LAUTECH Journal of Engineering and Technology 20 (2) 2026: 115-125
10.36108/laujet/6202.02.0241

Optimization of Process Variables on Yield of Biosurfactant Derived from a Mutant Acinetobacter Sp.

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Article Info

Article history:

Received: June 18, 2026
Revised: July 9, 2026
Accepted: July 13, 2026

Keywords:

Biosurfactant, Acinetobacter sp., Mutagenesis, Process Optimization, Response Surface Methodology, Tertiary Oil Recovery, Surface and Interfacial Tension, Atmospheric Room Temperature Plasma (ARTP)

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ABSTRACT

Microbial Enhanced Oil Recovery (MEOR) utilizes microorganisms to improve oil extraction from hydrocarbon reservoirs, with its success hinging on selecting potent strains that can produce high-performance biosurfactants under extreme reservoir conditions. This study, therefore, developed and optimized a hyperactive mutant strain of a novel Acinetobacter species isolated from reservoir formation water. To enhance its temperature, pH and salinity tolerance along with metabolic yield, the wild-type strain isolated from formation water was subjected to Atmospheric and Room Temperature Plasma (ARTP) using a helium plasma jet at a radio-frequency input power of 120 W. Exposure for 30 seconds resulted in a cellular lethality rate of 90.08% and yielded a positive mutation rate of 60%. Thermal stability evaluations across a temperature range of 45–95 °C over 10 days were assessed based on maximum Optical Density (OD) 550nm, emulsification indices (E24, E72), Oil Displacement Test (ODT), Surface Tension (ST), and Interfacial Tension (IFT) dynamics. The selected hyper-producing isolate was subsequently subjected to multi-objective numerical optimization to evaluate its yield limits across varying pH (7.20–10.52) and salinity (15–35%) levels under extreme thermal stress (95 °C). The Biosurfactant Yield (BY) of 1.858 mL, ODT of 7.985 mm, ST of 49.985 dyne/cm, IFT of 41.362 dyne/cm, and emulsification indices of 20.533% E24, 11.955% E48, and 6.568% E72 were obtained. The ARTP mutagenesis provides a highly efficient strategy for generating robust, stress-tolerant bio-agents for tertiary oil recovery applications.

INTRODUCTION

Microbial synthesis of biosurfactants relies heavily on media optimization, as the composition, concentration, and specific components of the nutritional substrate dictate the types of structural metabolites generated. These surface-active molecules span five primary chemical categories: lipopeptides, phospholipids, glycolipids (such as rhamnolipids, trehalose lipids, and sophorolipids), fatty acids, and neutral lipids. Cultivation methods frequently leverage low-cost, nutrient-rich agricultural byproducts and waste streams like molasses, beef extract, or cheese whey (Sari *et al*., 2019). However, this specific investigation selects a highly regulated fermentation broth composed of glucose and mineral salts as the primary carbon and nutrient source. This targeted selection establishes a stable, reproducible nutritional environment necessary to precisely control microbial growth kinetics and maximize the yield of target high-performance biosurfactants.

The primary challenge limiting the potential area of application, specifically enhanced oil recovery (EOR), is the massive volume of petroleum remaining trapped within tight reservoir rock pores due to capillary forces and hydrostatic pressure. Chemical flooding often fails to resolve this problem because standard surfactants face extreme adsorption and binding on the subsurface rock matrices, which rapidly depletes the active chemical agents before they can mobilize the oil. While surfactants are divided into anionic, cationic, amphoteric, and non-ionic categories, the anionic

group is highly preferred for subterranean applications due to its significantly lower rock adsorption rates (Aboelkhair *et al*., 2022). Nevertheless, maintaining a stable balance of low rock retention while achieving profound interfacial tension reduction and rock wettability alteration under harsh reservoir conditions remains a severe technical obstacle (Kumar *et al*., 2020).

