 10^{-4} dilution was spread, on Bennett's agar containing 0.1% yeast extract, 0.1% beef extract, 0.2% casein enzymic hydrolysate, 1% dextrose and 2% agar (pH 8) supplemented with nalidixic acid (50 μgml^{-1}) and nystatin (50 μgml^{-1}) followed by incubation at 30°C for two weeks. Morphologically distinct strain was selectively segregated and maintained by sub culturing on yeast extract malt extract dextrose (YMD) agar medium at 4°C for further study.

Identification

Metabolites of the promising actinomycete strain VSM-2 showed significant antimicrobial activity when compared to other tested isolates. The strain was identified as *S. halotolerans* VSM 2 by polyphasic taxonomy and by16S rDNA gene sequence (Gen Bank No: KT901294). Pure culture was maintained on Yeast Extract Malt Extract Dextrose (YMD) agar medium at 4°C for further study.

Experimental Design and Statistical analysis

Central composite design (CCD) of RSM has been enforced to evaluate the interaction effects among the variables and to design optimized conditions of the variables to predict the compelling values against the responses.[15] Five effective variables from the classical optimization approach OFAT (one-factor-at-a-time) were selected. The variables selected include, Time of incubation, pH, Temperature, concentration of maltose and meat extract for the production of bioactive metabolites by *S. halotolerans* VSM 2 and its effect (antimicrobial activity) against the five responses, *Shigella flexneri* (MTCC 1457), *Serratia marcescens* (MTCC 118), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 35218) (Zone of inhibition measured in mm). The experimental design consisted of 50 experimental trials and each of the selected variables has been analysed at three levels, low, medium, and high coded as (*-1*, *0* and *+1*) (Table 1).

The following equation represents the coded process variables

$$x = (X_i - X_0)/\Delta X \qquad \text{(Eq-1)}$$

The interactive effect of the process variables for bioactive metabolite production was studied for the three values for each factor (minimum, mean and maximum) by full factorial design. The number of experiments *n* for *k* factors is given as n=3*k*. The experimental runs have been randomized to reduce the unexpected variability of the observed responses. Experiments were designed according to CCD using a 2^5 full factorial design for five variables that consists 32 factorial points, 10 axial points and 8 replicates. 8 replicates at centre points were used for each categorical variable which incorporates a total of 50 experiments (Table S1) calculated from Eq- 1.[16]

A second order polynomial regression model was employed in the present study in Eq-2. The application of the polynomial equation is linear and quadratic.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_1 X_i + \left(\sum_{i=1}^{n} \beta_{ii} X_i\right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad \text{(Eq-2)}$$

Where Y is the predicted response, β_0 is intercept coefficient, β_i is the linear coefficient, β_{ij} are the interaction coefficients, β_{ii} are the quadratic coefficients, X_i and X_j are coded values of the five additive variables.

Statistical Analysis

Design-Expert version 7, was used for the design of experiments, and analysis of variance (ANOVA) was applied to analyse the interactive effects of the variables (Time of incubation, pH, Temperature, Concentration of maltose and meat extract) for the bioactive metabolite production by VSM 2 and its effect against the responses (antimicrobial activity). The analysis of ANOVA summarizes regression coefficients (RC) Eq. (2), sum of squares (SS), Standard error (SE), F-value (F), and P-value. The statistical significance of the model is analyzed by the probability *p*-value. The *p*-value should be <0.05 and <0.01 for 95% and 99% confidence levels for statistical significance of the effects. Lower the value of *p*, the significance of the corresponding coefficient is more.[17] The fit and quality of the polynomial model was expressed with the value of correlation coefficient (R^2) and adjusted R^2. If the model coefficient determination (R^2) and adjusted (R^2) values are >0.9, suggests that there is a high correlation between the experimental and model predicted values and indicates that the regression model explains the relation between the independent variables and responses.[18] 3D plots were generated by varying two variables with the experimental range with the other variable constant at the central point.[19] 3D response surface analysis determines the optimal regions of the independent and structured variables.[20] Additionally, numerical optimization was executed to determine the

optimum values of the independent variables. Further, a meaningful way of analyzing the kinetic behavior of the cell growth, substrate utilization and product formation in the fermentation process is executed through estimation of its kinetic parameters.

