An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

OPTIMIZATION OF PROCESS VARIABLES USING RESPONSE SURFACE METHODOLOGY (RSM) FOR BIOSURFACTANT PRODUCTION BY THE ISOLATE *STREPTOMYCES COELICOFLAVUS* (NBRC 15399^T)

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ARTICLE INFO

Key words:

Response surface methodology,
Submerged fermentation,
Quadratic regression,
Biosurfactant,
Culture media
optimization.

ABSTRACT

Surfactants produced by microorganisms are known as biosurfactants. The aim of this study is to optimize the process variables using response surface methodology (RSM) using Box-Benkhen design for biosurfactant production by the isolate *Streptomyces coelicoflavus* (NBRC 15399^T). Biosurfactants produced by the actinomycetes strain *Streptomyces coelicoflavus* (NBRC 15399^T) isolated from soil contaminated with oil near Naval dockyard, in seven day submerged fermentation medium using olive-oil, $NaNO_3$, level of inoculum concentration as independent variables. Biosurfactant production was depicted adopting a statistically second-order quadratic regression model. Optimum values for these independent variables to achieve the maximum predicted value of 467.762 µg/ml were derived as olive oil (3.008% v/v), $NaNO_3$ (0.102% w/v) and level of inoculum (10.676% v/v). Closeness of predicted values by statistical analysis and experimental results suggested reliability of regression model adopted. An increase of 2.38 fold in biosurfactant production was observed with the optimized production medium during experiments, 495.724 µg/ml compared to predicted 467.762 µg/ml, further confirmed significant positive effect of optimized physical and nutritional parameters of culture medium.

1. INTRODUCTION

Biosurfactants, synthesized by a variety of microorganisms, are a heterogeneous group of secondary metabolites having various surface active properties.[5] A great deal of interest in recent times about these surface active biosurfactants [13,33,43] can be attributed to a number of potential advantages over their chemically manufactured counterparts, including lower toxicity and biodegradability,[44] effectiveness at extreme temperatures, pH and salinity.[27] The functional properties of these surface active microbial compounds mainly comprise of emulsification, phase separation, wetting, foaming, surface activity and viscosity reduction of heavy crude oils [12, 26]

And their potential applications can be envisaged in several industries including agriculture, food and beverages, textiles, cosmetics, pharmaceutical preparations, petrochemical and petroleum production. The aim of this work is to statistically optimize the values of supplementary nutrients to enhance the biosurfactant production in submerged fermentation process. Experimental factorial design [11] and Response Surface Methodology (RSM) [10, 30] have been successfully applied in other fields, and to optimize the media and culture conditions in some fermentation processes for the production of primary and secondary metabolites.[8,14,16,29,34,35,37] Further, this study aims to evaluate the main and interaction effects of various parameters on the production of

biosurfactant. To support this, a 2n factorial Box-Benkhen design (BBD) and RSM were used in this study [2,3,4,21,22,42]. Optimization of the culture medium by classical methods involves changing of one independent variable each time (nutrient, pH, temperature, etc.), while fixing all others at a fixed level. This is extremely time consuming and is often expensive to study the effect of a large number of variables. To overcome this difficulty, experimental factorial design and Response Surface Methodology were employed in this study to optimize various physical and nutritional parameters of the culture medium.

2. MATERIALS AND METHODS

2.1 selection of soil samples

The oil contaminated soil sample was collected in sterile plastic bags from Naval dockyard in Visakhapatnam, India. This soil sample was found to be rich in fats and oils, and hence was used for the screening of biosurfactant producing microorganisms.

2.2 selective isolation and growth conditions of actinomycetes

The collected soil sample was air dried at room temperature for one week, then preheated at 55ºC in a hot air oven for three hours, and was stored at room temperature in sterile bags labelled 'NDYS'. One gram of soil sample was serially diluted and 100 µl aliquot was applied to Humic-acid-Salts-Vitamin-agar plates [15], with pH adjusted to 7.0. Humic acid used here was synthesised in the laboratory using modified Essington method.[32] These plates were then supplemented with 50 µg/ml of rifampicin and cycloheximide, and were incubated at 28ºC for seven days for the growth of actinomycetes colonies. On eighth day of incubation, actinomycetes colonies were preliminarily selected based on colony morphology, and a small portion of them was streaked on the Bennets agar medium.