Biosurfactants have recorded widespread operational success across diverse industrial 0solubility, and dispersion of insoluble organic compounds at fluid interfaces (Rafiu *et al*., 2019; Singh *et al*., 2007). In EOR frameworks, these amphiphilic agents assemble into structural micelles at the boundaries of immiscible fluids, lowering interfacial tension and altering the rock's wetting behavior to physically drive trapped oil into a flowing water phase (Aboelkhair *et al*., 2022). Documented successes include deploying specialized microflora that consume heavy oil as a carbon source, thereby shortening long carbon chains to lower oil viscosity and raise quality, as well as utilizing kerosene-driven microbial pathways to successfully decouple and release bitumen from tight tar sands (Sari *et al*., 2019).

Problem Statement

The specific problem that necessitated this investigation calls for the high sensitivity of microbial metabolite synthesis to varying nutrient configurations, paired with the rapid loss of standard surfactants through rock matrix adsorption, which limits the efficient mobilization of oil trapped by capillary forces. However, diverse and unrefined waste materials (like cheese whey or molasses) can support microbial growth; there is an unfulfilled need to investigate how a highly controlled, synthetic medium (glucose and mineral salts) can optimize the production of low-adsorption (anionic) biosurfactant yields that remain stable enough to alter rock wettability and lower interfacial tension and other active surface properties under demanding reservoir conditions.

METHODOLOGY

Materials, Reagents, Apparatus, and Equipment

Materials and reagents included crude oil, petroleum formation water, *Acinetobacter* species, distilled water, ethanol, and nutrient agar alongside broth ingredients like glucose, peptone, yeast extract, urea, ammonium sulphate, potassium hydrogen phosphate, magnesium sulphate, and sodium chloride. Apparatus comprised essential consumables, glassware, tools, pipettes, Petri dishes, and a density bottle. Critical laboratory instrumentation utilized was an autoclave, oven, water bath, incubator, refrigerator, shaker incubator, vortex mixer, Allegra X-30R centrifuge, weighing balance, pH meter, UV-2550 spectrophotometer, and dissecting microscope. Analytical measurements were performed using a Du Noüy tensiometer, surface tensiometer, TX-500C interface tensiometer, and an RS-300 Haake Viscometer.

Laboratory Setup

The laboratories of the Departments of Chemical Engineering, LAUTECH, Ogbomoso, Oyo State; Microbiology Department, Osun State University, Osogbo; and Department of Petroleum Engineering, University of Ibadan, Ibadan, Oyo State, all in Nigeria, were used. In these laboratories, the laboratory benches were thoroughly cleaned with disinfectants (85% ethanol) before and after each bench work to prevent contamination of samples and to ensure a safe working environment.

Development of Mutant Strain from Novel *Acinetobacter* Species

The novel *Acinetobacter* species isolated from formation water was subjected to Atmospheric and Room Temperature Plasma (ARTP) genetic mutation to obtain a hyperactive type of

Acinetobacter mutant strain with enhanced biosurfactant production activity and tolerance to very high temperature and salinity. The development of the mutant strain was performed as provided in the following sections.

Culture Preparation of *Acinetobacter* Species

The novel *Acinetobacter* sp. was purified and activated on a blood agar plate that consisted of 15 g/L of tryptone, 5 g/L of soy peptone, 5 g/L of NaCl, 5% defibrillated sheep blood, and 3 g/L of agar (pH 7.3) at 37 °C for 24 h (Bodour and Miller-Maier, 1998; Chotard *et al*., 2022; Islam and Sarma, 2021). The purified and activated novel *Acinetobacter* bacterial strain was then cultured according to the methods of Hajibagheri *et al*. (2021) and Ziwei *et al*. (2021). The bacterial strains were suspended and cultured in the Luria-Bertani (LB) broth, which is made up of 5.0 g/L of yeast extract, 10.0 g/L of tryptone, and 10 g/L of NaCl (pH 7.0) and incubated in an incubator shaker that is agitated at 220 rpm for 24 h at 30 °C. The culture broth was further enhanced by transfer into another culture medium that consisted of 10 g/L of KH_2PO_4, 10 g/L of $Na_2HPO_4{\cdot}12H_2O$, 10 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 10 mL of trace element solution SL-4, and 20 g/L of glucose (pH 7.0). This was incubated at 30 ℃ with an agitation of 220 rpm.