Unstructured Model Development

The proliferation of marine actinomycete with restricting carbon substrates impacts the metabolite production. A set of mathematical and unstructured kinetic models, significantly define the substrate usage and growth-related production formation kinetics in a batch system that were studied by many researchers.[21-22]

Models of Logistic and Luedeking-Piret were used to simulate the cellular increase and antibacterial metabolite production of *S. halotolerans* VSM 2. The statistics acquired from the models had been used to calculate the specific cell growth rate (μ_{max}), d^{-1} and unique production rate of bioactive metabolite, d^{-1}. Under desirable growth conditions with no effects of substrate and product inhibition, growth kinetic model of VSM 2(*X*) (as per Malthus's law), in a batch fermentation is ultimately described as Logistic function:[23]

$$\frac{dx}{dt} = \mu_{max} X \left(1 - \frac{X}{X_m}\right) \quad \text{(Eq-3)}$$

In combination with the equation (Eq-3) leads to the Logistic (L) - type model equation that correlates increased growth of cell:

$$X(t) = \frac{X_0 e^{\mu_{max} t}}{1 - \frac{X_0}{X_m}(1 - e^{\mu_{max} t})} \quad \text{(Eq-4)}$$

X represents biomass concentration (g/L) μ_{max} portrays the maximum specific cell growth rate, d^{-1}, and *Xm* depicts the maximum biomass concentration (g/L).

Antibacterial metabolite production can be obtained from growth limiting substrate (optimized media ingredients) and the substrate utilization kinetics may be taken from Modified Leudeking-Piret (MLP) equation:

$$-\frac{dS}{dt} = r_s = \gamma\left(\frac{dX}{dt}\right) + \eta X \quad \text{(Eq-5)}$$

In combination with the above equation results Logistic Incorporated Modified Leudeking-Piret (LIMLP) equation:

$$S(t) = S_o - \gamma\left[\frac{X_o e_{max} t}{1 - \left(\frac{X_o}{X_m}\right)(1 - e^{\mu_{max} t})} - X_o\right] + \frac{\eta X_m}{\mu_{max}} \ln\left[1 - \left(\frac{X_o}{X_m}\right)(1 - e^{\mu_{max} t})\right] \quad \text{(Eq-6)}$$

Constant of non-growth associated substrate intake, η, in equation (Eq-6) can be calculated from stationary phase data

$$\left(\text{Where } \frac{-dS}{dt} = 0\right); \eta = \frac{-\left(\frac{dS}{dt}\right)_{\text{stationary phase}}}{X_{max}} \quad \text{(Eq-7)}$$

Considerable bioactive metabolite production occurs in late-logarithmic phase of cell growth and its kinetics follows Leudeking-Piret equation [24] as:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad \text{(Eq-8)}$$

Logistic Incorporated Leudeking-Piret (LILP) equation derived from integration of above equation results:

$$P(t) = P_0 + \alpha\left[\frac{X_0 e^{\mu_{max} t}}{1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})} - X_0\right] + \frac{\beta X_m}{\mu} \ln\left[1 - \left(\frac{X_0}{X_m}\right)(1 - e^{\mu_{max} t})\right] \quad \text{(Eq-9)}$$

Non-growth associated product formation constant, β, can be determined from stationary phase data (where):

$$\beta = \frac{\left(\frac{dP}{dt}\right)_{\text{stationary phase}}}{X_{max}} \quad \text{(Eq-10)}$$

Experimental statistics acquired from batch shake-flask fermentations was used to simulate the use of the equations (Eq-4), (Eq-6) and (Eq-9).

RESULTS AND DISCUSSION

A randomized run of 50 experiments were executed by applying Central composite design of RSM. The influence of the model design matrix on the five variables to produce bioactive metabolite by VSM 2 and its effect on the five responses (zone of inhibition in mm) is represented in supplementary Table 1. The advised sequential model analyses the sum of squares and lack of fit tests for the best outcome quadratic model, for all the five responses. Experimental values of the five responses acquired were in close agreement with the predicted values that indicate the model is gratifying according to the experimental design (Table S1).

CCD analysis of bioactive metabolite production

CCD of RSM optimized conditions for the bioactive metabolite production by VSM 2 which executes the highest antibacterial activity were found to be 12 days for time of incubation, pH 8, temperature 25 °C, concentration of maltose 1% and meat extract was 1%. The maximum effect of the bioactive metabolite against the five responses (measured as zone of inhibition in mm) were recorded as 17 mm for *S. flexneri*, 17 mm for *S. marcescens*, 16 mm for *P. vulgaris*, 17 mm for *P. aeruginosa* and 18 mm for *E. coli*.

