ORIGINAL ARTICLE

Maximizing Biosurfactant Yield from *Betaproteobacteria* through Response Surface Optimization

Nalamalapu Konda Reddy[1], Santhi Priya Amarthaluri[2], Suryanarayana Veeravilli[3], Lakshmi Sri Madala[4], Tejaswi Gundapaneni[4], Lavanya Gunisetti[4], Trisha Gundla[4], Katiboina Indu Priya[4], V. N. Sai Krishna Rakshitha Iruganti[4]

[1]Department of Mathematics, KLEF University, Vaddeswaram, Andhra Pradesh, India, [2]Department of Biotechnology, Sri Venkateshwara College of Pharmacy, Hyderabad, Telangana, India, [3]Department of Chemistry, Aditya University, Surampalem, Andhra Pradesh, India, [4]Department of Biotechnology, KLEF University, Vaddeswaram, Andhra Pradesh, India

Abstract

Background: Microbial biosurfactants are eco-friendly alternatives to synthetic surfactants with diverse industrial applications. However, maximizing their production requires systematic optimization of process parameters. **Objectives:** The objectives of this study were to optimize biosurfactant production by *Betaproteobacteria* using response surface methodology (RSM) and evaluate its stability and antimicrobial potential. **Materials and Methods:** Thirty bacterial isolates were screened using emulsification index, oil spreading assay, and hemolysis test. A high-performing *Betaproteobacteria* strain was selected for optimization using central composite design under RSM, considering glucose concentration, temperature, pH, and incubation time as independent variables. **Results:** Optimal conditions were identified as 20 g/L glucose, 40°C, pH 6, and 10 days of incubation. The biosurfactant exhibited 83% foaming activity, thermal stability up to 40 min across 30–80°C, and pH stability between 4 and 9. In addition, it showed significant antibacterial activity against *Escherichia coli*. **Conclusion:** The study demonstrates that RSM effectively enhances biosurfactant yield and functional properties. The produced biosurfactant shows strong potential as a stable and efficient emulsifier for industrial applications.

Keywords: *Betaproteobacteria*, emulsification index, *Escherichia coli*, hemolytic activity, response surface methodology

INTRODUCTION

Microorganisms such as bacteria, yeasts, and fungi produce biosurfactants, which are eco-friendly, biodegradable surface-active compounds with applications in bioremediation, cosmetics, pharmaceuticals, and petroleum.[1] *Betaproteobacteria*, including *Achromobacter* and *Burkholderia*, are notable producers, useful in processes like enhanced oil recovery (EOR) and environmental remediation due to their ability to reduce surface tension and stabilize emulsions.[2-4] However, achieving high, cost-effective production remains challenging because yields are sensitive to environmental and nutritional factors such as temperature, pH, carbon source, and incubation time.[5-8] Traditional methods often fail to maximize production, limiting industrial scalability.[6] Response surface methodology (RSM), combined with approaches such as central composite design (CCD) and omics technologies, offers a powerful way to optimize these parameters, enhancing biosurfactant yield and sustainability.[4,9,10] This study applies RSM to maximize production from *Betaproteobacteria* by systematically optimizing temperature, pH, glucose concentration, and incubation time, aiming to bridge laboratory efficiency and industrial viability.[3,5,7,11] By analyzing interactions between environmental and nutritional factors, this strategy provides a scalable, cost-effective framework for improving biosurfactant synthesis, supporting more efficient and economical industrial applications.[12-17]

Address for correspondence:
N. Konda Reddy, Department of Mathematics, KLEF University, Vaddeswaram, Andhra Pradesh, India.
E-mail: kondareddymamatha@gmail.com

Received: 11-04-2026
Revised: 02-06-2026
Accepted: 18-06-2026

MATERIALS AND METHODS

Microorganism and culture conditions

Silt and oil-contaminated soil from Vaddeswaram, Andhra Pradesh, was used to isolate biosurfactant-producing bacteria. Samples were cultured in a defined mineral medium (pH 7.0) sterilized at 121°C for 20 min.[18] A potent isolate was purified by streak plating, characterized morphologically and biochemically (oxidase, catalase), identified through 16S rRNA sequencing and phylogenetic analysis, and maintained at 4°C with monthly subculturing.[15,19]

Hemolysis test

Cultures were inoculated on blood agar and incubated at 37°C for 24–48 h. Clear zones around colonies indicated hemolytic activity and potential biosurfactant production.[20]

Oil spreading

Biosurfactant activity was measured by adding 10 µL cell-free supernatant to 20 µL oil on 40 mL water.[21] The resulting clear zone ("oil displacement") indicated activity; area was calculated as $\pi(d/2)^2$.

Emulsification index (E24)

Equal volumes of biosurfactant and oil were vortexed. After 24 h, E24 (%) was calculated as the emulsified layer height relative to total height.[4,22]

Saponification test

Measured biosurfactant's ability to hydrolyze ester bonds by determining alkali consumption.[23]

Anthrone test

Carbohydrate content was quantified colorimetrically through spectrophotometry using anthrone reagent.[24]

Foaming activity

Foam stability was assessed by shaking 50 mL supernatant for 2 min, recording initial foam height, and measuring retention after 30 min.[20,25-30]

Extraction and purification

After 10 days, cultures were centrifuged at 10,000 rpm for 20 min, supernatant neutralized, distilled to reduce 50–75%, concentrated below 60°C under reduced pressure,[31-35] cooled, and stored at 4°C.