ARTP Genetic Mutagenesis of Novel A*cinetobacter* sp.

ARTP genetic mutagenesis was executed utilizing an ARTP-IIS breeding system (Wuxi TMaxTree Biotechnology Co., Ltd., China). The non-thermal plasma stream, operating via radio-frequency energy under atmospheric pressure with high-purity helium, generated reactive oxygen, nitrogen, and hydroxyl species to induce target microbial DNA damage (Li *et al*., 2025). Mid-exponential-phase *Acinetobacter* sp. cells were harvested, washed thrice with sterile physiological saline to eliminate background medium, and resuspended in sterile 10% glycerol (1:1 v/v) to establish a concentration of 10^7-10^8 CFU/mL. A 10 µL aliquot (~1.0×10^8CFU/mL) was spread uniformly on a sterile copper slide and positioned 2 mm from the plasma emitter nozzle. The operating parameters were maintained at a radio-frequency input power of 120 W and a helium gas flow rate of 10 L/min for a fixed 30-second exposure duration (Hajibagheri *et al*., 2021; Ziwei *et al*., 2021; Zhou *et al*., 2025). Post-mutagenesis, the sample was homogenized for 1 min, serially diluted tenfold, and plated in triplicate on blood agar for colony enumeration. Surfactant screening and subsequent lethality and positive mutation rate determinations were carried out using Equations 1 and 2 (Zhou *et al*., 2025):

$$Lethality\ Rate\ (\%) = \frac{Untreated\ viable\ count - Treated\ viable\ count}{Untreated\ viable\ count} \times 100 \quad (1)$$

A lethality plot was constructed based on this calculation. Mutations with enhanced biosurfactant surface-active properties are defined as positive mutations as follows:

$$Positive\ Mutation\ Rate\ (\%) = \frac{Number\ of\ enhanced\ biosurfactant\ surface-active\ mutants}{Total\ viable\ bacteria\ for\ bacteriostatic\ titer\ determine} \times 100 \quad (2)$$

Qualitative Analysis for Biosurfactant Surface-active Properties

The biosurfactant produced was subjected to the following surface activity qualitative analyses or measurements: oil displacement (ODT), interfacial tension (IFT), surface tension (ST) and emulsification.

ODT: 50 mL of distilled water and 10 µL of crude oil were mixed in a 150 mm diameter Petri dish. Thereafter, a 10 µL aliquot of the extracted biosurfactant solution was added to the surface of the oil (Rafiu, 2019). The diameter of the formed

oil-displaced circle was measured in millimetres (mm).

E_{24}: 2 mL of kerosene was added to 2 mL of biosurfactant solution and the mixture was mixed using a vortex mixer at 5000 rpm for 2 minutes. The mixture was allowed to stand for 24 and 72 hours, after which the calculation was performed as the percentage of the emulsion layer relative to the mixture layer.

ST/IFT: In the tensiometer (model K100), the sample was filled into a glass container and put in a measurement zone. After the measurement ring was inserted, it was zeroed in the air. The ring was then dragged down until it was hanging on the liquid's surface. Then, the fine adjustment of the tensiometer was set to zero, and the measurement began and ended when the upward pulling force on the ring just balanced the downward force exerted by the fluid. For the interfacial tension (IFT), the procedure was the same as that of surface tension measurement, except that the measurement was carried out between biosurfactant and water/crude oil.

Yield Determination

The isolates were cultured at different temperatures ranging from 45°C to 95°C to determine the optimum pH and salinity for biosurfactant production. The test was conducted at a pH and salinity range of 7.2 – 10.52 and 1.5 – 3.5 %, respectively. All adjusted media were incubated with the isolates inside a shaker operating at 200 rpm and 95 °C for 10 days (Qazi *et al.,* 2013). After incubation, growth of the isolate at varied pH and salinity was determined by absorbance at 550 nm using a UV-visible spectrophotometer (Spectrumlab 22). The broth cultures were then centrifuged at 4,000 rpm for 10 minutes to obtain the supernatant.

