

# Characterization And Optimization Of Biosurfactant Produced By *Bacillus Cereus* SnaU01 And Their Application In Hydrocarbon Contaminants Removal From Soil

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**Abstract:** The biosurfactant producing strain *Bacillus cereus* SNAU01 was isolated from hydrocarbon contaminated soil, Tamilnadu, India. From the preliminary screening test (blood haemolysis test, oil displacement, drop collapse test) potent biosurfactant producing strain was selected. The isolated strains *Bacillus cereus* was identified by morphological, biochemical and 16S rRNA gene sequencing. The biosurfactant produced by *Bacillus cereus* SNAU01 was able to reduce the surface tension of media to 34.64 mN/m. Using FT-IR spectroscopy, the chemical structure of the purified biosurfactant was identified as lipopeptide. To enhance the biosurfactant production, optimization was employed by central composite design (CCD) in response surface methodology (RSM). In the optimization study, glucose as carbon source, yeast extract as a nitrogen source, pH and salinity (NaCl gL<sup>-1</sup>) were assigned as a factor. The maximum emulsification index of *Bacillus cereus* SNAU01 was obtained under the optimal condition of 20.10 gL<sup>-1</sup> glucose, 2.45 gL<sup>-1</sup> yeast extract, pH 6.97 and salinity 57.96 gL<sup>-1</sup> NaCl. The optimised production of biosurfactant yield was approximately increased to 2.2 folds. The biosurfactant produced by *Bacillus cereus* SNAU01 recovered 93% of used engine oil adsorbed to a sand sample, suggested the potential application in microbial enhanced oil recovery and bioremediation.

**Keywords:** Biosurfactant, FT-IR, RSM-CCD, Emulsification index, Lipopeptide.

## 1. Introduction

Biosurfactants are a group of surface-active molecules with hydrophilic and hydrophobic moieties. As alternative surfactants, biosurfactants have outstanding advantages, such as high biodegradability, low toxicity, environmental compatibility, high selectivity and specific activity at extreme temperatures, pH, and salinity, among others. Biosurfactants have been shown to have a variety of applications, including enhancing crude oil recovery from oil reservoirs, mobilizing heavy crude oil transport in pipelines, and cleaning oil sludge from oil storage facilities. They are also used in soil/sand bioremediation, remediation of organics and metals, and as emulsifiers in agriculture and medicine in biological control [1].

The biosurfactant are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc. Since the last decade, increasing attention has been paid to the isolation of biosurfactant producing organisms [2]. Biosurfactant has advantages in comparison with synthetic compounds for use in soil remediation as they are natural compounds that will have a low environmental impact [3]. While the screening of new biosurfactant-producing species carries on, widening and variegating the groups of biosynthetic surfactants (which may have special quality or be capable of commercial production), optimization of cultural conditions also plays an important role in its production yield. The

statistical approach could overcome the limitations of classical medium optimization [4]. Response surface methodology (RSM) designs, helping designers to quantify the relationship between one or more measured response and the vital input factors, were successfully applied by many researchers to build reliable models and find the optimal medium for a greater biosurfactant yield [5]. Among bacterial species, recent studies are focused on *Bacillus* genus for their ability to produce lipopeptides, a class of biosurfactants with antimicrobial effects. Lipopeptides are low molecular mass biosurfactants (including surfactin, iturin, lichenysins, mycosubtilin, arthrofactin etc.) Which exhibit surface-active properties, and antimicrobial activities [6]. The objectives of this study were to isolate, characterize and optimize the media composition for biosurfactant producing microorganism from hydrocarbon contaminated soil from Cuddalore district, Tamilnadu, India. In the present study, we have used central composite design (CCD) of response surface methodology to optimize the media compositions enhance biosurfactant production by *Bacillus cereus* SNAU01 and recovered 92% of used engine oil adsorbed to a sand sample, suggested the potential application in microbial enhanced oil recovery and bioremediation.