2.3 production medium for biosurfactant activity

Selected actinomycetes was inoculated into a 500 ml Erlyn mayor flask containing 100 ml of Kim's medium for biosurfactant production,[23] containing 3% olive oil as the sole carbon source, $NaNO_3$: 1.0 g/l, KH_2PO_4: 0.1 g/l, $MgSO_4.7H_2O$: 0.1 g/l, $CaCl_2$: 0.1 g/l, Yeast extract: 0.2 g/l, and pH: 6.0. Then, the broth cultures were incubated at 30 ± 2ºC on a reciprocal shaker at 120 rpm for five days. This culture broth was then tested for the production of extracellular biosurfactants.

2.4 Analytical methods

At the end of fermentation process, Orcinol assay method was used for the direct assessment of the amount of biosurfactant present in the sample. For this, 400 µl of cell free supernatant was taken and its pH was adjusted to 2.0 by adding 2N HCl. Addition of HCl results in the separation of biosurfactant. Then, 750 µl of diethyl ether was added to this mixture to extract biosurfactant into an organic layer. This procedure of solvent addition and extraction was repeated twice, and diethyl ether fractions were dried by evaporation. Then, 400 µl of pH 8.0 adjusted phosphate buffer was added to the remaining precipitate. Further, 300 µl of this precipitate was measured out, and 2.7 ml of orcinol was added to it. Test tubes containing this precipitate were then boiled in water for 20 min, and were kept in darkness for 35 min to cool them down to room temperature. The Absorbance of the extracted biosurfactant was estimated by measuring the optical density of the precipitate at 421 nm against blank for various concentrations of the standard L-Rhamnose, and is shown in Fig.1. using standard graph of L-Rhamnose.[6]

2.5 optimization of selected nutrients using response surface methodology (RSM)

RSM was used with Box-Behnken design to optimize the selected media constituents: olive oil, $NaNO_3$, and level of inoculum for enhanced biosurfactant production by *Streptomyces coelicoflavus* (NBRC 15399^T). These three medium components (independent variables) were studied at three different levels: (-), (0) and (+) for low, intermediate and high concentrations, respectively as shown in Table.1. These variables were optimized between the range of values, olive oil: 2.8 to 3.2 (% v/v); $NaNO_3$: 0.08 to 0.12 (% w/v); and level of inoculums: 8 to 12 (% v/v), respectively.

This experiment was carried out in 17 trails, as shown in Table. 2., with five replicates at the centre point. The values of responses were the mean of two replications. In developing the regression equation, the test factors were coded according to the following equation.

$$xi = Xi - X0 / \delta X \; i = 0,1,2,3,.....n \quad (1)$$

where xi is the coded value of the ith independent variable, Xi the natural value of the ith independent variable, X0 the natural value of the ith independent variable at the centre point, and δX is the step change value.

For predicting the optimal point, a second order model was fitted to correlate the relationship between independent variables and the response. The behaviour of the system was explained by the following quadratic equation.

$$Y = \beta 0 + \Sigma \beta iXi + \Sigma \beta ijXiXj + \Sigma \beta iiXi2 \quad (2)$$

Where Y is predicted response, β0 is intercept term, βi is linear coefficient, βij is quadratic coefficient, βii is interaction coefficient, and XiXj represent independent variables. Design Expert trail package (version 9.0) was used for the experimental design and the regression analysis of the data obtained. The statistical significance of the model was verified by applying the analysis of variance (ANOVA). Overall model significance was determined using Fisher's F-test and its associated probability P(F). The lack of fit was also applied to estimate the model. Lack of fit values lower than 0.05 indicated that there might be a contribution to the variables response relationship that the model does not take into account. Quality of the polynomial model equation was judged statistically by coefficient of determination (R2) and adjusted R2. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots, to illustrate the relationship between the responses and the experimental levels of each independent variable [22]. Design Expert's numerical optimization method was employed to optimize the level of each variable for maximum response.

2.6 Experimental validation

The combination of different optimized variables, which yielded the maximum response, was experimentally validated by culturing *Streptomyces coelicoflavus* NBRC 15399^T) in optimized and unoptimized production medium. The cell free culture broths were collected and extracted with equal volume of diethyl ether and the top organic layer was dried for further analysis. The dried diethyl ether extracts were resuspended in phosphate buffer with pH 8.0 and were assayed for biosurfactant production.