Regression analysis of the experimental data was performed, and the model was found to be significant with the *p*- value (<0.0001) for all the five responses. The lower the *p* value the more significant is the model. The experimental data of each response followed the second-order polynomial equation.

Table 1: Experimental range of factors studied using CCD in terms of coded and actual factors.

Symbols	Independent Variables	Actual levels of coded factors		
		Low (-1)	Medium (0)	High (+1)
A	Time of Incubation, days	11	12	13
B	pH	7.00	8.00	9.00
C	Temperature, ^{0}C	20	25	30
D	Concentration of Maltose, % w/v	0.50	1.00	1.50
E	Concentration of Meat Extract, % w/v	0.50	1.00	1.50

Table 2: Sequential model fitting for all the responses (in terms of inhibition zone produced by bioactive metabolite).

Model parameter	*S. flexneri*	*S. marcescens*	*P. vulgaris*	*P. aeruginosa*	*E. coli*
Sequential model sum of squares- Quadratic vs 2FI (suggested)					
Sum of squares	75.50	116.4	61.64	75.6	176.78
Degrees of freedom	5	5	5	5	5
Mean square	15.10	23.28	12.33	15.12	35.36
F-value	122.29	263.89	190.0	168.98	308.83
p-value (Prob > F)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Model summary statistics- Quadratic (suggested)					
Standard Deviation	0.35	0.30	0.25	0.30	0.34
R^2	0.9584	0.9799	0.9727	0.9701	0.9821
Adjusted R^2	0.9296	0.9661	0.9538	0.9494	0.9697
Predicted R^2	0.9447	0.9350	0.9083	0.8878	0.9349
Adequate Precession	16.641	23.200	19.282	20.105	23.570
CV %	2.37	2.07	1.81	1.97	2.27

Table 3: ANOVA variance to test the adequacy of the model.

Statistics	Response				
	Shigella flexneri	*Serratia marcescens*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Escherichia coli*
R^2	0.9584	0.9799	0.9727	0.9701	0.9821
Adj-R^2	0.9296	0.9661	0.9538	0.9494	0.9697
Pred- R^2	0.8599	0.9350	0.9083	0.8878	0.9949
Adequate Precession	16.641	23.200	19.282	20.105	23.570
CV %	2.37	2.07	1.81	1.97	2.27

Checking the Model adequacy

ANOVA variance has been applied to analyze the adequacy of the model at a confidence level of 99% (Table 3). The coefficient of determination (R^2) and the adjusted R^2 of all the five responses was found to be >0.9 (Table 3). The Coefficient variation (CV) is the standard deviation expression as percentage (%) of Mean and need to be less than 10%. CV of the five responses is illustrated in Table 3. Adequate precision measures the signal to noise ratio and must be greater than 4 for the model to be significant. The outcome of adequate precision is given in Table 3. The CV and the adequate precision of the model executed were found to be significant.

The Fisher's statistical test was employed to determine the importance of each factor where the significance degree was ranked based on the *F*-ratio value (Table 2). The *p*- value of the model for the five responses were statistically significant with the probability F-Value that is <0.0001. ANOVA variance analysis reveals most of the significant factors, incubation time (days), pH, temperature, concentration of maltose and concentration of meat extract influence the maximum production of the bioactive metabolite by VSM 2 that effect the five responses (inhibition of growth of the pathogenic microorganisms).

Table 4: Estimated kinetic parameters using L, LILP, LIMLP model equations.

Kinetic Parameters	*S. flexneri*	*S. marcescens*	*P. vulgaris*	*P. aeruginosa*	*E. coli*
Logistic (L) Model Parameters					
μ_{max} (d^{-1})			0.72		
R^2			0.99		
X_0 (g/L)			0.005		
X_m (g/L)			0.191		
Logistics incorporated Modified Luedeking-Piret (LIMLP) Model parameters					
γ (g.S/g.X)			17.75		
R^2			0.95		
η (g.S/(g.X.d))			0.5235		
Logistics incorporated Luedeking-Piret (LILP) Model parameters					
α (mm/g.X)	78.37	73.17	68.97	70.22	78.50
R^2	0.986	0.964	0.989	0.981	0.983
β (mm/(g.X.d))			8.658		

Table 5: Comparison of zones of inhibition (mm) from shake-flask experiments and from model.

