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Formulation And Statistical Tools Used To Optimization Of Media Components For Enhancement Of Antibiotic Production By Streptomyces Geysiriensis Lk8.

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Abstract

Antibiotic resistance in bacteria presents a major challenge to public health, necessitating the search for new antibiotics. *Streptomyces geysiriensis* lk8, isolated from the saline desert of the Rann of Kutch, India, shows potential for antibiotic production. This study aimed to optimize the cultural and nutritional conditions for antibiotic production using Response Surface Methodology (RSM) and Design Expert software.

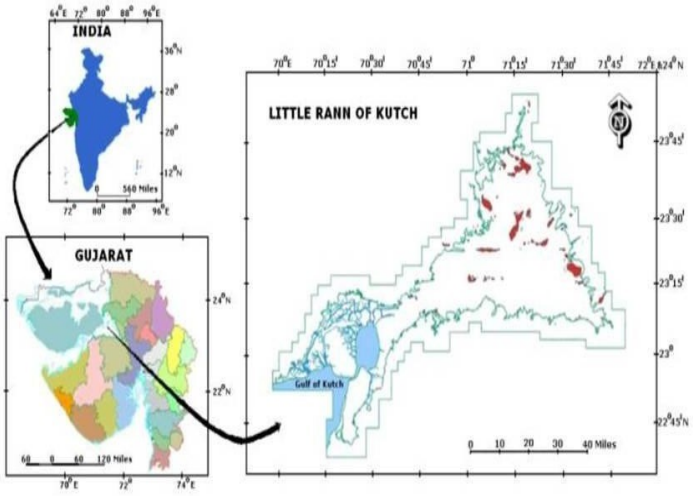
INTRODUCTION

The emergence and evolution of antibiotic-resistant bacteria have become a significant global health challenge. Once bacteria acquire resistance traits, they maintain these traits even in the absence of antibiotics, complicating efforts to reverse resistance simply by reducing antibiotic use (Fedorenko et al., 2015). Horizontal gene transfer, both interspecies and intraspecies, is a primary mechanism by which Gram-negative and Gram-positive bacteria become multidrug-resistant (MDR) (Tanwar et al., 2014). MDR occurs through two main mechanisms: the accumulation of multiple resistance genes within a single cell, often on resistance plasmids, or the increased expression of genes coding for multidrug efflux pumps, which expel a wide range of drugs (Nikaido et al., 2009).

The World Health Organization (WHO) has reported high rates of resistance in various bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Nontyphoidal Salmonella*, *Shigella* species, and *Mycobacterium tuberculosis* (Tanwar et al., 2014). These bacteria are responsible for common infections such as urinary tract infections, pneumonia, and bloodstream infections, and they contribute significantly to hospital-acquired infections.

Staphylococcus aureus, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), is a notable example. Methicillin was first used in 1959 to treat penicillin-resistant *S. aureus* infections, but resistance to methicillin was reported by 1961 in the UK (Enright et al., 2002). MRSA remains a significant threat in healthcare settings and increasingly in community environments (Sakoulas et al., 2008).

Actinobacteria, particularly from the order Actinomycetales, are filamentous bacteria that inhabit diverse environments, predominantly soil. These microorganisms are prolific producers of secondary metabolites, including antibiotics, and have been the source of many therapeutically important compounds (Hayakawa et al., 2008). Environmental factors such as soil temperature, pH, and organic matter content influence the diversity and quantity of actinomycetes present in a given region. The Little Rann of Kutch, a unique ecological zone in Gujarat, India, is an underexplored area from a microbial diversity and biotechnological standpoint. This saline desert covers an area of approximately 5,100 square kilometers and features diverse habitats that could harbor novel actinomycetes with potential antibiotic-producing capabilities (Patel et al., 2015). The region's extreme conditions make it an intriguing site for bioprospecting for novel bioactive compounds (Hug et al., 2018; Mohan et al., 2018).



This study aims to optimize the production of antibiotics by *Streptomyces geysiriensis* lk8 using Response Surface Methodology (RSM). RSM, a collection of mathematical and statistical techniques, is employed to optimize cultural and nutritional conditions to enhance antibiotic production. This approach not only identifies the optimal conditions for maximum antibiotic yield but also reduces the cost of production by optimizing the use of key nutrients. The success of this method can provide a cost-effective strategy for mass production of antibiotics and contribute to the development of new antimicrobial agents to combat MDR pathogens.

Methodology

3.1 Optimization of Antibiotic Production by Response Surface Methodology Using Design Expert Software

The optimization of fermentation conditions for antibiotic production by *Streptomyces geysiriensis* lk8 was conducted using Response Surface Methodology (RSM) with a central composite design (CCD) framework, facilitated by Design Expert software. This approach enables the identification of optimal cultural and nutritional conditions to maximize antibiotic yield. The methodology is structured as follows:

3.2 Experimental Design and Data Analysis

The statistical software Design Expert was utilized for the experimental design and subsequent data analysis, following the procedures outlined by Wang et al. (2010). This software facilitated the systematic evaluation of significant variables influencing antibiotic production, namely carbon source, nitrogen source, NaCl concentration, and pH.