Single and Interactive Effects of pH and Salinity on Biosurfactant Production by Mutagenized *Acinetobacter* cells

Salinity and pH are the independent factors, while biosurfactant yield (BY), oil displacement (OD), surface tension (ST), interfacial tension (IFT), emulsification index (E24) and density are the dependent response factors. The pH of the fermentation culture broth was varied at 7.2, 8.75, 9.80, and 10.52, while salinity was varied at 15%, 20%, 25%, 30%, and 35%. At these different pH and salinity levels, the fermentation culture broth was inoculated with the mutagenized cells and incubated at the optimum temperature for 9 days. After the incubation, the sample was collected and the Biosurfactant Yield (BY) at OD_{550} was determined (Rafiu, 2019).

Effect of Temperature

To test the thermal stability of the biosurfactant-producing strains, axenic isolates were cultured in the optimized fermentation broth and incubated at 45, 55, 65, 75, 85, and 95 °C (Rafiu, 2019; Rafiu *et al*., 2024). The growth at 10 days was determined by measuring the absorbance of the broth culture at 550 nm wavelength using a UV-visible spectrophotometer (Spectrumlab 22). Each of the broths at different temperatures was centrifuged at 4,000 rpm for 10 minutes to obtain the cell-free supernatant (Qazi *et al.,* 2013; Ainon *et al.,*2013). The biosurfactant yield potential and surface-active properties (E24, ODT, surface tension, and interfacial tension) were determined. The isolate with the highest biosurfactant production at 95 °C (optimal temperature), as determined by E_{24}, E_{72}, ODT, surface tension and interfacial tension data, was selected for the pH and salinity optimization (Rafiu *et al*., 2024).

RESULT AND DISCUSSION

Mutant Screening, Isolation and Characterization

The wild-type *Acinetobacter* sp. was subjected to ARTP mutagenesis, yielding a 90.08% lethality rate at 30 seconds, as shown in Figure 1. Screening of 50 randomly selected isolates from 200 post-mutagenesis colonies revealed a 60% positive mutation rate. Successive screening isolated three mutants with a >15% enhancement in surface-active properties, as shown in Figure 2, ranked by performance: 30s-38, 30s-41, and 30s-25. This enhancement boosts MEOR efficacy via strengthened microbiota regulation as a result of improved surface active properties (ODT, E_{24}, ST and IFT). These results align with literature positioning ARTP as a robust strain improvement tool, which has achieved a >60% positive mutation rate as stated in the Equation 2, in *Ligilactobacillus salivarius* (Zhou *et al*., 2025), a 1.8-fold increase with Docosahexaenoic Acid (DHA), an omega-3 Polyunsaturated Fatty Acid (PUFA), highly valued for human eye and brain development (Zhao *et al*., 2018), a 40% avermectin B1a increase and enhanced hydrocarbon degradation (Hua *et al*., 2010).

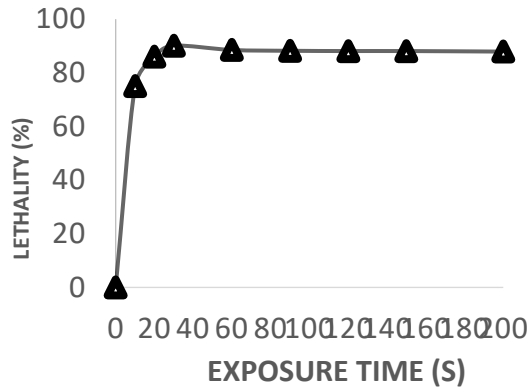


Figure 1: Novel *Acinetobacter sp*. wild-type lethality following ARTP treatment.