Maximum Zone of Inhibition (mm)	*S. flexneri*	*S. marcescens*	*P.vulgaris*	*P. aeruginosa*	*E. coli*
Experimental	27	26	25	25	27
Model fitted	25.97	25.01	24.23	24.46	25.99

Interaction effects of the variables

Three-dimensional (3D) surface plots of the five responses illustrated gives the interactive effects of the process variables for maximizing the production of bioactive metabolites. Figure 1-5, represents the 3D plots of the zone of inhibition versus two varying parameters with a fixed value of the third operating parameters. Analysis of the 3D plots revealed that all the five parameters showed a positive effect. Further increase in the concentration and values of the variables showed decrease in the production of bioactive compounds by *S. halotolerans* VSM 2 and decrease in the diameter of the zone of inhibition against the responses. The highest production of the bioactive metabolite by *S. halotolerans* VSM 2 was obtained when the time of incubation was 12 days, pH 8, Temperature 25°C concentration of Maltose and Meat Extract at 1%.

Unstructured kinetic modelling

In this study, for fitting of experimental data with unstructured logistic models, nonlinear regression using least-square method was applied with the help of Microsoft Excel Solver 2010. The profiles of *S. halotolerans* VSM 2 growth limiting substrate utilization results obtained from shake flask experiments and model kinetics were compared in Figure 6(f). Figure 6 (a) - (e) shows the comparison of experimental versus model predicted zones of inhibition of produced bioactive metabolite on media, inoculated with *S. flexneri*, *S. marcescens*, *P. vulgaris*, *P. aeruginosa* and *E. coli* strains over the time. From all the profiles, it was observed that model predicted, and experimental obtained values show very good fit. Biokinetic parameters used in the mathematical model equations were

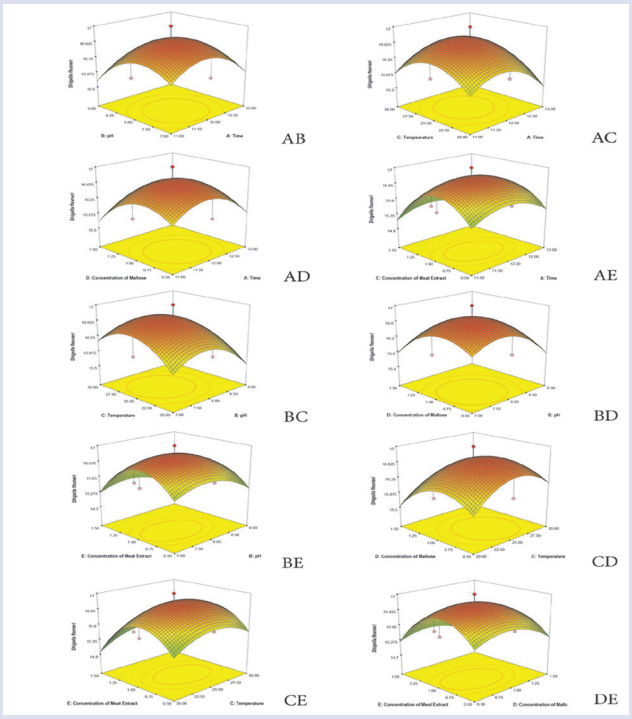

Figure 1: 3D Response surface plots showing interactive effects of selective variables on zone of inhibition (mm) of the bioactive compound production by *S. halotolerans* VSM-2 against *S. flexneri*.