2.7 Identification of actinomycetes

The molecular identification and characterization of the actinomycetes NDYS-4[28] was carried out by 16S rRNA gene sequencing performed at Institute of Microbial Technology (IMTECH), Chandigarh (India). The similarity search was conducted insilico using the Basic Local Alignment Search Tool (BLAST) database of National Centre for Biotechnology Information (NCBI) of United States of America. The scanning electron microscope of the actinomycetes was done by Ruska Laboratory at the College of Veterinary Sciences, Sri Venkateswara Veterinary University, Rajendra nagar, Hyderabad (India).

3 RESULTS

3.1 Statistical condition for optimization of biosurfactant production by *streptomyces colicoflavus* (nrbc 15399^t) using rsm

Biosurfactant production by *Streptomyces coelicoflavus* was optimized using Box-Behnken design and RSM by varying the concentrations of medium components, especially olive oil, $NaNO_3$ and level of inoculum. The parameters with fixed central points of olive oil 3% v/v, $NaNO_3$ 0.1% w/v, level of inoculum 10% v/v were taken in the methodology. The range and the levels of variables used in the Box-Behnken design are shown in the Table 1.

With the help of RSM, the relationship between dependent variables (biosurfactant production) and independent variables (medium components such as olive oil, $NaNO_3$ and level of inoculum) was evaluated, and the observed and predicted values of the biosurfactant production by *Streptmyces coelicoflavus*(NBRC 15399^T) is shown in Table. 2. The reliability of the model can be seen comparing the observed and predicted values. Results of the second order response surface model in the form of analysis of variance (ANOVA) are given in Table. 3. The Fisher F-test with a very low probability value (Pmodel > F = 0.0001) demonstrate a high significance for the regression model.[2, 22] Fit of the model was checked by the determination coefficient (R^2).

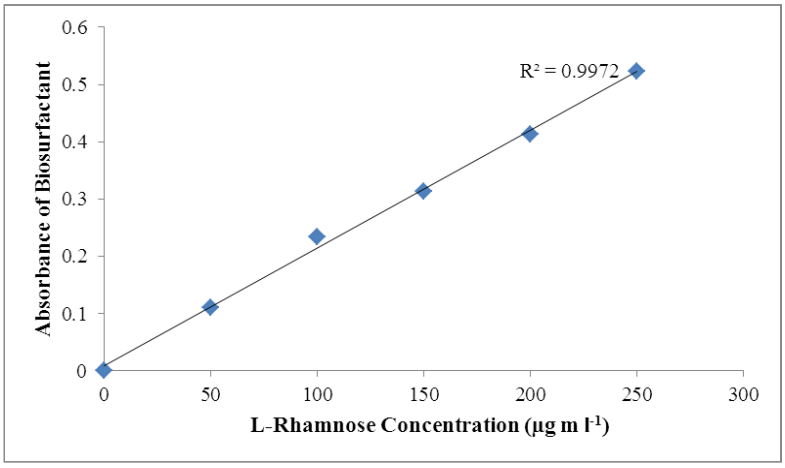


Fig.1. Standard graph of L-Rhamnose

Variables	Range and levels		
	-1	0	+1
Olive oil (% v v-1)	2.8	3.0	3.2
NaNO3 (% w v-1)	0.08	0.1	0.12
Level of inoculum (% v v-1)	8	10	12

Table. 1. Experimental range and the levels of the variables

Table. 2. Box-Behnken design matrix along coded and uncoded independent variables and with experimental and predicted responses (Biosurfactant activity) of *Streptomyces coelicoflavus*

Run	O (Code)		N (Code)		I (Code)		Biosurfactant activity (µg ml/1)	
	Value (% v/v)		Value (% w/v)		Value (% v/v)		Observed response	Predicted response
1	-1	2.8	-1	0.08	0	10	305.00	306.258
2	1	3.2	-1	0.08	0	10	314.236	315.286
3	-1	2.8	1	0.12	0	10	310.069	309.019
4	1	3.2	1	0.12	0	10	332.222	330.964
5	-1	2.8	0	0.1	-1	8	315.138	315.859
6	1	3.2	0	0.1	-1	8	303.333	304.262
7	-1	2.8	0	0.1	1	12	339.861	338.932
8	1	3.2	0	0.1	1	12	382.222	381.501
9	0	3	-1	0.08	-1	8	352.777	350.798
10	0	3	1	0.12	-1	8	342.291	342.621
11	0	3	-1	0.08	1	12	383.888	383.558
12	0	3	1	0.12	1	12	408.194	410.173
13	0	3	0	0.1	0	10	464.513	462.847
14	0	3	0	0.1	0	10	461.875	462.847
15	0	3	0	0.1	0	10	464.722	462.847
16	0	3	0	0.1	0	10	459.375	462.847
17	0	3	0	0.1	0	10	463.75	462.847