Surfactant aggregation concentration

Critical micelle concentration (CMC) was determined using serial dilutions (10–1000 mg/L) of biosurfactant at pH 8.0 ± 0.1 and measuring surface tension[36,37] at 25°C.

Antibacterial activity

Agar well diffusion assessed efficacy against *Escherichia coli*. Wells received 50 µL biosurfactant; inhibition zones after 24 h at 37°C indicated activity.[38,39]

Microbial identification

16S rRNA sequencing identified the potent isolate as a *Betaproteobacterium*, confirmed through GenBank (accession MK079349.1).[40,41]

Fourier transform infrared (FTIR) spectroscopy

Functional groups in purified biosurfactant were analyzed using KBr pellets over 4000–400 cm^{-1}, identifying peaks for carboxyl/ester (~1700–1735 cm^{-1}), alkyl (~2920 cm^{-1}), and hydroxyl (~3400 cm^{-1}) groups.[42-46]

Gas chromatography-mass spectrometry (GC-MS) profiling

Biosurfactant (1–10 mg/mL) in methanol/chloroform was analyzed through GC-MS (helium carrier, electron impact, Durabond-5 column, 60–250°C) to identify compounds by retention times and spectra.[47-51]

Experimental design

Central composite RSM assessed effects of temperature (25–35°C), pH (6–8), incubation (3–7 days), and glucose (5–15 g/L) on biosurfactant production. Thirty runs were designed in Design-Expert v7.0, with E24 as the response variable.[4,9,10,52-55]

The significance of the cultural conditions was evaluated by analysis of variance (ANOVA) ($P < 0.05$). Four replicates were used to validate the predicted ideal conditions, and the findings were presented as mean ± standard deviation.

Temperature and pH stability

Biosurfactant solutions (1–10 mg/mL) were tested for stability and activity across pH 4–9 and 30–80°C over 7 days, using E24 and surface tension measurements. Conditions were optimized for mesophilic growth and refined through RSM.[56-58]

RESULTS AND DISCUSSION

Thirty bacterial isolates were screened for biosurfactant production using hemolysis, oil spreading, emulsification (E24), and foaming tests. Isolate KLUBT28 showed the highest activity across all assays – strongest hemolysis, largest oil displacement (36.3 cm^2), highest E24 (66.9%), and greatest foaming (49.8%) – making it the most promising for commercial applications.

KLUBT29 and KLUBT30 also demonstrated significant activity, though lower than KLUBT28, while KLUBT01–KLUBT03 showed minimal biosurfactant production with small oil displacement areas (1.8–2.4 cm²) and low foaming/emulsification. These findings align with prior studies showing Bacillus, Pseudomonas, and marine isolates from oil-contaminated environments possess strong emulsification, foaming, and oil displacement abilities.[56-59] Overall, KLUBT28 emerged as the most effective producer, with other isolates showing potential for further investigation in industrial and bioremediation applications.

Table 1: Range of variable levels for response surface methodology

Factor	Symbol	High	Low
Temperature (°C)	A	35	25
pH	B	8	6
Incubation period (days)	C	7	3
Glucose concentration (g/L)	D	15	5

Identification of the most effective biosurfactant-producing strain

KLUBT28, a Gram-negative *Betaproteobacteria*, was identified using Bergey's Manual.[59] It was gelatinase- and catalase-positive but negative for amylase, casein, and indole, with optimal growth at 35–55°C.

Biochemical tests [Table 3] confirmed its rod shape, fermentation of glucose, trehalose, and xylose, and enzymatic ability to hydrolyze fat, starch, and gelatin. Catalase and

Table 2: Screening biosurfactant-producing isolates using initial and supplementary methods

Isolate number	Hemolytic assay	Oil displacement technique (cm²)	Emulsification index (%)	Foaming activity (%)
KLUBT01	-	2.4	0	10.8
KLUBT02	-	1.8	0	11
KLUBT03	-	2.1	0	10.9
KLUBT04	+	3.8	14.8	12.5
KLUBT05	+	5.9	16.9	13
KLUBT06	+	6.9	18.8	14
KLUBT07	++	12.8	28.9	19
KLUBT08	+	7.5	0	8.9
KLUBT09	+	7.8	24.9	10.9
KLUBT10	+	8.5	23.9	12.5
KLUBT11	+	9.3	28.9	13.9
KLUBT12	+	8.9	32.5	15.8
KLUBT13	-	1.5	6.9	28.9
KLUBT14	-	2.3	7.5	32.8
KLUBT15	-	1.9	6.5	33
KLUBT16	-	2.5	9.8	35
KLUBT17	-	2.3	10.3	38
KLUBT18	+++	25.8	35.9	29
KLUBT19	++	19.8	31.8	29
KLUBT20	-	1.2	11.5	35
KLUBT21	-	1.4	12.8	36
KLUBT22	-	2.9	15.8	37
KLUBT23	+++	22.3	38.9	35
KLUBT24	++	18.9	41.5	32.8
KLUBT25	+++	23.9	32.8	36.9
KLUBT26	++	17.9	32.8	37
KLUBT27	-	3.2	29.8	38
KLUBT28	+++++++	36.3	66.9	49.8
KLUBT29	+++	27.4	41.8	32
KLUBT30	++	24.5	38.9	35