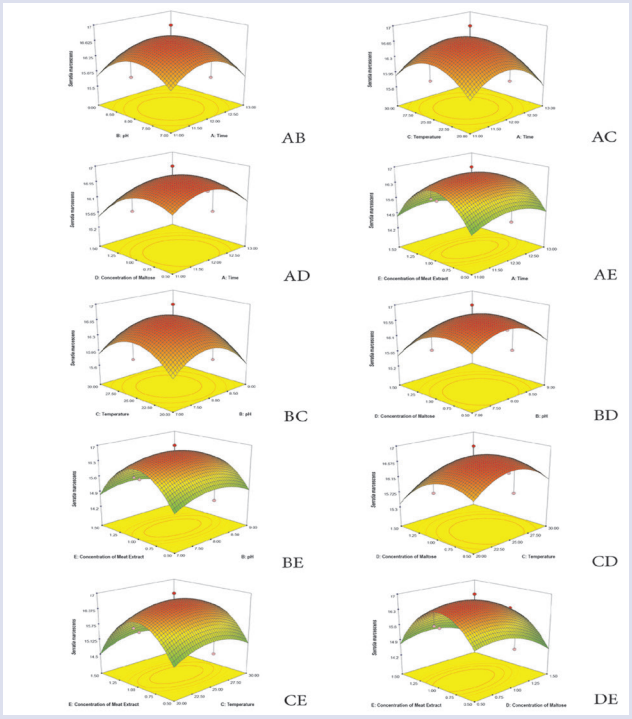

Figure 2: 3D Response surface plots showing interactive effects of selective variables on zone of inhibition (mm) of the bioactive compound production by *S. halotolerans* VSM-2 against *S. marcescens*.

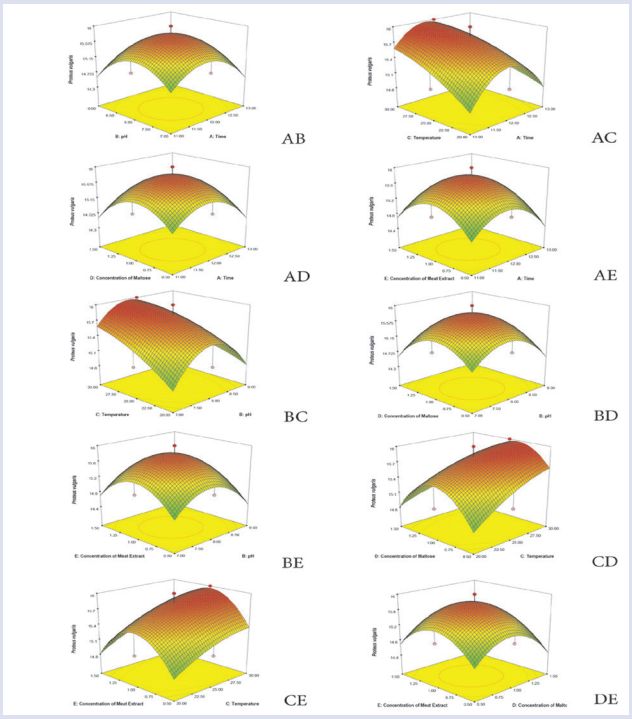

Figure 3: 3D Response surface plots showing interactive effects of selective variables on zone of inhibition (mm) of the bioactive compound production by *S. halotolerans* VSM-2 against *P. vulgaris*.

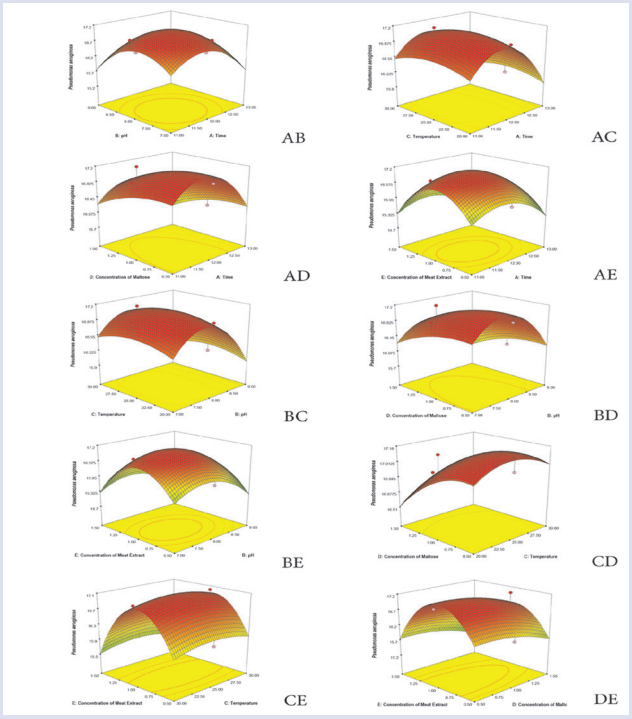

Figure 4: 3D Response surface plots showing interactive effects of selective variables on zone of inhibition (mm) of the bioactive compound production by *S. halotolerans* VSM-2 against *P. aeruginosa*.