(O: olive oil; N: $NaNO_3$; I: level of inoculum)

Table. 3. ANOVA for the quadratic response surface model

Source	Sum of Squares	Degrees of Free-dom	Mean Square	F Value	(p-value) Probability > F
Residual model	36.30	7	5.19		
Lack of Fit	22.83628	3	5.40	1.07	0.4546
Pure Error	42.85194	4	5.03		
Total correlation	55761.44	16			

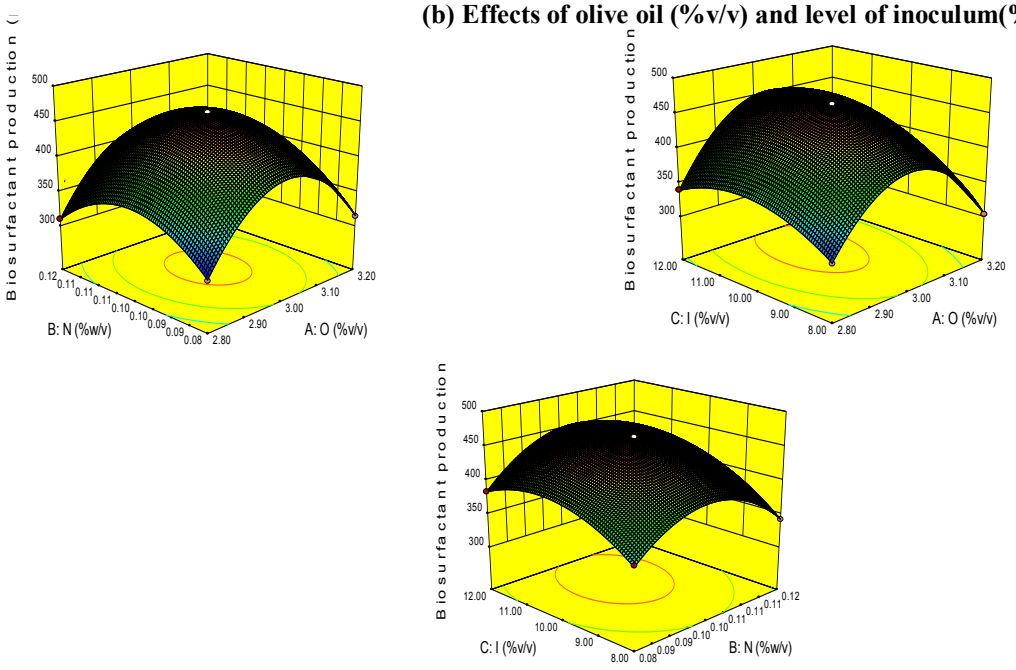
R^2 = 0.9994, Adjusted R^2 = 0.9987, Coefficient variance % = 0.60, Adequate precision = 90.805

Table. 4. Coefficient of the model for biosurfactant production

Source	Sum of squares	Df	Mean Square	F Value	p-value Prob > F	Remarks
Model	65910.98	9	7323.44	1412.419	< 0.0001	Significant
O	479.65	1	479.65	92.50619	< 0.0001	Significant
N	169.97	1	169.97	32.78101	0.0007	Significant
I	5031.35	1	5031.35	970.3596	< 0.0001	Significant
O*N	41.71	1	41.71	8.044732	0.0252	Significant
O*I	733.49	1	733.49	141.4627	< 0.0001	Significant
N*I	302.62	1	302.62	58.36427	0.0001	Significant
O*O	35682.17	1	35682.17	6881.759	< 0.0001	Significant
N*N	12926.57	1	12926.57	2493.053	< 0.0001	Significant
I*I	5351.67	1	5351.67	1032.137	< 0.0001	Significant

(a) Effects of olive oil (%v/v) and $NaNO_3$ (%w/v)

(b) Effects of olive oil (%v/v) and level of inoculum(%v/v)



(c) Effect of $NaNO_3$ (w/v) and level of inoculum (%v/v)