"-": No hemolysis observed, "+": Slight hemolysis, "++": Moderate hemolysis, "+++": Strong hemolysis, "+++++": Very strong hemolysis

Table 3: Biochemical characterization of *Betaproteobacteria*

Experimental test	Observation
Bacterial staining profile	Negative (–)
Cell morphology	Rod-shaped bacterium
Carbohydrate metabolism	
Ribitol	Negative (–)
Dextrose	Positive (+)
Galactosyl glucose	Negative (–)
α-D-Glucopyranosyl-(1→1) -α-D-glucopyranoside	Positive (+)
Pentose sugar	Positive (+)
Chemical decomposition by water	
Polysaccharide carbohydrate	Positive (+)
Protein hydrogel	Positive (+)
Glycerol ester	Positive (+)
Hydrogen peroxide decomposing enzyme	Positive (+)
Cytochrome c oxidase	Positive (+)
Nitrate-to-nitrite conversion	Positive (+)
Litmus paper test	P
Oxidative decarboxylation	
2-Amino-5-guanidinopentanoic acid	Positive (+)

(+): Positive, (-): Negative

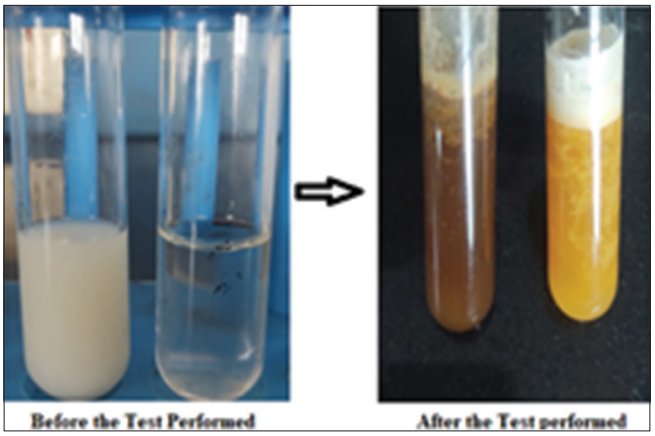

Figure 1: Anthrone test

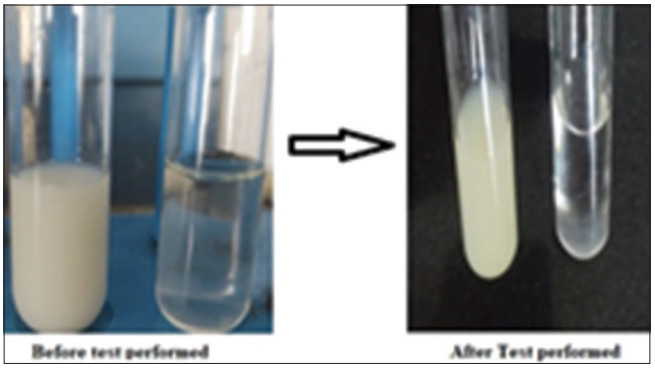

Figure 2: Saponification test

oxidase activity indicated aerobic respiration, while positive litmus, nitrate reduction, and arginine decarboxylation demonstrated adaptability to diverse environments.

The Anthrone method was used to quantify carbohydrates in biosurfactant samples [Table 1 and Figure 1]. Initially colorless or slightly turbid, samples turned green to dark brown after reaction, indicating carbohydrate presence. This sensitive colorimetric assay, commonly used for biosurfactants like *Pseudomonas aeruginosa*, *Bacillus subtilis*, and rhamnolipids,[59,60] suggests polysaccharides or glycoproteins that enhance emulsification and support bioremediation and oil recovery. Results in Figure 1 validate this conventional characterization method.

The saponification test for *Betaproteobacteria*-derived biosurfactants [Tables 2,4 and Figure 2] showed clear phase separation in some tubes, confirming micelle formation and soap-like activity essential for emulsification and reducing surface tension. KLUBT28 exhibited the highest carbohydrate content (27%), while KLUBT07 had the lowest (12.5%), indicating variability in biosurfactant structure and activity across isolates. Positive saponification was observed in strains KLUBT01, KLUBT02, KLUBT04, KLUBT06, KLUBT08, KLUBT09, KLUBT11, KLUBT13, KLUBT15, KLUBT17, KLUBT19, KLUBT21, KLUBT23, KLUBT25, and KLUBT28, whereas other isolates showed minimal or no activity. These findings align with previous reports linking carbohydrate content to foam stability and oil displacement[61-63] and highlight the potential of *Betaproteobacteria* biosurfactants for biotechnological and industrial applications in emulsification, environmental remediation, and oil recovery.