## 2. Materials And Methods

### 2.1 Sampling Site And Isolation Of Biosurfactant Producing Microorganism

For isolation of biosurfactant producing bacteria, the samples of hydrocarbon-contaminated soil were collected from Cuddalore district, Tamilnadu, India. From these soil samples ten strains were isolated in Mineral medium salt (MSM) using diesel as carbon source. The cultures were incubated at 30°C. The isolates were evaluated by hemolytic activity, oil-spreading test, drop collapse and surface tension test in order to analyse their capability as biosurfactant producers. The best strain was selected for further studies.

### 2.2. Bacterium Identification And 16S Rrna Analysis

The isolated bacterium was identified biochemically according to Bergey's Manual. Molecular identification was carried out by 16S rRNA gene phylogenetic approximation. DNA from the selected bacterium was extracted with microbial DNA extraction kit (InstaGene™ Matrix, Bio-Rad). PCR amplification of the 16S rRNA were performed with universal bacterial primers. The sequencing products were resolved on an Applied Biosystems model 3730 XL automated DNA sequencing system (Applied BioSystems, USA). The 16S rRNA gene sequence was compared to the Gen Bank nucleotide database (NCBI) using BLAST and BLASTX algorithms. The sequence alignments and the phylogenetic tree construction was conducted in MEGA software version 5.2 [7]. The phylogenetic tree was constructed using a neighbor joining method and assessed with 1000 bootstrap replications.

### 2. 3. Biosurfactant Production

The biosurfactant producing bacterium was transferred to medium containing (gL<sup>-1</sup>) glucose 20; Yeast extract 2; NaCl 6; Na<sub>2</sub>HPO<sub>4</sub> 2.2; K<sub>2</sub>HPO<sub>4</sub> 1.4; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.6; FeSO<sub>4</sub>.7H<sub>2</sub>O 0.01; CaCl<sub>2</sub>.2H<sub>2</sub>O 0.02, trace elements (1mLL<sup>-1</sup>). Five milliliters of the inoculum were transferred to 45ml of medium contained in a 250ml Erlenmeyer flask and incubated for 72h at 37°C on a rotary shaker at 200rpm. The Emulsification index was used to quantify the biosurfactant production. The percent ratio of the height of emulsified zone total height after 24h gives the emulsification index (%EI<sub>24</sub>) as given below equation.

$$\%EI_{24} = \frac{\text{Height of emulsified zone}}{\text{Total height of liquid (sum of aqueous, oil and emulsified zone)}} \times 100$$

### 2.4. Biosurfactant Isolation

The cell free supernatant was acidified with 6N hydrochloric acid solution to pH 2.0. The precipitate contained biosurfactant was allowed to settle down and kept overnight at 4°C. The precipitated biosurfactant was collected by centrifugation at 15,000rpm for 20 min. The precipitate was neutralised and recentrifuged at 12000 rpm for 10 min. The precipitate was freeze dried and stored.

## 2.5. Characterization Of Biosurfactant

The ionic charge of the biosurfactant was determined by agar diffusion method according to Julina [8]. A FT-IR spectrum were recorded on AVATAR-NICOLAT system with a spectral resolution and wave number accuracy was  $4000-400\text{ cm}^{-1}$  and  $0.01\text{ cm}^{-1}$ , respectively. KBr pellet was used as background reference. GC-MS were performed for the purified samples in a GC Clarus 500 Perkin Elmer, using helium gas as a carrier at a flow rate of 1ml/1min and 70eV of energy. The negative ion mode used throughout and scanning was done at 45-450 m/z range.

## 2.6. Optimization Of Culture Medium

RSM is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously [9]. RSM is not only used for optimization of culture parameters in the fermentation process, but also for studying the combined effects of medium components[10]. In this regard, carbon source, nitrogen source, pH and salinity ( $\text{NaCl gL}^{-1}$ ) were considered as independent variables for the emulsion index ( $E_{24}$ ) in the culture media. The specific codes for each independent variable and range of the variables used for this experiment are given in Table 2. The experiment was performed using central composite design (CCD). In CCD, a total 30 treatment combinations were generated using designer expert 7.0 software (Stat-Ease Inc. Minneapolis, USA).