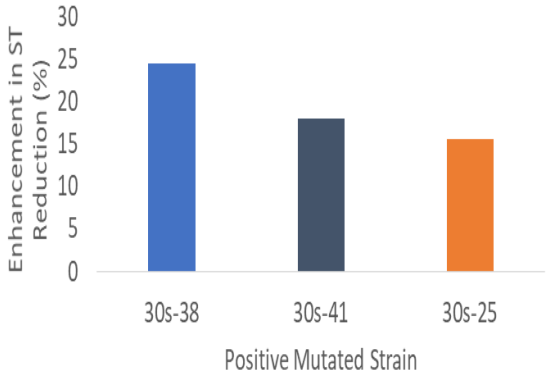


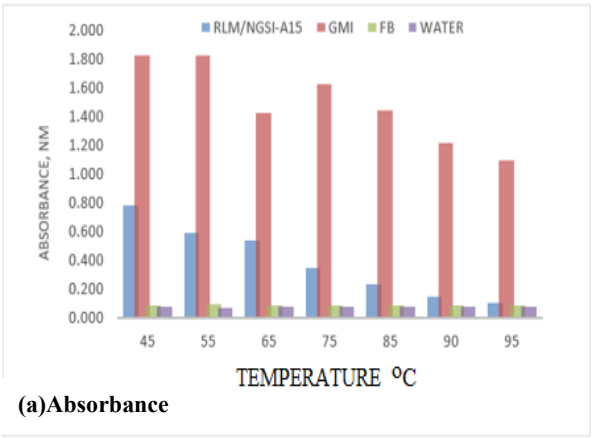
Figure 2: Mutagenesis of wild-type novel *Acinetobacter* by ARTP and the biosurfactant surface-active properties' enhancement ability of mutant strains.

Effects of temperature

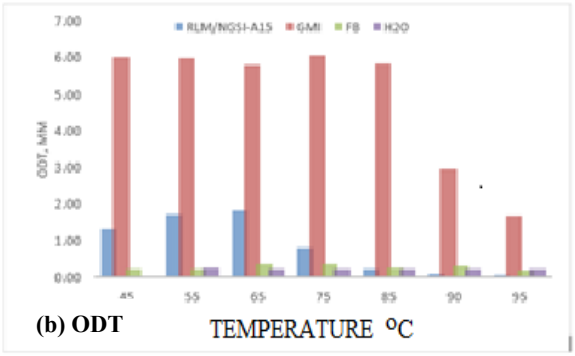
Temperature Effects on Biosurfactant Performance

Temperature fundamentally governs microbial growth kinetics and metabolic efficiency (Samuel and Noble, 2015). Both *Acinetobacter* strains exhibited temperature-dependent declines in biomass absorbance (OD_{550}) between 45 °C and 95 °C, as shown in Figure 3a. However, the ARTP mutant maintained superior optical density and biosurfactant yields, reflecting enhanced thermal tolerance (Putra and Hakiki, 2019). The wild type remained stable up to 65 °C (9.80% absorbance loss) but collapsed thereafter, dropping from 0.400 to 0.001 at 95 °C, whereas the mutant retained metabolic activity due to cellular adaptations. Oil displacement tests (ODT) mirrored the stability trends as displayed in Figure 3b. The mutant biosurfactant demonstrated superior oil displacement across 45–85 °C, producing a robust halo zone (>5 cm) indicative of high functional titers (Zhou *et al*., 2015). ST and IFT dynamics confirmed this disparity as exhibited in Figures 3c and 3d. 20 µL of supernatant biosurfactant at a critical micelle concentration (CMC) measured at OD_{550}, the wild type reduced ST by ~19% (72 to 58.5 dyne/cm) at 65 °C, while the mutant achieved