Fig. 2. Response surface plots showing individual and interactive effects of variables on biosurfactant production by *Streptomyces coelicoflavus*

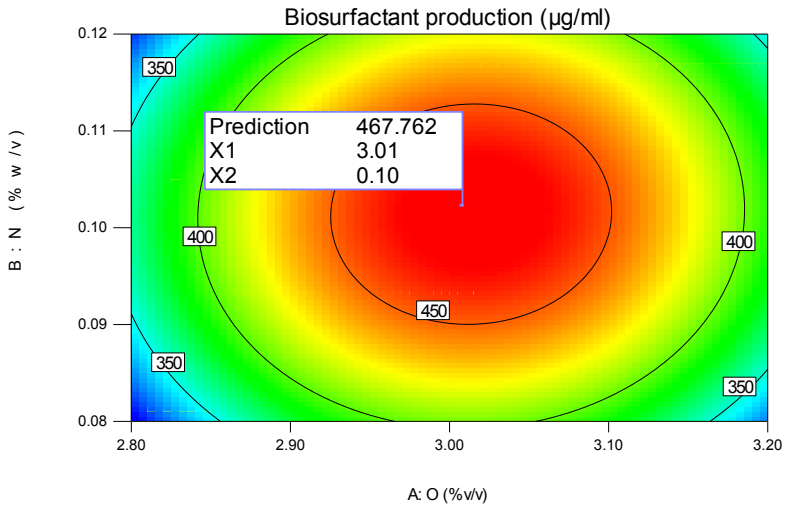


Fig. 3. Contour plot showing the maximum biosurfactant production at optimum values of the various variables

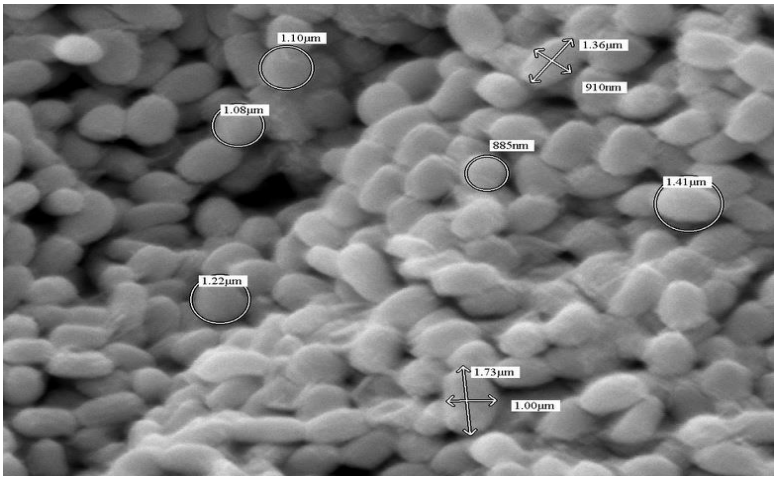


Fig. 4. Scanning electron image of the isolate *Streptomyces coelicoflavus*

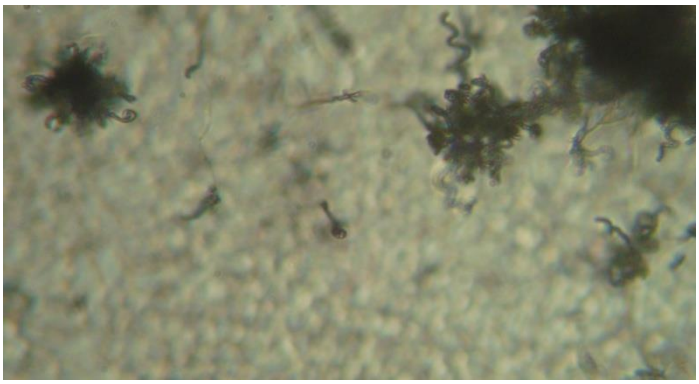


Fig. 5. Microscopic morphology of isolate *Streptomyces coelicoflavus* observed under 400X 455 magnification

