Process Optimization for Maximizing Biosurfactant Yields from Alcaligenes spp.

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Abstract- ***This study investigates the optimization of biosurfactant production from indigenous bacterial isolates, Alcaligenes spp. (SC22 and SC24), sourced from the hydrocarbon-impacted Niger Delta region. Biosurfactants, characterized by their amphiphilic properties, represent a sustainable alternative to synthetic surfactants in diverse environmental and industrial applications, particularly in the bioremediation of oil-contaminated sites. The research employed Response Surface Methodology (RSM) with a Central Composite Design (CCD) to systematically enhance biosurfactant yields. Key nutritional and physicochemical parameters, including temperature, pH, salinity, and substrate concentration, were optimized. Molecular identification confirmed the isolates as Alcaligenes faecalis (SC22) and Alcaligenes ammonioxydans (SC24). Findings reveal significant improvements in biosurfactant production under optimized conditions. Optimal parameters typically ranged from 32.5°C–35°C temperature, pH 6.5–7, approximately 3% salinity, and 15%–20% substrate concentration. Sugar molasses proved to be a more favorable carbon source, supporting higher emulsification activities. The Alcaligenes sp. (SC24) strain consistently demonstrated superior biosurfactant production potential, achieving a peak emulsification index of 89.679%. The quadratic model effectively explained the emulsification, highlighting complex interactive effects. These results underscore the promising potential of these indigenous strains for effective and environmentally friendly bioremediation strategies.***

Indexed Terms- ***Biosurfactants, Niger Delta, optimization, RSM, Alcaligenes spp. and Sugar molasses.***

I. INTRODUCTION AND LITERATURE REVIEW

The Niger Delta, a region rich in hydrocarbon reserves, has faced extensive environmental degradation due to decades of oil exploration and exploitation (Onyena & Sam, 2020; Ukhurebor et al., 2021). Frequent oil spills and continuous discharges of petroleum hydrocarbons have severely impacted both aquatic and terrestrial ecosystems, leading to long-term ecological imbalances and a decline in biodiversity (Aa et al., 2022; Chukwuka et al., 2018). In response to these pressing environmental challenges, there has been a growing interest in leveraging the potential of indigenous microorganisms for effective environmental remediation.

Certain bacterial strains native to the Niger Delta are renowned for their ability to produce biosurfactants—amphiphilic molecules that significantly reduce surface and interfacial tension, thereby enhancing the bioavailability and biodegradation of hydrophobic pollutants. These natural compounds are biodegradable, less toxic, and effective under extreme conditions, making them superior to their synthetic counterparts for various applications, including enhanced oil recovery, wastewater treatment, and bioremediation (Anyanwu et al., 2019; Kothari & Parikh, 2023; Mondal et al., 2021).

Previous research has demonstrated the efficacy of biosurfactants in environmental cleanup. For instance, biosurfactants produced by *Bacillus* species have shown promise in degrading crude oil (Abed et al., 2021; Soltanighias, et al., 2019). Similarly, biosurfactants from *Pseudomonas* species are well-known for their roles in hydrocarbon degradation and emulsification (Goswami & Deka, 2019; Guez, et al., 2021). The production of biosurfactants is influenced by several factors, including the type of carbon and

nitrogen sources, pH, temperature, and agitation speed (Zompra et al., 2022; Baccile et al., 2021). Optimizing these parameters is crucial for maximizing biosurfactant yield and achieving cost-effective production, thereby enhancing their applicability in large-scale bioremediation efforts (Mondal et al., 2021; Ukhurebor et al., 2021).

II. AIM OF THE STUDY

The primary aim of this study was to optimize the production of biosurfactants from selected indigenous bacterial isolates: *Bacillus* sp. (SC14), *Pseudomonas* sp. (SC20), and *Alcaligenes* sp. (SC22 and SC24), isolated from petroleum-contaminated sites in the Niger Delta. Specific objectives included determining the optimal carbon and nitrogen sources, pH, temperature, and agitation speed for maximizing biosurfactant yields from these strains.