From the experimental data according to this design, a second-order polynomial regression model equation was derived.

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC + \beta_{14}AD + \beta_{23}BC + \beta_{24}BD + \beta_{34}CD.$$

Where Y: predicted response (Emulsion Index, %),  $\beta_0$ : intercept, A: Carbon source, B: Nitrogen source, C: pH, D: Salinity,  $\beta_1, \beta_2, \beta_3$  and  $\beta_4$  are the linear coefficients;  $\beta_{11}, \beta_{22}, \beta_{33}$ , and  $\beta_{44}$  are the squared coefficients;  $\beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$  are the interaction coefficients;  $A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, CD$  are the interaction between the variables as significant terms.

## 2.7. Statistical Analysis

This data was analysed by analysis of variance (ANOVA) technique to find out which factors had the most effective interactions for higher biosurfactant production [11].

## 2.8. Application Of Biosurfactant In Hydrocarbon Contaminants Removal From Soil

The suitability of biosurfactant for enhancing oil recovery was carried out using artificial contaminated sand with 10% used engine oil according to method described by Aparna [12].

# 3. Results and Discussion

## 3.1. Isolation, Selection And Identification Biosurfactant Producing Microorganism

In the present study, we have isolated twelve morphologically distinct microbial colonies and evaluated for haemolytic activity ( $\beta$  hemolysis), drop collapse positive, oil displacement test (8.5) and reduction in surface tension (value below  $34.64\text{mN/m}$ ) in Mineral salt medium. Ultimately one of the isolate which was initially named SNAU01 had highest biosurfactant production and activity was selected for the further studies. The best biosurfactant producer strain SNAU01 was identified by biochemical test and 16S rRNA analysis. The strain SNAU01 was identified by 16S rRNA analysis, suggested that the strain belongs to *Bacillus cereus* cluster. The SNAU01 nucleotide sequence was deposited in GenBank (NCBI) under accession number KC560768.

## 3.2. Characterization Of Biosurfactant

The molecular composition and structural analysis of the purified biosurfactant was evaluated by FT-IR analysis. The spectrum was presented in fig1. The peak at  $3287.74\text{ cm}^{-1}$  show the presence of amide N-H stretch; wavenumber  $1652.39\text{ cm}^{-1}$ , resulting from the stretching mode C=O bond and wavenumber  $1537.63\text{ cm}^{-1}$  resulting in the deformation mode of N-H bond combined with C-N stretching mode. An ester carbonyl group (wave number  $1731.51\text{ cm}^{-1}$ , ester C=O), suggesting the presence of lactone ring. Wave number  $3000\text{ cm}^{-1}$  to  $2800\text{ cm}^{-1}$ , C-H stretching mode suggests the presence of an aliphatic chain. A band corresponding to an ester carbonyl group (-CO) was observed wavenumber  $1730\text{ cm}^{-1}$ . These results were strong evidences that the

product contains aliphatic and peptide moieties might be lipopeptide compound [13]. FT-IR analysis confirmed the biosurfactant produced by *Bacillus cereus* SNAU01 as lipopeptide derivative compound.

### 3.3. Optimization Of Biosurfactant Production

The carbon source, nitrogen source and salinity are supplemented with the media which influences the cell growth and synthesis of various metabolic products like biosurfactant and extracellular enzymes. pH plays an important role in most of microorganisms for cell growth and secondary metabolite production. Hence, optimization of these input parameters can improve the efficiency of the bacteria for growth [11]. The selected variables, glucose as carbon source, yeast extract as a nitrogen source, pH and salinity (NaCl concentration  $\text{gL}^{-1}$ ) as input parameters. The coded values of each parameter are presented in Table 1. The model was built with the result of the 30 treatments (runs) presented, actual and predicted value of the % E<sub>24</sub> in Table2. A second-order polynomial equation was used to determine the influence of individual input parameter on the production of biosurfactant through multiple regression analysis. After regression analysis, the second order response model was obtained which is given in equation 1.

$$\text{Emulsification Index} = +68.85 + 0.53A - 1.00 B - 0.34 C - 1.54D - 0.71 AB + 2.83AC + 1.47AD + 2.80 BC - 0.15BD - 0.37 CD - 7.27 A^2 - 8.23 B^2 - 7.68 C^2 - 5.46D^2 \quad \text{--Eq (1)}$$

Where, A: glucose, B: yeast extract, C: pH and D: salinity and A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>, D<sub>2</sub>, AB, AC, AD, BC, CD were identified as significant terms.