3.3 Central Composite Design (CCD)

A central composite design was employed to explore the interactions between the four critical factors and their respective levels. A total of 30 experimental runs were generated by the CCD, with each run being conducted in triplicate to ensure reliability and accuracy of the results. The CCD approach included a 2^n factorial design, augmented with additional center and axial points to provide sufficient data for a robust quadratic model.

3.4 Experimental Procedure

3.4.1 Preparation of Media and Inoculation:

- The media for fermentation was prepared according to standard protocols, with variations in the concentrations of the selected factors (carbon source, nitrogen source, NaCl, and pH) as specified by the CCD.
- *Streptomyces geysiriensis* lk8 was inoculated into each prepared medium and incubated under controlled conditions.

3.4.2 Fermentation and Antibiotic Production:

- Fermentation was carried out in a batch culture system, with periodic monitoring to ensure optimal growth conditions.
- After the incubation period, the culture broth was harvested, and the antibiotic was extracted using appropriate solvent extraction methods.

3.4.3 Bioassay and Response Measurement:

- The concentration of the produced antibiotic was determined using standard bioassay techniques.
- The zone of inhibition was measured to assess the antimicrobial activity of the produced antibiotic against selected test organisms.

3.5 Data Analysis and Model Validation

- The response data from the bioassay (zone of inhibition) was fed into the Design Expert software for analysis.
- 3D contour plots were generated to visualize the interactions between the factors and to identify the optimal concentrations for maximum antibiotic production.
- The validity of the chosen quadratic model was tested using the point prediction feature of the Design Expert software, and experimental runs were conducted to confirm the predicted optimal conditions.

Result and Discussion

Optimization is crucial for scaling up antibiotic production at an industrial scale. Response Surface Methodology (RSM) was used to examine the effect of multiple factors on the fermentation process, allowing for the determination of nutritional demands and circumventing unnecessary components in the fermentation medium. This study focused on optimizing antibiotic production by *Streptomyces geysiriensis* lk8 using a Central Composite Design (CCD) within the RSM framework. The CCD generated a total of 30 combinations of production media, as detailed in Table 4-1.

Table 4-1 Variables used in RSM-CCD for antibiotic production

Variable	Unit	Low value	High Value
Peptone	% (w/v)	0.1	0.5
Lactose	% (w/v)	1.0	7.0
NaCl	% (w/v)	1.0	5.0
pH		7.0	9.0
Zone of inhibition	mm	12.0	24.0

4.1 Statistical Analysis for the Antibiotic Production

The significance of the RSM model was evaluated using F-test and ANOVA analysis of the statistical response (Table-4.2). In this study, the model F-value of 11.09 and P-value of <0.0001 proved that model is significant for antibiotic production. There is only a 0.01% chance that a greater F-value results due to noise effect. Moreover, A, C, AB, BC, BD, A^2, B^2, C^2, D^2 were found to be significant terms in the RSM model. Noticeably, the lack of fit for the F-value of 0.925 indicates that model is not significant when comparedto the pure error of the model. The non-significant lack of fit signifies good response for antibiotic production. Since the difference is less than 0.2, the predicted R squared value of 0.716 is in almost close with the adjusted R squared value of 0.829, which indicates that the model is in good agreement with the observed and predicted response. (Table-4.2). The predicted R-squared value and the adjusted R-squared value should be within 0.20 of each other.

Central Composite Design for Antibiotic Production

Run	Variable	Actual	Predicted
1	A	24.00	21.00

2	B	16.00	15.04
3	C	13.00	13.46
4	D	21.00	21.00
5	A	14.00	13.96
6	B	12.00	12.33
7	C	16.00	15.38
8	D	20.00	21.00
9	A	12.00	10.71
10	B	17.00	16.29
11	C	22.00	21.00
12	D	14.00	13.96
13	A	16.00	15.88
14	B	12.00	13.67
15	C	13.00	13.83
16	D	12.00	11.83
17	A	14.00	15.13
18	B	19.00	21.00
19	C	15.00	14.00
20	D	17.00	17.50
21	A	14.00	14.33
22	B	20.00	21.00
23	C	12.00	12.21
24	D	15.00	14.54
25	A	12.00	12.29
26	B	17.00	16.21
27	C	18.00	18.00
28	D	16.00	16.00
29	A	15.00	16.00
30	B	24.00	22.00

Response Surface Analysis

A second-order polynomial equation was obtained for the prediction of antibiotic production:

Antibiotic Production=+15.38−2.50A+1.62B−1.12C−2.25D−1.25AB−1.25AC−1.25BC+2.67BD+4.21A2−1.58B2+2.04C2+2.17D2\text{Antibiotic Production} = +15.38 - 2.50A + 1.62B - 1.12C - 2.25D - 1.25AB - 1.25AC - 1.25BC + 2.67BD + 4.21A^2 - 1.58B^2 + 2.04C^2 +

2.17D^2Antibiotic Production=+15.38−2.50A+1.62B−1.12C−2.25D−1.25AB−1.25AC−1.25BC+2.67BD+4.21A2−1.58B2+2.04C2+2.17D2

where A, B, C, and D represent the factors (peptone, lactose, NaCl, and pH, respectively).

The adequacy and significance of the regression model were confirmed with an R-squared value of 0.9041. The adjusted R-squared value of 0.8285 and predicted R-squared value of 0.7160 demonstrated a good fit between the observed and predicted responses.

Table 4-3 Analysis of variance (ANOVA) for the quadratic model of response antibiotic production

Source	Sum of Squares	df	Mean Square	F-value	p-value	Model validation
Model	**282.12**	**14**	**20.15**	**11.09**	**< 0.0001**	**Significant**
A-pH	6.00	1	6.00	3.30	0.0892	
B-NaCl	0.1667	1	0.1667	0.0917	0.7661	
C-Lactose	6.00	1	6.00	3.30	0.0892	
D-Peptone	2.67	1	2.67	1.47	0.2444	
AB	6.25	1	6.25	3.44	0.0834	
AC	1.0000	1	1.0000	0.5505	0.4696	
AD	1.0000	1	1.0000	0.5505	0.4696	
BC	**9.00**	**1**	**9.00**	**4.95**	**0.0418**	
BD	**9.00**	**1**	**9.00**	**4.95**	**0.0418**	

CD	0.2500	1	0.2500	0.1376	0.7159	
A^2	100.76	1	100.76	55.47	< 0.0001	
B^2	88.05	1	88.05	48.47	< 0.0001	
C^2	114.33	1	114.33	62.94	< 0.0001	
D^2	29.76	1	29.76	16.38	0.0011	
Residual	27.25	15	1.82			
Lack of Fit	11.25	10	1.13	0.3516	0.9250	
Pure Error	16.00	5	3.20			**Not significant**
Cor Total	282.12	14	20.15	11.09	< 0.0001	

4.2 Final Equation in Terms of Coded Factors:

Antibiotic production (Zone of inhibition, mm) =+21.00+0.50008*A +0.0833*B-0.5000*C -0.3333*D-0.6250*AB +0.2500*AC-0.2500*AD +0.7500*BC -0.7500*BD +0.1250*CD -1.92*A^2-1.79*B^2-2.04*C^2-1.04*D^2

The higher points of the factors implied as +1 and the low levels showed as -1. The coded equation detects the relative impact of the factors by comparing with the factor of the coefficients.

4.3 Predicted vs. Actual values for the antibiotic production

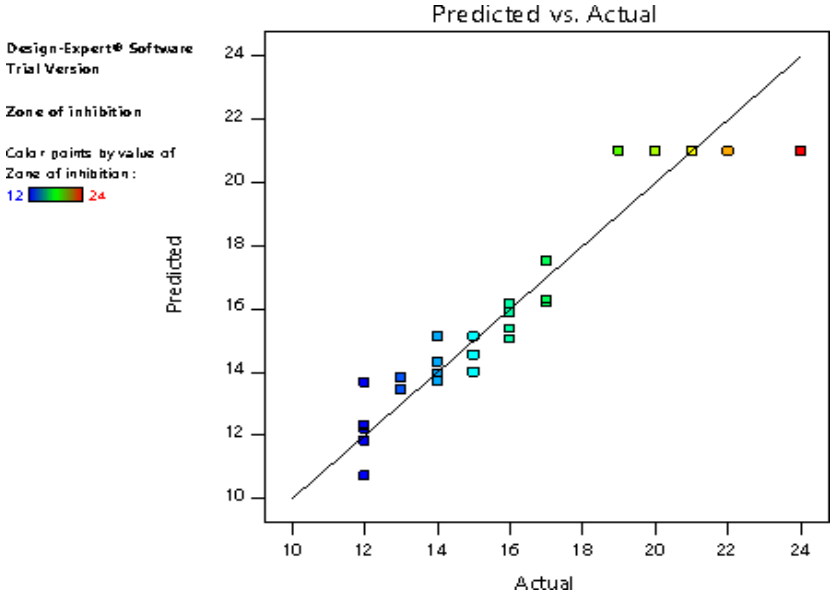


Figure 4-1 Predicted vs Actual value for the antibiotic production

The predicted vs. actual values for antibiotic production demonstrated that the values closely aligned with the linear regression line, validating the model's response for antibiotic production. (Figure-4.1).