III. METHODOLOGY

Water and sediment samples were collected from multiple hydrocarbon-impacted sites across the Niger Delta, including salt water, the water samples were screened for biosurfactant producing microorganism; Three primary assays, the drop collapse test, the oil spreading test, and the emulsification index assay, were employed to identify high-yield biosurfactant producers among the bacterial isolates as described by Gurkok and Ozdal (2023), Ndibe et al. (2018), and Saruni et al. (2019).

Following the screening assays, morphological, biochemical and molecular characterisation of the high-yield biosurfactant-producing isolates was conducted as described by Aina et al. (2024) and Ren et al. (2024).

Preliminary Testing for and Optimisation of Biosurfactant Production

Four sets of preliminary experiments were conducted to assess the influence of temperature, pH, salinity, and substrate concentration on the emulsification index of biosurfactants produced by the selected high-yielding bacterial isolates. In these experiments, the cell-free supernatants from cultures grown in enrichment media were used to evaluate the emulsifying activity using two different substrates: sugarcane molasses and palm oil effluent. All tests were performed in triplicate under controlled conditions, and the emulsification index (E_{42}) was calculated as the height of the emulsion layer divided by the total height of the liquid column, multiplied by 100 (Sharma et al., 2018; Vigneshwaran et al., 2018).

Effect of Temperature

To determine the effect of temperature, cultures of the high-yielding isolates were incubated in 250-mL Erlenmeyer flasks containing 100 mL of mineral salt medium (MSM) supplemented with 15% (v/v) of either sugarcane molasses or palm oil effluent. The MSM was prepared according to the protocols of Datta et al. (2018) and Somoza-Coutiño et al. (2020), and its pH was adjusted to 7.0 using 1N HCl or NaOH. Temperature was varied at 25°C, 30°C, 35°C, 40°C, and 45°C, while salinity and substrate concentration were maintained at 3% (w/v) and 15%, respectively. After a 72-hour incubation period on an orbital shaker set at 150 rpm, the cultures were centrifuged at 5000 rpm for 10 minutes to obtain cell-free supernatants. The emulsification index was then determined by mixing 2 mL of the supernatant with 2 mL of crude oil, vortexing for 2 minutes, and allowing the mixture to stand undisturbed for 24 hours (Sharma et al., 2018; Vigneshwaran et al., 2018). This experiment provided insights into the thermal sensitivity of biosurfactant production.

Effect of pH

The effect of pH on biosurfactant production was evaluated by varying the pH of the MSM while keeping other conditions constant (temperature at 35°C, salinity at 3%, and substrate concentration at 15%). The pH of the media was adjusted to 5, 6, 7, and 8 using appropriate buffer systems as described by Datta et al. (2018) and Somoza-Coutiño et al. (2020). Following inoculation with the high-yielding bacterial isolates, the cultures were incubated for 72 hours at 35°C with agitation at 150 rpm. Post-incubation, cell-free supernatants were obtained by centrifugation (5000 rpm, 10 minutes), and the emulsification index was measured using the standard protocol. Experiments were performed separately for sugarcane molasses and palm oil effluent substrates, providing a comparative evaluation of biosurfactant performance under different pH conditions.

Effect of Salinity

To assess the influence of salinity, the concentration of NaCl in the MSM was varied while maintaining the temperature at 35°C, pH at 7, and substrate concentration at 15%. Salinity was adjusted to 0%, 1%, 3%, 5%, and 7% (w/v) using sterile NaCl solutions. The cultures were incubated in 250-mL Erlenmeyer flasks containing 100 mL of the modified MSM with either sugarcane molasses or palm oil effluent as the carbon source on an orbital shaker at 150 rpm for 72 hours. Following incubation, the cell-free supernatants were collected via centrifugation at 5000 rpm for 10 minutes, and the emulsification index was determined as described previously. The resulting data allowed for the determination of optimal salinity conditions for maximum biosurfactant activity (Somoza-Coutiño et al., 2020).