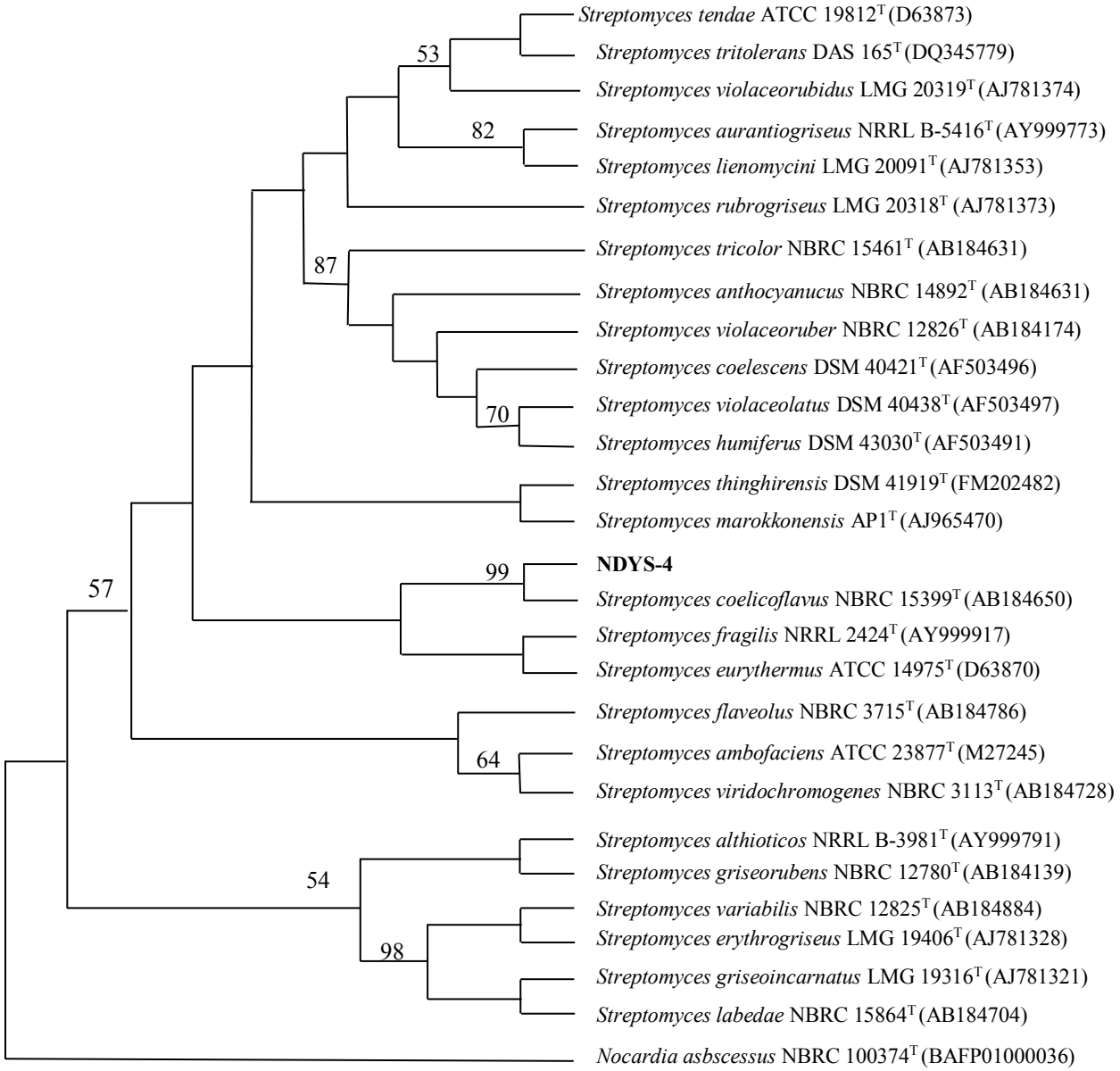


Fig. 6. Neighbour-joining Phylogenetic Tree of the isolate NDYS-4 made by IMTECH

In this case, value of the determination coefficient (R^2 = 0.9994) indicate that only 0.06% of the total variations could not be explained by the model. The value of the adjusted determination coefficient (adjusted R^2 = 0.9987) is also high to advocate a high significance of the model.[2, 22] A higher value of the correlation coefficient (R = 0.9994) justifies an excellent correlation among the independent variables.[9] At the same time, a relatively lower value of the coefficient of variation (CV = 0.60%) indicate a better precision and reliability of the experiments carried out.[2, 3] The application of RSM[3,4,21,22] yielded the following regression equation, which is an empirical relationship between the values of biosurfactant production yields and test variables in coded units.