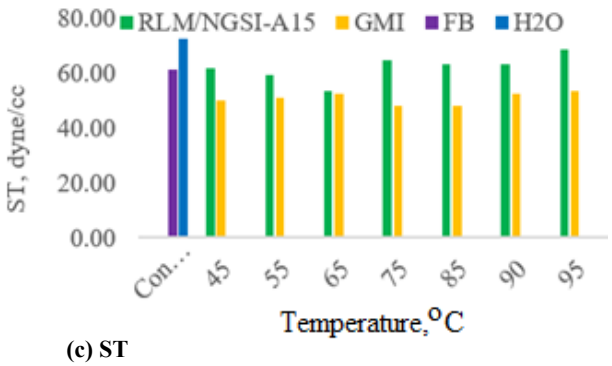
a ~38% reduction in both ST and IFT up to 95 °C. This efficiency aligns with typical CMC dynamics (Putra and Hakiki, 2019). Minor deviations from benchmarks, like *Bacillus* sp. reducing ST below 40 dyne/cm at 50 mg/L (Zhou *et al*., 2015), are attributable to extract purities. Emulsification activity (E_{24}) at 15% (w/v) salinity and pH 8.50 established clear thermal thresholds in Figure 3e. Wild-type emulsification failed at 65 °C. Conversely, the mutant biosurfactant sustained robust emulsification up to 85 °C, declining gradually at extreme temperatures.



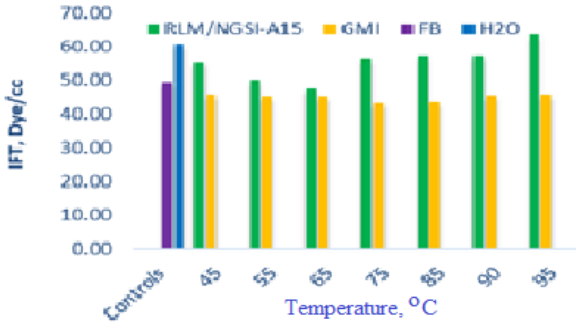
(a)Absorbance



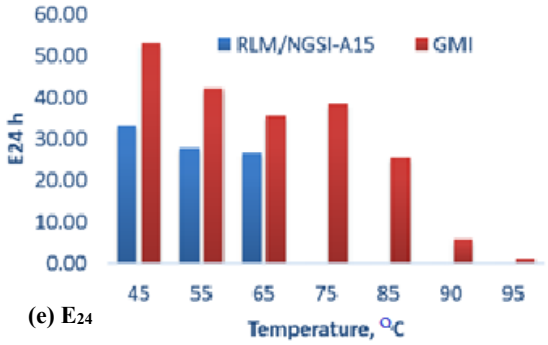
(b) ODT



(c) ST



(d) IFT



(e) E_{24}

Figure 3: Effect of temperature on (a) absorbance, (b) ODT, (c) ST, (d) IFT, and (e) E_{24} h of biosurfactant produced by wild-type and mutant novel *Acinetobacter* bacterial strains at pH 8.75 and a salinity of 15% (w/v) of NaCl.

Single and Interactive Effect of Salinity and pH

Moderate salinity stabilizes osmotic balance and enzyme activity, boosting biosurfactant synthesis in halotolerant strains, whereas excessive levels suppress bacterial growth and yield (Dadrasnia and Ismail, 2015). Most producers (e.g., *Pseudomonas*, *Bacillus* and *Rhodococcus*) thrive at pH 6.50–8.00

(Ibrahim *et al*., 2020). Table 1 demonstrates that broth salinity (15–35%) and pH (7.20–10.50) significantly affect oil displacement, surface/interfacial tension, emulsification, and biomass density. Variations in cell density reflect bacterial growth and light scattering (Wacogne *et al*., 2024); poor growth limits biosurfactant production and impairs surface tension reduction capabilities.

Figure 4 illustrates the interaction effects of pH and salinity on the biosurfactant yield of the *Acinetobacter* sp. mutant-derived biosurfactant. At high pH (10.52) and low salinity (15%), synergistic impacts show an increase in the biosurfactant yield.