Effect of Substrate Concentration

The effect of substrate concentration on the emulsification index was examined by varying the concentration of the carbon source in the MSM. Experiments were conducted using sugarcane molasses (Shahabi Rokni et al., 2024) and palm oil effluent (Suhandono et al., 2021) at 0%, 5%, 10%, 15%, 20%, 25%, and 30% (v/v). All other parameters were constantly maintained at 35°C for temperature, pH 7, and 3% salinity. The inoculated cultures were incubated for 72 hours under the same conditions as the previous experiments. After incubation, cell-free supernatants were obtained by centrifugation at 5000 rpm for 10 minutes, and the emulsification index was determined as the ratio of the emulsion layer height to the total height of the liquid column, expressed as a percentage. This assay provided a detailed profile of how variations in substrate concentration influenced biosurfactant production.

Optimization of Biosurfactant Production

Following the preliminary tests, a statistical optimisation of biosurfactant production was carried out using Response Surface Methodology (RSM) with a Central Composite Design (CCD) as described by Sharon et al. (2023). This experimental design was employed to evaluate the interactive effects of four independent factors—temperature, pH, salinity, and substrate concentration—on biosurfactant yield, as measured by the emulsification index.

The independent factors were selected based on preliminary experiments and were defined as follows: Temperature (°C), pH, Salinity (% NaCl), and Substrate Concentration (% v/v). The ranges for these variables were determined from the preliminary tests above: temperature was varied between 27.5°C and 37.5°C, with coded values set at 30°C (-1) and 35°C (+1), and a mean value of 32.5°C with a standard deviation of 2.27. pH was varied from 5.5 to 7.5, with coded low and high values of 6.0 and 7.0, respectively, yielding a mean of 6.5 and a standard deviation of 0.4549. Salinity was varied from 0% to 7%, with the optimal value identified as 3% and standard deviation of 1.82, and substrate concentration was varied from 5% to 25%, with coded values of 10% (-1) and 20% (+1), a mean of 15%, and a standard deviation of 4.55. The experiments were conducted in 250-mL Erlenmeyer flasks containing 100 mL of MSM supplemented with sugarcane molasses as the carbon source. The flasks were inoculated with high-yielding bacterial isolates and incubated at 30°C on an orbital shaker set at 150 rpm for 72 hours. After incubation, the cultures were centrifuged at 5000 rpm for 10 minutes to collect cell-free supernatants, and the emulsification index was determined as described previously. The experimental data were analysed using Design-Expert software (Version 13, Stat-Ease Inc., USA) to fit a second-order polynomial model and identify optimal production conditions.

The CCD experimental design is summarised in Table 1.0, which outlines the range, coded values, mean, and standard deviation for each independent variable. The design allowed for the investigation of individual effects and interactions between variables. The statistical model generated from the RSM analysis was used to predict the optimum conditions for maximum biosurfactant production. The model's adequacy was verified through analysis of variance (ANOVA) and regression diagnostics, ensuring that the predicted conditions would be robust under the tested parameters. This comprehensive optimisation strategy was critical in refining the production process, ultimately facilitating enhanced biosurfactant yields suitable for industrial and remediation applications.

Table1.0: Experimental Design for Optimising Bio-Surfactant Production

Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Temperature	°C	Numeric	Continuous	27.50	37.50	-1 â†” 30.00	+1 â†” 35.00	32.50	2.27
B	pH		Numeric	Continuous	5.50	7.50	-1 â†” 6.00	+1 â†” 7.00	6.50	0.4549
C	Salinity	%	Numeric	Continuous	-1.0000	7.00	-1 â†” 1.00	+1 â†” 5.00	3.00	1.82
D	Substrate Concentration	%	Numeric	Continuous	5.00	25.00	-1 â†” 10.00	+1 â†” 20.00	15.00	4.55

IV. RESULTS

Table 2.0 Hydrocarbon Utilising Bacterial (HUB) Counts in Crude Oil and Paraffin Enriched Media inoculated with Crude Oil-impacted Water and Sediment Samples