Y = - 21315.82237 + 13428.01562 * O + 23338.09375 * N + 67.48975 * I + 807.31250 * O * N + 33.85375 * O * I + 217.45000 * N * I – 2301.42812 * O * O – 1.38520E + 005 * N * N - 8.91284* I * I

Where Y is the biosurfactant production, and O, N, and I are the coded values of the test variables olive oil, $NaNO_3$, and level of inoculum, respectively. The coefficient of the model (parameter estimation) and the corresponding P values are presented in Table. 4. The significance of regression coefficients was

considered at 95% significance level. The P values of the regression coefficients suggest that among the independent test variables, linear, quadratic, and interaction effects of olive oil, $NaNO_3$, and level of inoculum are highly significant. In this study, O, N, I, O^2, N^2, I^2, ON, OI, and NI signify model terms for biosurfactant production. Thus, statistical analysis of all the experimental data had shown that olive oil, $NaNO_3$, and level of inoculum had significant effect on biosurfactant production in this study. The three-dimensional (3D) response surface plot figures of the experimental data of Box-Behnken design had shown a relationship between the individual and interactive effects of olive oil, $NaNO_3$, and level of inoculum on biosurfactant production by *Streptomyces coelicoflavus* (NBRC 15399^T). Each 3D plot presented the effects of two variables while the remaining variable was held at middle level. Fig. 2(a) showed the interaction of olive oil and $NaNO_3$ with fixed coded values of level of inoculum as mentioned earlier, the predicted biosurfactant production of 422.263 µg/ml with olive oil 3.09% (v/v) and $NaNO_3$ 0.12% (w/v) was obtained. Fig. 2(b) showed the interaction of olive oil and level of inoculum keeping $NaNO_3$ constant, the biosurfactant production obtained was 428.669 µg/ml with olive oil 3.01% (v/v) and level of inoculum 11.48% (v/v). Fig. 2(c) showed the interaction of $NaNO_3$ and level of inoculum keeping olive oil constant, the biosurfactant production obtained was 435.779 µg/ml with $NaNO_3$ 0.1% (w/v) and level of inoculum 11.14% (v/v).

3.2 Optimization and experimental validation

On the basis of numerical optimization, the quadratic model predicted that the maximum biosurfactant production would be 464.72 µg/ml, with optimal test factor values of olive oil 3.008% (v/v), $NaNO_3$ 0.102% (w/v) and level of inoculum 10.676% (v/v) respectively as shown in Fig. 3. Validation of the statistical results using the optimized medium was accomplished by carrying out shake-flask experiments in triplicate. The maximum biosurfactant production obtained experimentally was found to be 495.724 µg/ml, an increase of 2.38 fold in biosurfactant production was achieved compared to the predicted value of 464.72 µg/ml. Hence, the developed model can be considered to be accurate and reliable for predicting the biosurfactant production by *Streptomyces coelicoflavus* (NBRC 15399^T). The final optimized medium contained 3.008% (v/v) olive oil, 0.102% (w/v) $NaNO_3$, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) $CaCl_2$, 0.2% (w/v) yeast extract and 10.676% (v/v) level of inoculum with pH 6.0.

3.3 Identification and characterization of the isolate pls-1

Outer surface of colonies were perfectly round initially, but later were developed into thin wavy mycelium. The colour of the aerial mycelium observed was white and colour of the substrate mycelium was light pink by studying the morphology (Fig. 5.) and SEM (Fig. 4.), 16s rRNA gene sequencing, homology and Phylogenetic tree (Fig. 6.); the isolated strain was found to be *Streptomyces coelicoflavus* (NBRC 15399^T).

4. DISCUSSION

Biosurfactants are biologically surface active agents produced as membrane components of secondary metabolites by various microorganisms, such as bacteria, yeast, fungi and actinomycetes. Biosurfactants constitute glycolipids, phospholipids, lipopeptides, polymeric compounds, mycolic acids and lipopolysaccharides.[9] In recent years, biosurfactants have gained much attention owing to several advantages such as low toxicity, biodegradability and ecological acceptability,[24] compared to toxic and polluting chemical surfactants.[7] Production of biosurfactants is encouraged in recent times to reduce the toxicity produced by chemical surfactants[44] in pharmaceutical and cosmetic industries[39], for medical purposes[43], and also for their usefulness as anticancer agents.[19] Many studies have reported the production of biosurfactant from *Rhodococcus erythropolis*, *R. aurantiacus* and surface active lipid from *Nocardia erythropolis*[25], but their production by actinobacteria is reported in very few cases. And, among actinomycetes species, very few studies have focused on *Streptomyces* genus to produce biosurfactants. According to Solanki *et al*. (2005), *Streptomycetes* isolated from largely unexplored coastal and marine habitats are potential producers of large number of bioactive molecules, having enormous biosynthetic potentials over other microbial groups.