Three-dimensional (3D) response surface plots between the input factors for antibiotic pdn

Combination effect between NaCl and Lactose for the antibiotic production (Figure4.2)

Figure 4-2 D response surface plot for interaction between NaCl and Lactose for antibiotic production

Combination effect between NaCl and Peptone for the antibiotic production (Figure4.3)

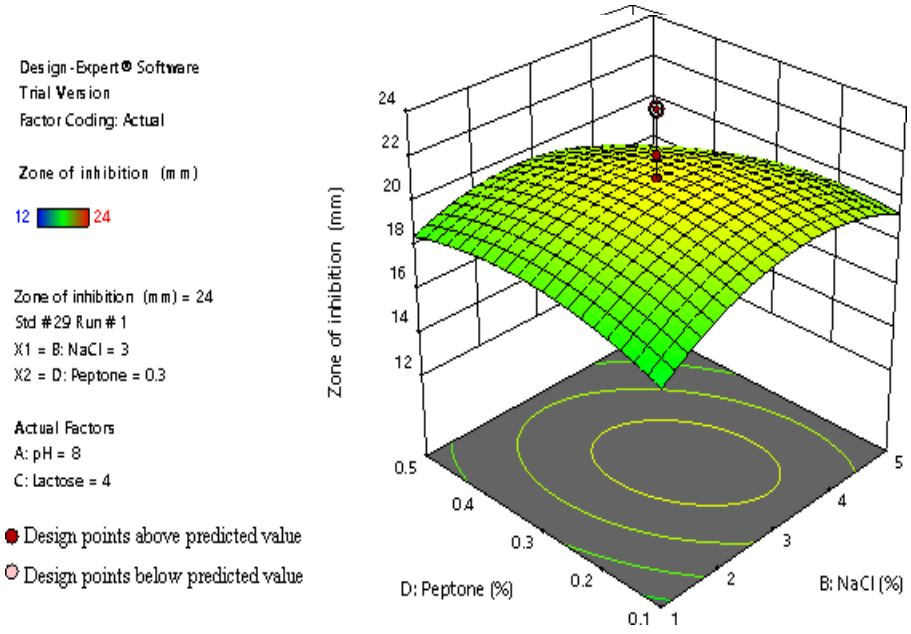


Figure 4-3 D response surface plot for interaction between NaCl and Peptone for antibiotic production

The 3D surface plots represent the interaction between the significant factors and theoptimal concentration of cultural and nutritional factors. The optimum predicted cultural conditions were as follow, pH = 8.0, NaCl = 3 %, lactose = 4 % and peptone = 0.3 %. Thesevalues predicted 21 mm of zone diameter in the RSM model. Likewise, the optimized conditions produced experimental antimicrobial activity of 24 mm zone diameter when SCBmedium amended with optimized conditions. The results suggested that experimental antimicrobial response was higher than predicted response that validates the model. This is in agreement with the RSM model prediction. Therefore, the model established reflected to be accurate and dependable for predicting the production of antibiotic by the isolate lk8.

4.4 Validation of RSM model for the antibiotic production

The cultural and nutritional conditions were optimized using response surface methodology to accomplish maximum antibiotic production by the *Streptomyces geysiriensis* (lk8) isolate. The optimized fermentation conditions such as NaCl, 3.0%, Lactose 4.0 %, Peptone 0.3% and pH 8.0 produced maximum antimicrobial activity in the model. The growth of the *Streptomyces* isolate lk8 under optimized condition resulted in the20% higher antimicrobial activity than that of non-optimized condition. The result suggested that fermentation medium had a significant effect on the production of antibiotic by the *Streptomyces geysiriensis* (lk8). The optimized fermentation conditions yielded 20% higher antimicrobial activity compared to non-optimized conditions. The RSM-optimized medium contained a moderately lower concentration of lactose, reducing the cost of sugar by 50% while increasing antimicrobial activity by 20%. This cost-effective optimization method is beneficial for further characterization of antibiotics and the development of antimicrobial agents to suppress or control MRSA activity.

4.5 Comparison with Previous Studies

A five-factor-three-level CCD was previously used by Ahsan *et al*. (2017) and Wang *et al*. (2010) for optimizing secondary metabolite production. Statistically based experimental design is an economical approach for dealing with numerous variables compared to traditional optimization techniques. Classical methods or PBD designs can identify significant factors but do not provide insights into interaction effects among medium components, which is critical for optimal antibiotic production (Ram *et al*., 2014).

CONCLUSION

The fermentation conditions developed using CCD of RSM and the validation of optimized conditions indicate a significant improvement in antibiotic production by *Streptomyces geysiriensis* lk8, supporting the feasibility of scaling up this process for industrial applications.

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