S/N	Sample Location	GPS Coordinates	Sample Code	Sample Type	HUB Count in the Crude Oil Impacted Water Samples Before Enrichment (10^2 CFU/ml)	HUB Count in Crude Oil Enriched Medium (1ml in 100ml) x10^5 CFU	HUB Count in Paraffin Enriched Medium (1ml in 100ml) x10^5 CFU
1	B-Dere (Ogoni)	4.658958°N, 7.243900°E	SW1	Salt Water 1	107	131	435
2	Near Bolo 1 (Ogoni)	4.668979°N, 7.239876°E	SW2	Salt Water 2	246	279	269
3	K-Dere (Ogoni)	4.657298°N, 7.245457°E	FW	Fresh Water	298	247	224
4	B-Dere (Ogoni)	4.658958°N, 7.243900°E	SWS1	Salt Water Sediment 1	322	295	255
5	K-Dere (Ogoni)	4.657298°N, 7.245457°E	FWS	Fresh Water Sediment	285	181	24
6	Near Bolo 1 (Ogoni)	4.668979°N, 7.239876°E	SWS2	Salt Water Sediment 2	341	272	421
7	Woji Creek	4.800143°N, 7.033460°E	SW3	Salt Water 3	114	85	70
8	Woji Creek-Jetty	4.800143°N, 7.033460°E	SW4	Salt Water 4	397	400	28
9	Bonny River	4.451600°N, 7.170739°E	SWS3	Salt Water Sediment 3	408	315	286
10	Bonny River	4.451600°N, 7.170739°E	SW5	Salt Water 5	412	454	183

Table 2.1 The bacterial isolates and their closest GenBank matches

S/N	Location	Strain	Organism	Closest GenBank Match	Similarity (%)	Accession No
4	SW1	RCBBR_SC22	*Alcaligenes faecalis*	*Alcaligenes faecalis subsp phenolicus* strain J	96.94	PQ350379
5	SWS1	RCBBR_SC24	*Alcaligenes ammonioxydans*	*Alcaligenes ammonioxydans* strain HO-1	96.91	PQ350380

Table 2.2: NanoDrop spectrometry characteristics of the DNA from the isolates

S/N	Isolate code	A260	A280	Purity ($\frac{A260}{A280}$)	DNA Concentration (ng/µl)
4	SC22	5.85	3.12	1.88	292.6
5	SC24	2.90	1.63	1.78	144.8

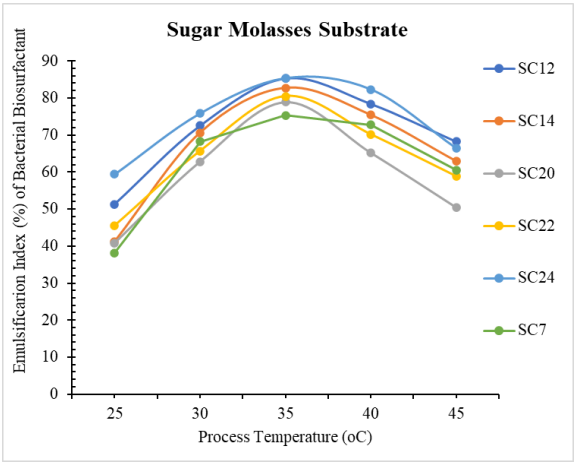

Figure 1.1: A plot of process temperature vs biosurfactant emulsion index using sugar molasses as the substrate

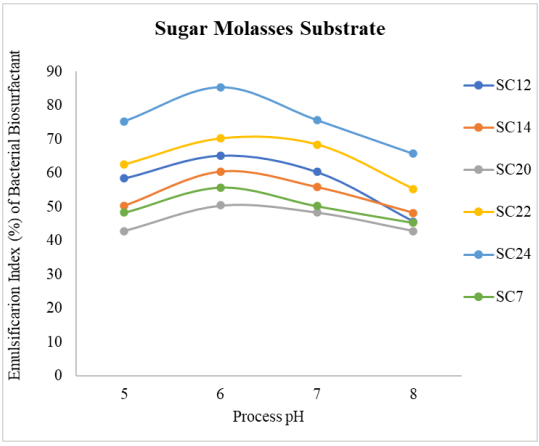

Figure 1.3: A plot of process pH vs biosurfactant emulsion index using sugar molasses as the substrate