The *Betaproteobacteria*-derived biosurfactant showed strong surface activity in the oil spreading assay, producing a 1.7 cm clear zone and a 36.31 cm² displacement area, comparable to Pseudomonas and Bacillus biosurfactants,[63-65] indicating potential for oil spill remediation and EOR. It exhibited 49.8% foaming activity and 66.9% E24, demonstrating stable foam formation and hydrocarbon emulsification, relevant for industrial and environmental applications.

The biosurfactant also showed antibacterial activity, producing a 3.5 cm inhibition zone against *E. coli*, suggesting membrane-disruptive effects similar to previously reported Pseudomonas and Bacillus biosurfactants.[66-68] Extraction and purification through distillation ensured high-purity recovery, suitable for industrial use.[69,70]

Surface tension and CMC analysis revealed that KLUBT28 had the lowest surface tension (37.5 mN/m) and highest biosurfactant concentration (310 mg/L), outperforming other isolates (CMC 125–190 mg/L), indicating strong emulsifying

Table 4: Analysis of carbohydrate content and saponification test results in biosurfactant-producing isolates of *Betaproteobacteria*

Sample ID	Anthrone test result			Saponification test result	Formation of foam
	Absorbance at 620 nm	Carbohydrate concentration (mg/g)	Carbohydrate content (% of total biosurfactant)		
KLUBT01	0.45	180	18	Positive	Yes
KLUBT02	0.5	200	20	Positive	Yes
KLUBT03	0.4	160	16	Negative	
KLUBT04	0.55	220	22	Positive	Yes
KLUBT05	0.48	190	19	Negative	
KLUBT06	0.6	240	24	Positive	Yes
KLUBT07	0.3	120	12	Negative	
KLUBT08	0.52	210	21	Positive	Yes
KLUBT09	0.47	185	18.50	Positive	Yes
KLUBT10	0.65	260	26	Negative	
KLUBT11	0.42	170	17	Positive	Yes
KLUBT12	0.58	235	23.50	Negative	
KLUBT13	0.37	150	15	Positive	Yes
KLUBT14	0.62	250	25	Negative	
KLUBT15	0.49	195	19.50	Positive	Yes
KLUBT16	0.41	165	16.50	Negative	
KLUBT17	0.36	145	14.50	Positive	Yes
KLUBT18	0.39	155	15.50	Negative	
KLUBT19	0.44	180	18	Positive	Yes
KLUBT20	0.54	215	21.50	Negative	
KLUBT21	0.31	125	12.50	Positive	Yes
KLUBT22	0.53	205	20.50	Negative	
KLUBT23	0.34	140	14	Positive	Yes
KLUBT24	0.59	240	24	Negative	
KLUBT25	0.38	155	15.50	Positive	Yes
KLUBT26	0.43	175	17.50	Negative	
KLUBT27	0.38	155	15.50	Negative	
KLUBT28	0.66	270	27	Positive	Yes
KLUBT29	0.46	185	18.50	Negative	
KLUBT30	0.61	255	25.50	Negative	

efficiency and industrial potential. FTIR analysis confirmed functional groups (C–H, O–H, C=O), with concentration-dependent spectral differences reflecting biosurfactant purity and activity, consistent with prior studies.[66,68,71-74] The corresponding results are presented in Figures 3 and 4.

Overall, KLUBT28 exhibits superior surface activity, foaming, emulsification, antibacterial effect, and low CMC, making it a promising candidate for biotechnological and industrial applications.

A dominating peak at RT 19.25 min is revealed by GC-MS analysis of the biosurfactant from *Betaproteobacteria* strain KLUBT28 [Figure 5], with important fragment ions at m/z 118, 133, 194, and 222.3-(benzoylamino)-2, 2-diphenylpropan-1-one is identified by the mass spectrum, and its structure is verified by comparing it to the NIST library. These results highlight the importance of GC-MS as a biosurfactant characterization tool for bioremediation and emulsification.

Partial sequencing

Figure 6 shows a 16S rRNA-based phylogenetic tree of *Betaproteobacteria*, illustrating evolutionary relationships and species-level divergence. Branching patterns reflect genetic distances, with color coding indicating taxonomic groups or clades. This method leverages conserved and hypervariable 16S regions, enabling clear delineation of

evolutionary connections, including hydrocarbon-degrading, nitrogen-fixing, and biofilm-forming clades.[72-74]

Clustering also reflects environmental adaptations, separating species from soil, freshwater, or marine niches.[75] The tree highlights metabolic and ecological distinctions within *Betaproteobacteria*, reinforcing the value of 16S rDNA sequencing for bacterial taxonomy.

In addition, CCD-based RSM optimization of %E24 [Table 5] confirmed model accuracy (errors 0.1–0.5%), showing that temperature and pH significantly influence biosurfactant production – for example, Runs 12 and 14 at 40°C produced 28.99% E24 at pH 9 versus 66.6% at pH6.