The yield decreases significantly as salinity increases at low pH, while at high pH, the yield increases insignificantly as pH increases. This is attributed to disruptions in molecular stability and interfacial behavior as alkaline environments alter the protonation state of ionizable functional groups, inhibiting micelle formation and weakening interfacial adsorption (Zhou *et al*., 2025). At a constant pH of 7.20, increasing salinity (15–35%) decreased biosurfactant yield capacity. The statistical significance of salinity, pH and their interaction was established using ANOVA. Table 2 shows the suitability of selected models for biosurfactant yield based on the coefficient of determination (R^2), probability (Prob > F), adjusted coefficient of determination (Adj. R^2) and predicted coefficient of determination (Pred. R^2). A quadratic regression model was suggested for the BY. The p-value of <0.0001 for the suggested model was less than 0.05, which indicates that the model terms were significant. The values of the correlation coefficient and adequate precision for various models indicate that the model is significant (Arinkoola *et al.*, 2022; Baghaee *et al*., 2015).

Table 1: Experimental design and properties of mutagenized *Acinetobacter* sp.-derived biosurfactant yield

	A: Salinity (%)	B: pH	BY(ml)
1	15	7.20	2.097
2	15	8.75	1.712
3	15	9.80	1.736
4	15	10.52	1.989
5	20	7.20	1.977
6	20	8.75	1.302
7	20	9.80	1.703
8	20	10.52	1.908
9	25	7.20	1.707
10	25	8.75	1.839
11	25	9.80	1.710
12	25	10.52	2.007
13	30	7.20	1.776
14	30	8.75	1.704
15	30	9.80	1.696
16	30	10.52	2.184
17	35	7.20	1.661
18	35	8.75	1.722
19	35	9.80	1.673
20	35	10.52	1.970

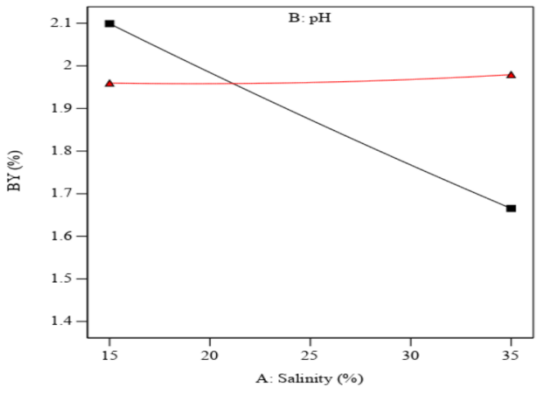


Figure 4: Interaction effects of pH and salinity on the capacity of the mutagenized *Acinetobacter* sp.-derived biosurfactant yield

Table 2: ANOVA of the developed model for the yield of the mutagenized *Acinetobacter* sp.-derived biosurfactant

	Sum of Squares	df	Mean Square	F-value	p-value	Remark
BY						
Model	0.2952	5	0.0590	64.54	< 0.0001	Significant
A-Salinity	0.0824	1	0.0824	90.06	< 0.0001	Significant
B-pH	0.0159	1	0.0159	17.40	0.0031	Significant
AB	0.0684	1	0.0684	74.77	< 0.0001	Significant
A^2	0.0002	1	0.0002	0.1913	0.6734	Significant
B^2	0.2113	1	0.2113	230.98	< 0.0001	Significant
Residual	0.0073	8	0.0009			
Cor Total	0.3026	13				
Cor Total	1051.55	19				

Similarly, there is a close agreement between experimental and predicted values since the difference between Adj. R^2 and Pred. R^2 is less than 0.2. Adequate precision is a comparison between the range of predicted values and the average prediction error. A signal-to-noise ratio larger than 4 validates that the model is adequate for navigating the design space. Modelling and simulation are essential for designing, analysing, and optimizing scientific and technical systems (Roy and Balch, 2012). The approximated experimental data is shown in Equation 3.

$$BY_{mutant} = 13.0491 - 0.0751487 * A - 2.3762 * B + 0.00683133 * AB + 8.55033e\text{-}05 * A^2 + 0.125942 * B^2 \quad (3)$$