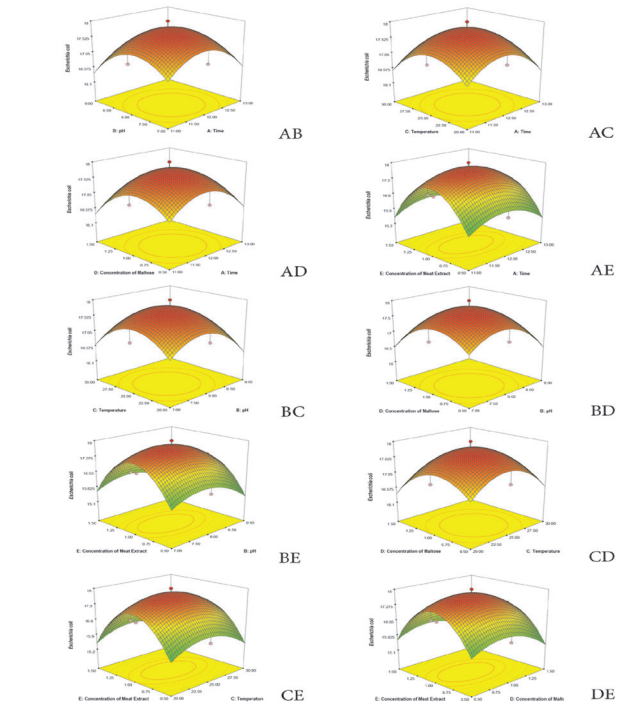

Figure 5: 3D Response surface plots showing interactive effects of selective variables on zone of inhibition (mm) of the bioactive compound production by *S. halotolerans* VSM-2 against *E. coli.*

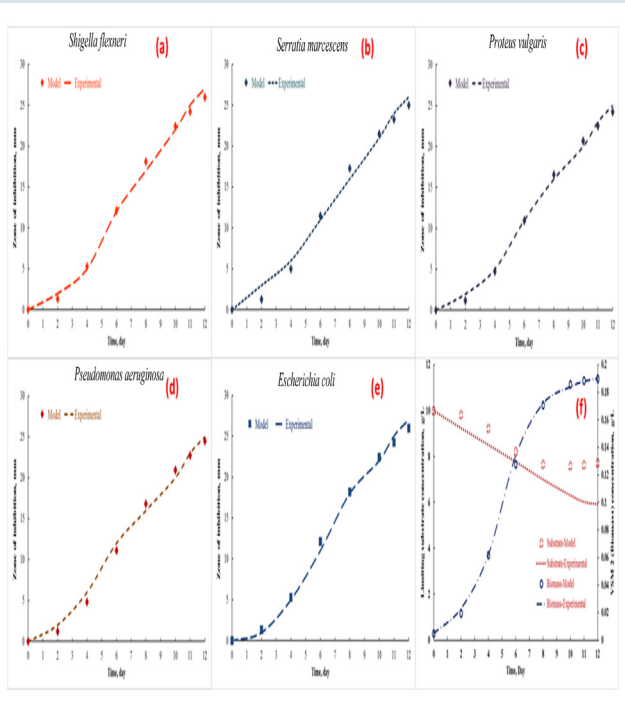

Figure 6: Comparison of experimental and model predicted kinetics (A-E): for zone of inhibition (mm); (F): for biomass growth (g/L), substrate utilization (g/L).

also estimated and are tabulated in Table 4. Table 4 also shows determination coefficient (R_2) values obtained by fitting Logistic (L), Logistic Incorporated Leudeking-Piret (LILP), and Logistic Incorporated Modified Leudeking-Piret (LIMLP) models to the experimental data were found to be high, thus revealing good precision of the models.

From the data of shake-flask used in this study, μ_{max}, X_0 and X_{max} were calculated from *S. halotolerans* VSM 2 growth kinetic profile using Logistic (L) model. Values of growth and non-growth associated product parameters, α and β, were estimated using Logistic Incorporated Leudeking-Piret (LILP) model and a higher α values than β confirmed that bioactive metabolite production by *S. halotolerans* VSM 2 is more growth associated than non-growth associated in shake flask. The simulated parameters, γ and η, of Logistic Incorporated Modified Leudeking-Piret (LIMLP) model are also in good agreement with the experimental values, implies that this model is more appropriate to represent limiting substrate utilization kinetics in bioactive metabolite production by *S. halotolerans* VSM 2. Further, zones of inhibition from agar diffusion tests are almost like model predicted values (Table 5).