Effect of incubation time and glucose

Higher glucose levels and longer incubation enhanced biosurfactant yield, with the highest E24 (66.6%) at 20 g/L glucose and 10 days. CCD optimization indicated maximal efficiency at pH 6–7, 30–40°C, >8 days incubation, and glucose >15 g/L, supporting scalable industrial applications. These trends align with prior studies linking carbon availability, incubation, and moderate conditions to improved emulsification.[71-75]

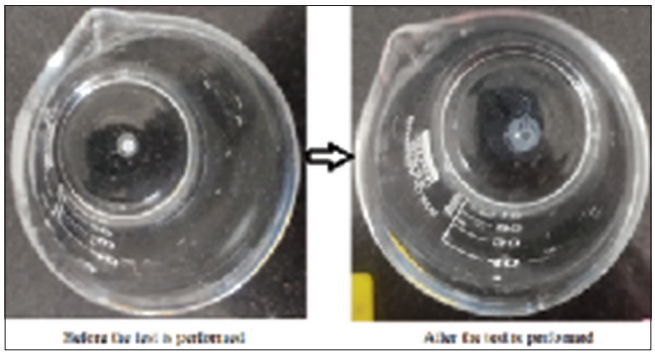

Figure 3: Oil spreading test

Model validation

ANOVA confirmed the model's high predictive accuracy ($P < 0.0001$, F = 1.450), with E24 strongly influenced by factors A–D, their interactions, and quadratic terms. Despite a significant lack of fit ($P = 0.0007$), the model explains 97.51% of response variability ($R^2 = 0.9751$), with adjusted and predicted R^2 closely matching, SD = 5.79, C.V. = 17.13%, and Adeq Precision = 26.206, demonstrating RSM's robustness for optimizing biosurfactant production.

Final equation in terms of coded factors

$$\text{Emulsification index} = 32.75 + 0.71 * A - 1.51 * B + 1.54 * C + 1.85 * D - 2.22 * A * B + 2.64 * A * C + 1.91 * A * D - 1.44 * B * C - 2.43 * B * D + 2.36 * C * D + 0.42 * A^2 + 0.25 * B^2 + 0.31 * C^2 + 0.32 * D^2$$

Where,
A: Incubation temperature (C);
B: pH;
C: Incubation period (days);
D: Glucose concentration (g/L).