The fit of the model was expressed with the coefficient of determination R<sup>2</sup> which was found to be 0.9932 and could be indicating that 99.32 % of variability in the response could be explained by this model. The adjusted R<sup>2</sup> value of the model was found to be 0.9868 and predicted R<sup>2</sup> value was 0.9749. The production of biosurfactant obtained as results of valid experimental design was found to be 30 % higher than that of the predicted value. The strain showed maximum biosurfactant production at 57.96  $\text{gL}^{-1}$  salinity (NaCl  $\text{gL}^{-1}$ ) supplement. Since it is a marine isolate it showed optimum activity in the high salt supplemented medium. Thus, the appropriate combination of glucose, yeast extract, pH and salinity (NaCl  $\text{gL}^{-1}$ ) could enhance the production of biosurfactant by strain *Bacillus cereus* SNAU01. Hence our results show that application of RSM enhances the biosurfactant production with the combination of inputs.

### 3.4. Application Of Biosurfactant In Hydrocarbon Contaminants Removal From Soil

Application of biosurfactant in enhanced oil recovery is one of the best techniques to remove and recover a significant amount of the residual oil. The results obtained demonstrated that the *Bacillus cereus* biosurfactant solution at 0.01% and 0.05% biosurfactant concentrations was capable to remove 86% and 93% of the oil adsorbed in the sand respectively. The distilled water (control) removed 48% and synthetic surfactant, sodium dodecyl sulphate (SDS) removed 62% of the contaminated oil respectively. In addition, 84% of used engine oil was removed using cell-free broth containing biosurfactant produced by *Bacillus cereus*. In the present study, we could observe that the isolated biosurfactant was more effective than the commercial available surfactant, SDS. Thus, cell free broth-containing biosurfactant can be directly used without purification steps, which would further reduce the cost of production of the biosurfactant. The biosurfactant produced by *Bacillus cereus* could be applied in enhanced oil recovery. Similar results were obtained by Abu-Ruwaida et al. [14].

## 4. Conclusion

In the present investigation indigenous strain of *B. cereus* was isolated from mangrove forest soil. The isolated *Bacillus cereus* SNAU01 is a very potent biosurfactant producing strain. The characterization study of SNAU01 viz., FT-IR, confirmed as lipopeptide. The optimization of a variable could increase the biosurfactant production at 20.10  $\text{gL}^{-1}$  glucose, 2.45  $\text{gL}^{-1}$  yeast extract, pH 6.97 and salinity 57.96  $\text{gL}^{-1}$  of NaCl. The production yield is approximately 2.2 fold increased than the original production. The conclusion of this study represented SNAU01 is the novel strain and used in industrial and environmental applications.