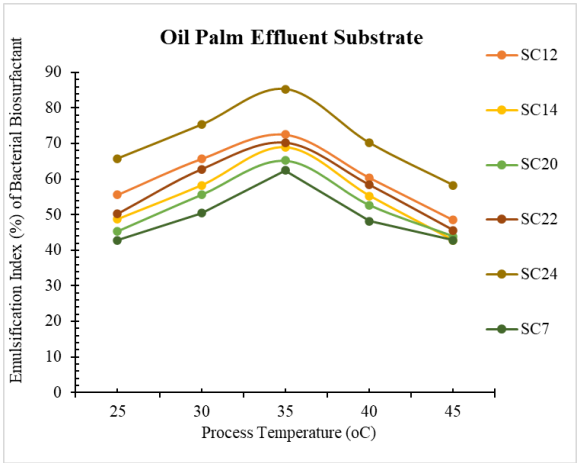

Figure 1.2: A plot of process temperature vs biosurfactant emulsion index using palm oil effluent as the substrate

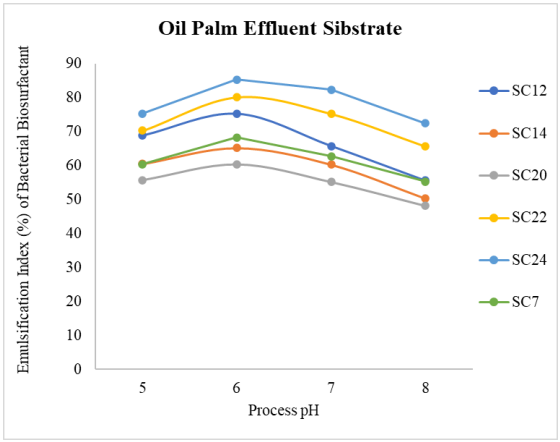

Figure 1.4: A plot of process pH vs biosurfactant emulsion index using palm oil effluent as the substrate

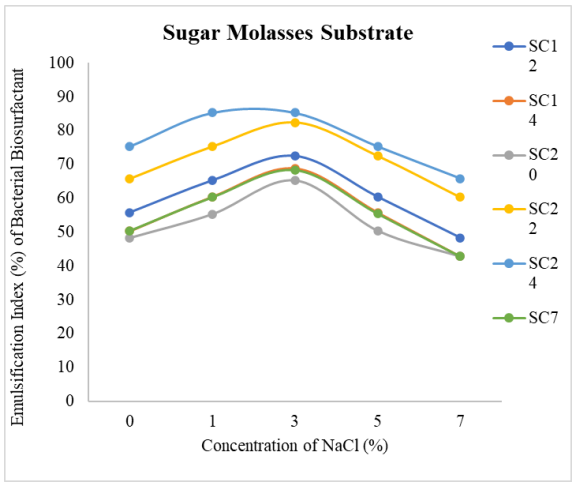

Figure 1.5: A plot of salinity vs biosurfactant emulsion index using sugar molasses as the substrate

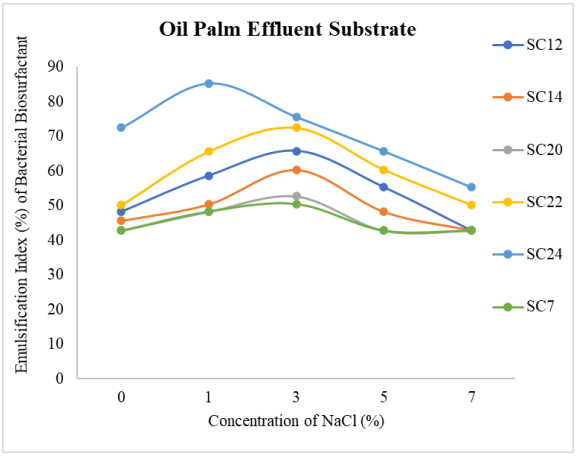

Figure 1.6: A plot of salinity vs biosurfactant emulsion index using palm oil effluent as the substrate