Response surface methodology is a statistical and mathematical technique applied for optimizing process parameters and analyzes the interactive effects among the process variables and its effect against the responses.[25] The CCD of RSM optimized conditions for the production of bioactive compounds by *S. halotolerans* VSM 2 were found to be time of incubation as 12 days, pH as 8, temperature as 25°C, concentration of maltose as 1% and meat extract as 1%. Zone of inhibition of the growth of the 5 responses by the bioactive metabolite produced by VSM 2 was recorded as 17 mm for *S. flexneri*, 17 mm for *S. marcescens*, 16 mm for *P.vulgaris*, 17 mm for *P.aeruginosa* and 18 mm for *E. coli*.

Regression analysis was employed to fit the empirical model with the generated response variable data.[26] The data obtained from the CCD of RSM was fitted into second order polynomial equation.[27] Considering only the significant independent factors (Table 2), the obtained model indicates the relationship between the predicted results are in agreement with the experimental results obtained.

ANOVA based computation of the predicted and experimental responses determine the polynomial expression of the responses statistically. Adequacy of the model check is essential to check whether the model is suited and to verify that it delivers an accepted approximation to the actual system.[28] The effect of bioactive metabolite against five responses *S. flexneri, S. marcescens, P. vulgaris, P.aeruginosa* and *E. coli* (zone of inhibition in mm) produced by the optimization of the independent variables have a significant quadratic effect ($p<0.0001$). The p value is taken in to account, to review the significance of each of the coefficients and the interactive strength of each parameter. If the p value is found to be less than <0.05, it suggests that the corresponding variables are more significant.[29] The validity of the model was also confirmed by the insignificant Lack fit test values obtained for the five responses ($p> 0.05$) (Table 2).

The coefficient of determination (R^2) shows the proportion of the total variability of the model which suggests the good fit of the model. R^2 should be close to the value 1 or at least should have a minimum value of not less than 0.80.[30] The coefficient of determination (R^2) of the five responses was found to be above 0.9 for all the five responses (Table 3). Problem with the fit of the model, which always increases with the increase in the added factors even though the factors are not significant. Hence the adjusted R^2 is used to evaluate the model adequacy since it is adjusted for the number of terms in the model.[31] R^2 is adjusted for the size of the model in a way it decreases the insignificant factors added to the model.[32] The value higher than 0.9 indicates that the regression model explained the procedure well. The coefficient of variation (CV) is the standard deviation expressed as a percentage of the mean and need

to be less than 10%.[28] Adequate precision measures signal to noise ratio, greater than 4 is considered as an adequate signal for the model.[33] The signal to noise ratio of the developed model of all the five responses is presented in Table 3. In addition, the coefficient of variation (CV<10%) found to be precise and reliable for the experiments. Three-dimensional (3D) surface plots of the five responses illustrated gives the interactive effects of the process variables. Evaluation of the 3D plots revealed that all the five parameters showed a positive impact.[34]

The profiles of *S. halotolerans* VSM 2 growth limiting substrate utilization results acquired from shake flask experiments and model kinetics of experimental versus model predicted zones of inhibition of produced bioactive metabolite on media, inoculated with *S. flexneri*, *S. marcescens*, *P. vulgaris*, *P. aeruginosa* and *E. coli* strains over the time. From all the profiles, it become determined that model predicted, and experimental obtained values exhibited very good fit. Values of growth and non-growth associated product parameters, α and β, were estimated using Logistic Incorporated Leudeking-Piret (LILP) model and a better α values than β showed that bioactive metabolite production by *S. halotolerans* VSM 2 is more growth associated than non-growth associated in shake flask. The simulated parameters, γ and η, of LIMLP model are also in accurate settlement with the experimental values, means that this model is more suitable to symbolize restricting substrate utilization kinetics in bioactive metabolite production by *S. halotolerans* VSM 2.

Further, the unstructured models provided a better approximation of kinetic profiles of bioactive metabolite production by *S. halotolerans* VSM 2 in submerged shake flask fermentations. To the best of our knowledge, this is the first report on the kinetic modelling for bioactive metabolite production (in terms of zones of inhibition studies) by *S. halotolerans* VSM 2.