## 5. References

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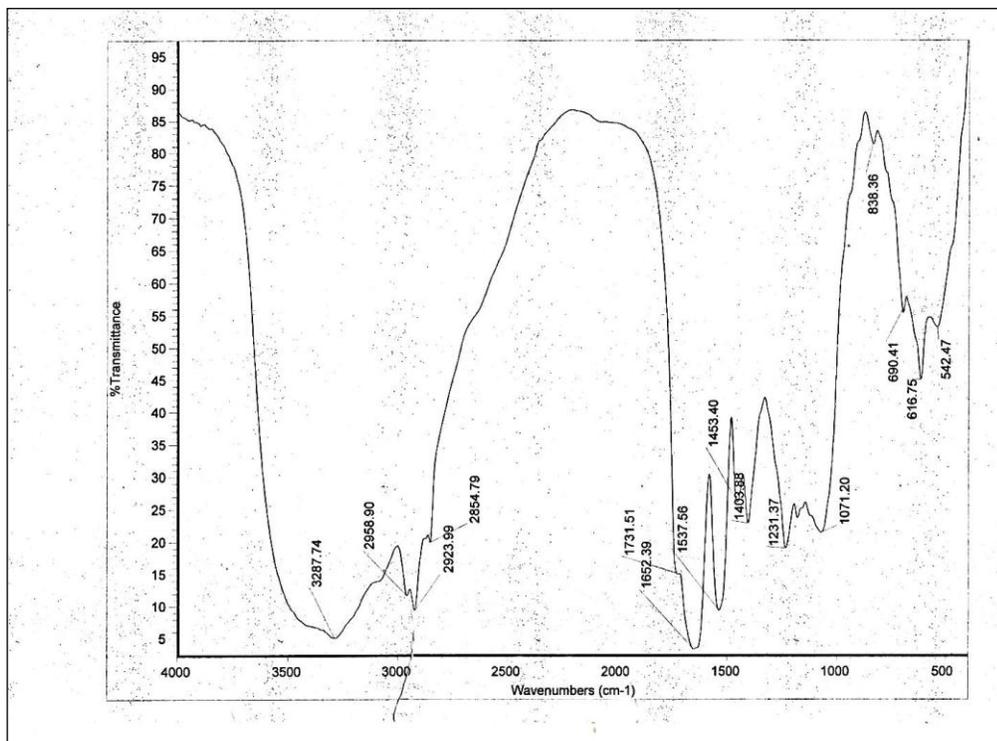


Fig. 1. Fourier transform infra-red spectrum of biosurfactant produced by *Bacillus cereus* SNAU01

TABLE I: Specific coded values of carbon source, Nitrogen source, pH and NaCl

S. No	Independent variable	Coded values				
		-2	-1	0	1	2
1	glucose (gL <sup>-1</sup> )	10	15	20	25	30
2	Yeast extract (gL <sup>-1</sup> )	0	1.75	2.5	3.25	4
3	pH	5	6	7	8	9
4	Salinity (NaCl gL <sup>-1</sup> )	30	45	60	75	90

TABLE II :Central Composite Design (CCD) matrix of independent variables and their corresponding experimental and predicted yields of emulsification activity (EI<sub>24</sub> %)

Run No	Media Components (Coded values)				Emulsification index (E <sub>24</sub> ) %	
	Carbon	Nitrogen	pH	Salinity	Experimental	Predicted
1	0	0	-2	0	37.89	38.81
2	1	1	-1	-1	33.42	33.58
3	-1	1	1	-1	42.15	42.55
4	0	0	0	2	43.21	43.94
5	1	1	1	-1	44.78	44.91
6	0	0	0	0	70.21	68.85
7	1	1	-1	1	33.57	33.87
8	-1	-1	-1	-1	49.10	48.41
9	0	0	2	0	37.14	37.45
10	-1	-1	1	1	31.45	30.77
11	1	-1	1	-1	42.81	42.44
12	1	-1	-1	-1	43.21	42.30
13	-1	-1	1	-1	38.31	37.22
14	-1	1	-1	-1	42.63	42.54
15	-1	1	-1	1	37.12	36.97
16	-1	1	1	1	35.36	35.46
17	0	0	0	0	70.24	68.85
18	0	-2	0	0	35.45	37.93
19	2	0	0	0	40.41	40.81
20	0	0	0	0	65.61	68.85
21	0	0	0	-2	49.56	50.10
22	-2	0	0	0	37.82	38.69
23	0	0	0	0	69.62	68.85
24	1	-1	-1	1	44.12	43.21
25	1	1	1	1	43.54	43.71
26	0	0	0	0	68.72	68.85
27	-1	-1	-1	1	44.37	43.45
28	0	0	0	0	68.82	68.85
29	0	0	2	0	35.12	33.92
30	1	-1	1	1	42.58	41.85