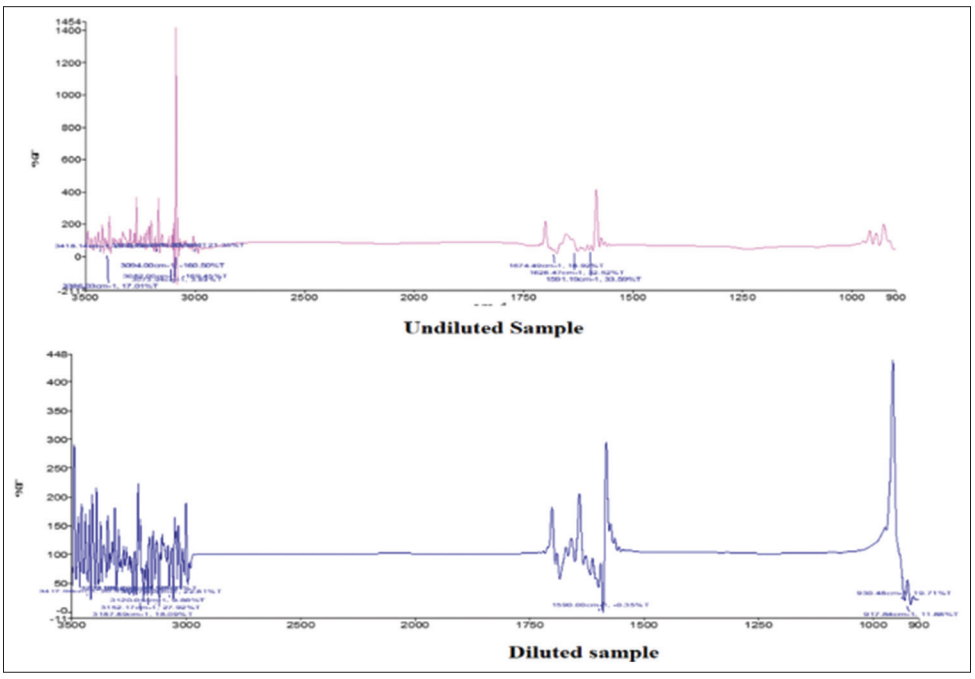

Figure 4: Fourier transform infrared spectroscopy spectrum of biosurfactant

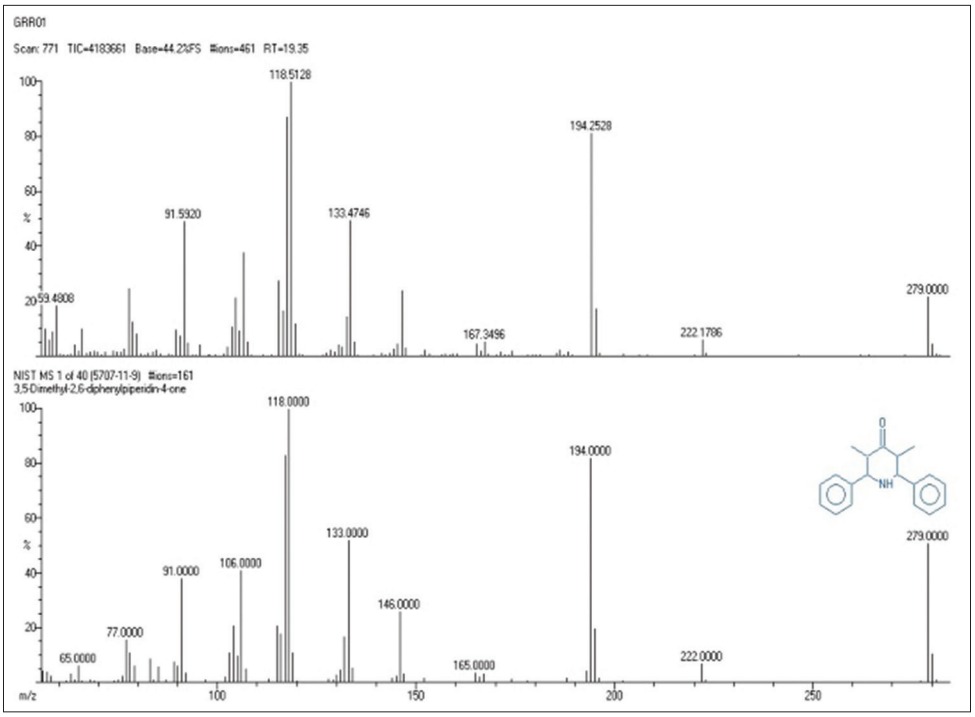

Figure 5: Gas chromatography-mass spectrometry analysis of biosurfactant

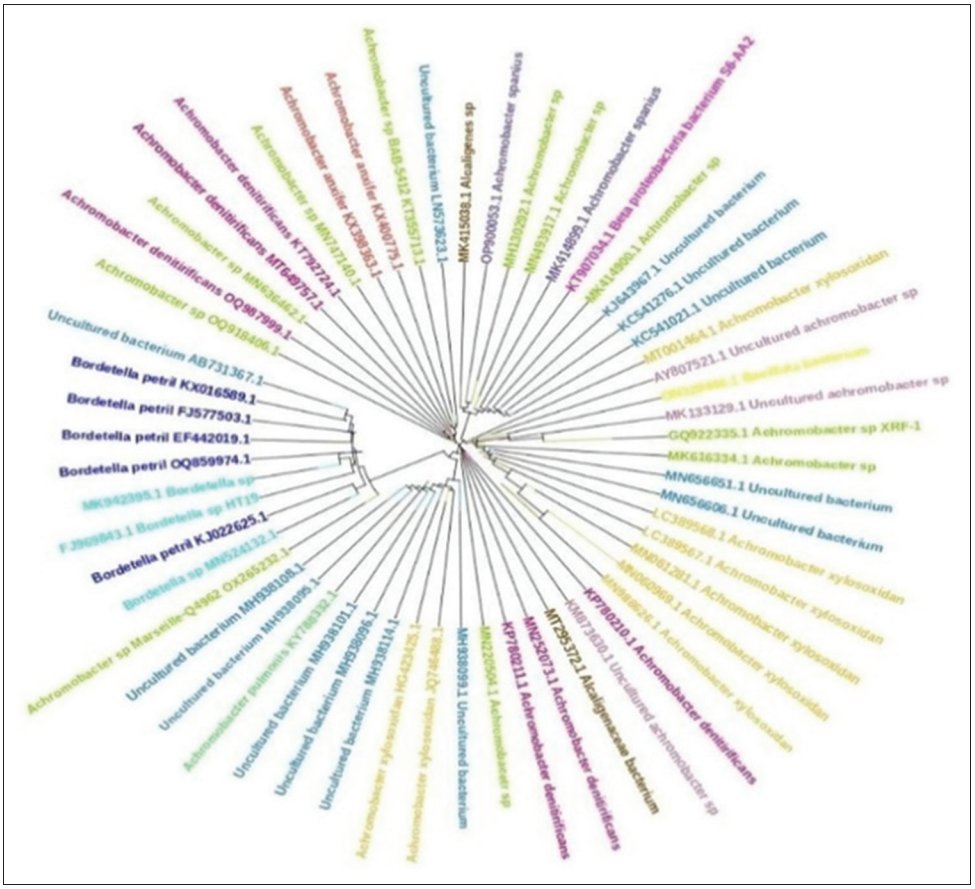

Figure 6: Phylogenetic tree derived from 16S rDNA gene sequencing

Table 5: ANOVA results for the produced emulsification index for the quadratic model