Hence, the present study aimed at production of biosurfactant by the actinomycetes *Streptomyces coelicoflavus* (NBRC 15399^T),

isolated from Naval dockyard soil contaminated with petroleum oils, and optimizing the fermentation medium to improve biosurfactant production. The selected isolate showed 100% homology with *Streptomyces violaceoruber* and *Streptomyces violaceolatus*, and 99% homology with *Streptomyces fradie*. The Basic Local Alignment Search Tool (BLAST) result and Phylogenetic analysis confirmed that their similarity to the respective species.

To improve the biosurfactant production, the effect of physicochemical and nutritional factors on the fermentation process was evaluated by using olive oil as a sole carbon source in this study, as compared to other carbon sources like glucose [20] and n-hexadecane.[18] The influence of olive oil (carbon source), $NaNO_3$ (nitrogen source) and level of inoculum on substrates consumption and product formation was evaluated using the Box-Behnken design and RSM. Further, a statistical model was used to optimize the culture medium components and other factors to increase the biosurfactant production. Similar experiments were reported in other studies on biosurfactant production, but many of them have mainly focused on bacteria and fungi, and not on actinomycetes.[20, 40] Using the RSM with Box-Behnken design, maximum biosurfactant production was predicted as 467.762 µg/ml, when the optimized medium constituents of the fermentation medium were set as follows 3.008% (v/v) olive oil, 0.102% (w/v) $NaNO_3$, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) $CaCl_2$, 0.2% (w/v) yeast extract and 10.676% (v/v) level of inoculum with pH 6.0. Analysis of the data obtained in this study had provided critical information about the effect of specific variables and their impact on the production of biosurfactant. It was observed during the experiments that, using olive oil as a carbon source had increased the biosurfactant production when $NaNO_3$ and level of inoculum were supplied in high concentrations. All of these three variables had shown significant influence on the biosurfactant production, and the same was confirmed by the 3D surface plots. It can be verified from the Figures 2(a), 2(b), and 2(c) that, with an increase in olive oil and level of inoculum concentrations, the biosurfactant production had increased, but later, it has slightly decreased with constant $NaNO_3$. The opposite effects were also observed when antimicrobial compound was supplemented at high concentrations, reducing the compound levels. Variations in the nutritional and physicochemical factors and corresponding increase in biosurfactant production was also reported.[17]

As a result of the optimized components of the culture medium, actual biosurfactant produced by *Streptomyces coelicoflavus* (NBRC 15399^T) was 495.724 µg/ml, indicating a 2.38 fold increase over the predicted maximum biosurfactant production of 467.762 µg/ml, with optimal test factor values of: olive oil 3.008% (v/v), $NaNO_3$ 0.102% (w/v) and level of inoculum 10.676% (v/v) respectively. This indicates that small manipulations in the culture medium composition can exert significant effect on secondary metabolite biosynthesis in microorganisms.[36] Advocating similar behaviour, Abolos *et al*. (2002) recorded an 18.7% increase in the biosurfactant production by *Pseudomonas aeruginosa* AT10, and, Nalini *et a*l. (2013) reported a 2.2 fold increase in the biosurfactant production by *Pseudomonas fluorescens* using RSM approach. The validity of the model was proven by fitting the values of the variables in the second order polynomial equation and by actually carrying out the experiment at those predicted values for the three independent variables: olive oil, $NaNO_3$ and level of inoculum. As shown in the results, the model is adequate for predicting the optimized production of biosurfactant within the range of experimental variables, and with the determination coefficient of $R^2 = 0.9994$, the model could not explain only 0.06% of the total variations.

5. CONCLUSION

Several researchers working on biosurfactant production have applied RSM as a statistical tool to recognize, manipulate and optimize the influencing medium constituents and have recorded the increased biosurfactant production. The methodology as a whole proved to be adequate for the design and optimization of the bioprocess. In addition, further studies are necessary to validate these results in good biosurfactant production for bioremediation and anticancer activity.

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