CONCLUSION

CCD of the RSM showed the effect against the five responses by the bioactive metabolite produced by *S. halotolerans* VSM 2. The predicted values of the impact of bioactive metabolite produced against the five responses had been well consistent with experimental values. High values of the adjusted and predicted R^2 and adequate precession along with low values of the coefficient of variation conclude that the models for responses suit the experimental data adequately. Process parameters optimized was time of incubation 12 days at pH 8, temperature 25°C and 1% concentrations of maltose and meat extract. Further, the unstructured models provided a better approximation of kinetic profiles of bioactive metabolite production by *S. halotolerans* VSM 2 in submerged shake flask fermentations. To the best of our knowledge, this is the first report on statistical optimization and kinetic modelling for bioactive metabolite production (in terms of zones of inhibition studies) by *S. halotolerans* VSM 2.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RSM: Response Surface Methodology; **CCD:** Central Composite Design.

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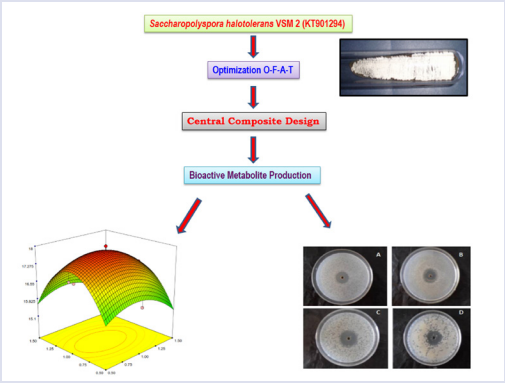
GRAPHICAL ABSTRACT



SUMMARY

- Research designed different process variables affecting the production of bioactive metabolites by *Saccharopolyspora halotolerans* VSM-2 and its effect against the 5 responses was executed in the laboratory. As a result, the application of RSM was handy and viable to study the effect of the important parameters on the production of bioactive metabolites by *Saccharopolyspora halotolerans* VSM-2 and its effect against the 5 responses. In addition the mathematical modelling of the process parameters have been executed to also study the process parametes. The accomplishment of the process optimization was feasible with the application of RSM and factorial design. The study clearly demark that RSM is most reliable design to optimize the operating conditions to escalate the production of bioactive metabolite. Full factorial central composite design (50 assays) was successfully operated for the experimental design and the result analysis. The predicted values of RSM are in agreement with the experimental values. Process parameters optimized was time of incubation 12 days at pH 8, temperature 25 °C and 1% concentrations of maltose and meat extract for the highest production of the bioactive metabolite. Further, the unstructured models provided a better approximation of kinetic profiles of bioactive metabolite production by *S. halotolerans* VSM 2 in submerged shake flask fermentations.

ABOUT AUTHORS

Dr. Ushakiranmayi Managamuri: Dr. Managamuri is a UGC-Women Post Doctoral Fellow, She obtained her Ph.D degree in Microbiology from Sri Krishnadevaraya University, Anantapur and Ph.D Degree in Biotechnology from Acharya Nagarjuna University.

Prof. Muvva Vijayalakshmi: Prof Muvva is a Professor in Department of Botany and Microbiology and Dean of Life Science. She had handled a good number of projects and has more than 200 publications

Dr. Deepa Indupalli: She has completed her Masters and Ph.D from Department of Microbiology, Acharya Nagarjuna University.

Dr. Venkat Siva Rama Krishna Ganduri: Dr Rama Krishna completed his B.Tech in Chemical Engineering from NIT Warangal, M.Tech Biotechnology from Anna University and Ph.D in Biotechnology from Acharya Nagarjuna University.

Dr. Satish Babu Rajulapati: Dr. Satish completed his B.Tech in Chemical Engineering from NIT Warangal, and M.Tech Biotechnology and Ph.D in Biotechnology form JNTU hyderabad. He is at present working as Associate Professor, Department of Biotechnology, NIT Warangal.

Dr. Sudhakar Poda: Dr. Poda obtained his Masters from Madras University and Completed his Ph.D form Sri Krishnadevarya University. He is at present working as Coordinator and Assistant Professor in Department of Biotechnology, Acharya Nagarjuna University.

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