Source	Sum of squares	Df	Mean square	F-value	*P*-value Prob >F	Model significance
Model	680.5565	14	48.61117774	1.450350837	<0.0001	Significant
A-incubation temperature	12.1695	1	12.16950417	0.363086256	<0.0001	
B-pH	54.934	1	54.93400417	1.638997091	<0.0001	
C-incubation period	56.703	1	56.70300417	1.691776529	<0.0001	
D-glucose concentration	82.47334	1	82.4733375	2.460653693	<0.0001	
AB	78.81001	1	78.81000625	2.351355466	0.0005	
AC	111.7778	1	111.7777563	3.334972939	0.0008	
AD	58.10251	1	58.10250625	1.73353172	0.0003	
BC	33.37951	1	33.37950625	0.995902528	0.0009	
BD	94.62426	1	94.62425625	2.82318544	0.0002	
CD	88.97206	1	88.97205625	2.654547827	0.0007	
A^2	4.879286	1	4.879286012	0.145577147	0.0006	
B^2	1.670286	1	1.670286012	0.049834232	0.0009	
C^2	2.6235	1	2.623500298	0.078274093	<0.0001	
D^2	2.839857	1	2.83985744	0.08472927	<0.0001	
Residual	502.7526	15	33.51684056			
Lack of Fit	494.9576	10	49.49576083	31.74840336	0.0007	Significant
Pure error	7.795	5	1.559			
Cor total	1183.309	29				

ANOVA: Analysis of variance

Final equation in terms of actual factors

Emulsificationindex

$$=14.37792-0.43679*IncubationTemperature+9.53750*pH-3.24017*IncubationPeriod-0.1415*Glucoseconcentration-0.14796*IncbationTemperature*pH+0.10573*IncubationTemperature*IncubationPeriod+0.038113*IncbationTemperature*Glucoseconcentration-0.38517*pH*IncubationPeriod-0.32425*pH*Glucoseconcentration+0.18865*Incubationperiod*Glucoseconcentration+0.00421771*IncubationTemperature^2+0.10968*pH^2+0.049483*Incubationperiod^2+0.012871*Glucoseconcentration^2$$

The effect of incubation temperature, pH, duration, and glucose concentration on biosurfactant synthesis (%E24) is shown in the response surface plot, as shown in Figure 7. To assess these interactions and determine ideal conditions, RSM analysis was used.

Emulsification was greatly impacted by the interplay between pH and incubation temperature. In accordance with known microbial growth preferences, the maximum %E24 values were found at moderate temperatures (30–40°C) and near-neutral pH (6–7.5), suggesting ideal conditions for microbial biosurfactant synthesis.

Similarly, there was a considerable link between the incubation period and the glucose levels. Extended incubation (~10 days) and higher glucose levels (15–20 g/L) improved emulsification, underscoring the significance of carbon availability and sustained microbial activity for biosurfactant synthesis. This implies that a major limiting factor that directly affects the yield of biosurfactants is glucose.

These results are supported by earlier research.[75] Maximized the synthesis of *P. aeruginosa* biosurfactants at neutral pH and between 30 and 35°C. Found that agro-industrial waste produced higher yields at 15–20 g/L glucose[76] and 35–40°C.[77] Discovered that high glucose levels and up to 10 days were necessary for *B. subtilis* to produce its maximum amount. These findings support the importance of pH, temperature, incubation duration, and glucose in the synthesis of biosurfactants.

Figure 8 presents the E24 of the biosurfactant across different pH and temperature conditions, highlighting its stability under varying environmental factors relevant to industrial applications. The results show a bell-shaped trend, indicating that specific pH and temperature ranges significantly influence stability.

pH stability

The biosurfactant exhibits maximum stability in neutral to slightly alkaline conditions, with the E24 reaching approximately 70% at pH 7–8 [Figure 8a]. This aligns with