Diagnostic Process via Parity Plots

Evaluation of model performance is a critical step in scientific research, especially in experimental design and data analysis. The normal probability plot, which indicates the distribution of the errors and the cross plot, which shows the discrepancy between experimental and predicted response values, are displayed as shown in Figure 5. The alignment of data points along the straight line on a normal probability plot confirmed that the model errors are normally distributed with a unified mean and standard deviation. Similarly, the closer the data points are to the 45 °C line on a parity plot indicates a minimum residual between the predicted and experimental values. As can be

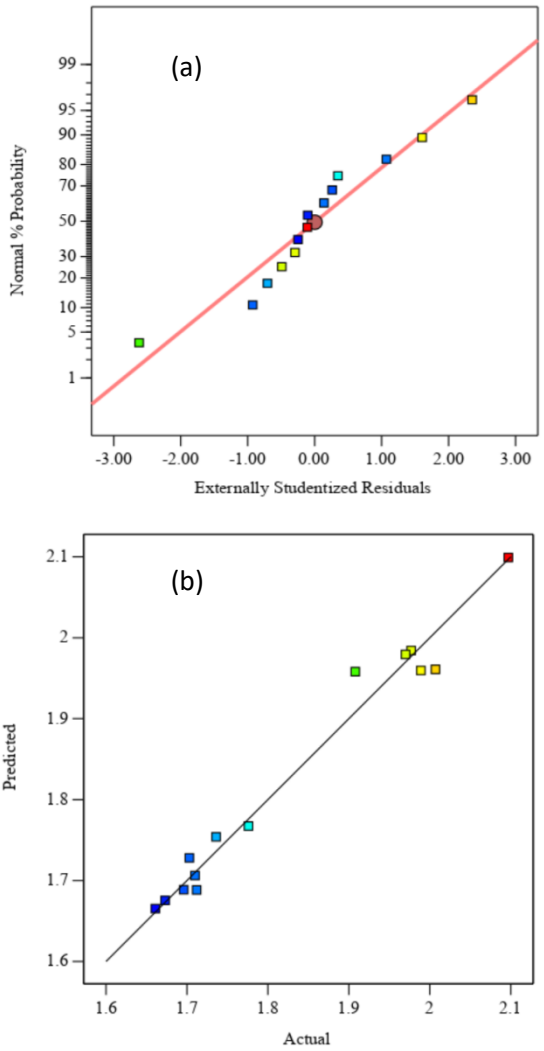


Figure 5. Diagnostic plots showing normal plots of residuals and cross plots of (a-b) BY.

observed, the residual points are dispersed equally around the line of equality, as are the actual

experimental and predicted values. This result showed that the developed models correctly capture the variability in the experimental data and are suitable for further use, especially to sample data during the optimization.

Numerical Multi-Objective Optimization

The analysis revealed that at the optimum condition (pH = 7.20 and salinity = 25.734%), the predicted BY is 1.858 nm. However, the confirmation results were obtained from experiments performed using the optimum values of the independent variables. The Percentage of Absolute Relative Error (PARE) obtained using Equation 4 is an indication of the reliability of the developed models. Thus, at the predicted optimum values of 1.86 ml at pH = 7.20 and salinity = 25.734%, corresponding experimental confirmation values of 1.79 ± 0.67 ml, with PARE of 3.91%, were obtained for the BY.

$$PARE = \left|1 - \frac{Simulated\ value}{Experimental\ value}\right| x\ 100 \quad (4)$$

CONCLUSION

The study revealed an optimized response surface methodology that established a pH of 7.20 and a salinity of 25.734% as the definitive parameters for maximum biosurfactant production. Under these optimum precise conditions, the mutant strain yielded a maximum biosurfactant volume of 1.858 ml, exhibiting optimized surface-active properties with an ODT of 7.985 mm, ST of 49.985 dyne/cm, IFT of 41.362 dyne/cm, and emulsification indexes of 20.533% (E_{24}), 11.955% (E_{48}), and 6.568% (E_{72}). Model validation confirmed high predictive accuracy and structural reliability, demonstrating a negligible Percentage of Absolute Relative Error (PARE) when cross-referenced with empirical experimental outputs.

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