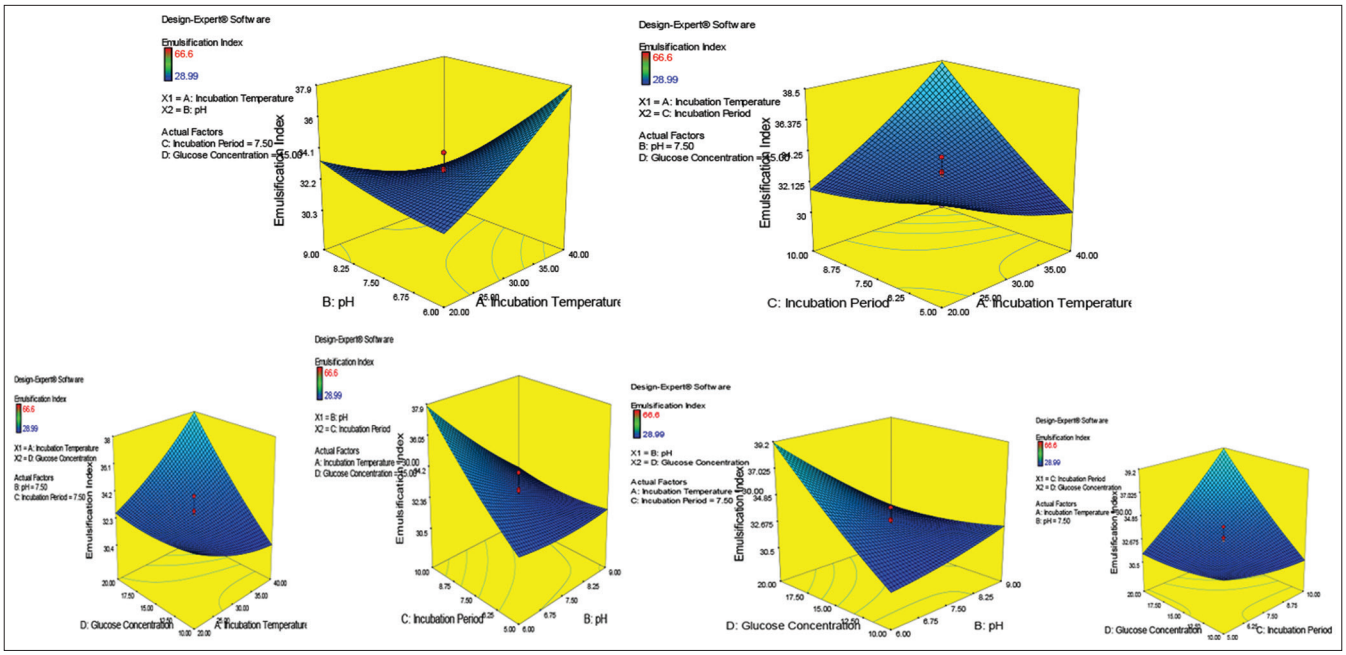

Figure 7: Response surface plot for the interactions between different selected factors

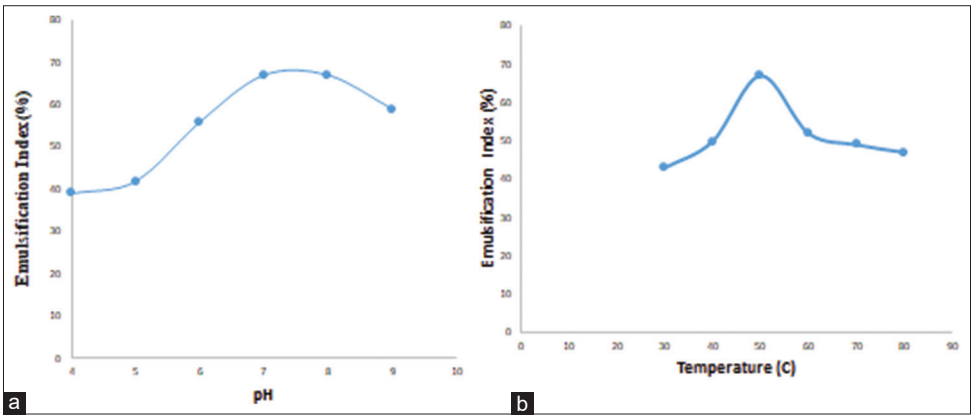

Figure 8: Biosurfactant stability at different pH (a) and different temperatures (b)

previous findings, where biosurfactants from *B. subtilis* and *P. aeruginosa* demonstrated optimal activity in similar pH ranges.[76-78] The sharp decline in the E24 at extreme pH values indicates sensitivity to highly acidic or alkaline environments, limiting its efficiency under such conditions.

Thermal stability

The biosurfactant maintains its highest stability at 50°C, with an E24 of about 70% [Figure 8b]. Stability declines at temperatures above 50°C, though some activity persists beyond 70°C. Similar thermal stability has been reported for biosurfactants from *Rhodococcus erythropolis*, which retain functionality up to 60°C before activity loss.[78,79] These findings suggest suitability for industrial applications requiring moderate thermal resistance, such as EOR and thermophilic bioprocesses. The observed stability trends are further supported by studies on *Acinetobacter calcoaceticus*, where biosurfactants exhibited maximum stability[65-70,79] between 45°C and 50°C.

The biosurfactant's stability profile suggests its potential for industrial applications, including wastewater treatment, bioremediation, and petroleum recovery. Its optimal performance at neutral pH and moderate temperatures enhances its applicability in dynamic industrial environments. These findings provide a basis for further optimization and scale-up of biosurfactant production for commercial use.

CONCLUSION

This study demonstrated the effectiveness of the model in optimizing biosurfactant production by *Betaproteobacteria* using the RSM methodology. The optimal conditions for maximum yield were identified as pH 6, incubation temperature 40°C, incubation duration 10 days, and

glucose concentration 20 g/L. Under these conditions, the biosurfactant exhibited 83% foaming activity, thermal stability between 30°C and 80°C for 40 min, pH stability from 4 to 9, and antibacterial activity against *E. coli*. These properties underscore its strong emulsifying capabilities and potential applications in industrial processes such as bioremediation, oil recovery, and wastewater treatment. This work provides a foundation for future research and large-scale biosurfactant production.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception, design, execution, and writing of this manuscript. Each author has read and approved the final version of the manuscript.

DATA AVAILABILITY

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Source of Support: Nil. **Conflicts of Interest:** None declared.