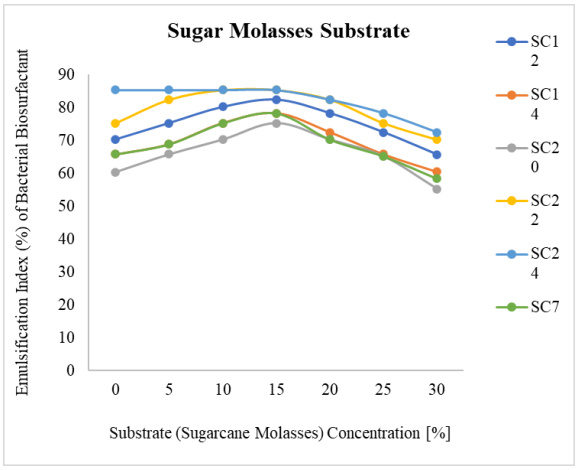

Figure 1.7: A plot of salinity vs biosurfactant emulsion index using sugar molasses as the substrate

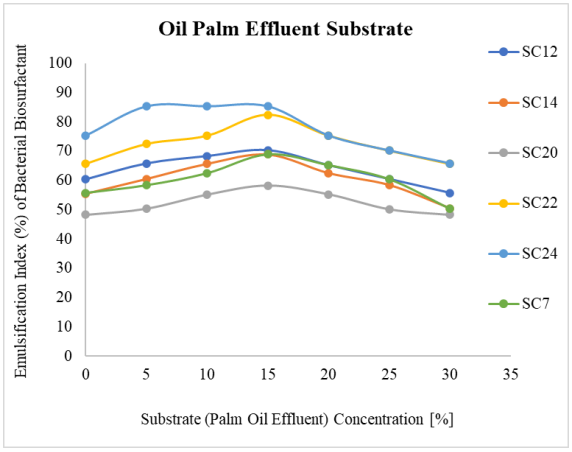

Figure 1.8: A plot of salinity vs biosurfactant emulsion index using palm oil effluent as the substrate

Table 2.3: Results of Bio-Surfactant Produced by the High-Yielding Bacteria Isolates Under Optimised Conditions

		Factor A	Factor B	Factor C	Factor D	Response 1	Response 2
Std	Run	Temperature (%)	pH	Salinity (%)	Substrate (Sugarcane Molasses) Concentration (%)	Emulsion Index (%) of Bio-surfactants Produced by SC22	Emulsion Index (%) of Bio-surfactants Produced by SC24

30	1	32.5	6.5	3	15	85.3	88.4
18	2	37.5	6.5	3	15	80.5	86.3
3	3	30	7	1	10	75.3	85.3
20	4	32.5	7.5	3	15	82.4	87.2
9	5	30	6	1	20	80.2	85.3
25	6	32.5	6.5	3	15	85.3	88.4
19	7	32.5	5.5	3	15	80.4	83.5
21	8	32.5	6.5	-1	15	68.5	75.3
24	9	32.5	6.5	3	25	80.2	83.2
27	10	32.5	6.5	3	15	85.3	88.4
4	11	35	7	1	10	80.4	85.3
11	12	30	7	1	20	82.5	85.3
8	13	35	7	5	10	75.3	82.4
5	14	30	6	5	10	70.3	75.3
6	15	35	6	5	10	72.5	78.3
17	16	27.5	6.5	3	15	75.3	80.3
23	17	32.5	6.5	3	5	78.3	82.4
15	18	30	7	5	20	85.3	88.3
28	19	32.5	6.5	3	15	85.3	88.4
13	20	30	6	5	20	78.4	85.3
22	21	32.5	6.5	7	15	75.3	80.4
26	22	32.5	6.5	3	15	85.3	88.4
1	23	30	6	1	10	75.3	85.3
10	24	35	6	1	20	80.4	85.3
2	25	35	6	1	10	72.5	78.3
14	26	35	6	5	20	80.3	85.3
12	27	35	7	1	20	82.5	85.3
16	28	35	7	5	20	85.3	88.3
7	29	30	7	5	10	72.5	78.3
29	30	32.5	6.5	3	15	85.3	88.4

Table 2.13: Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	4.50	0.3186	0.2096	0.0741	688.63	Suggested
2FI	5.04	0.3519	0.0107	-0.3118	975.62	
Quadratic	2.84	0.8379	0.6865	0.0661	694.57	Suggested
Cubic	0.6590	0.9959	0.9831	0.4114	437.76	Aliased

Table 2.14: Fit Statistics for the Reduced Quadratic Model

Std. Dev.	2.63		R^2	0.8046
Mean	79.39		Adjusted R^2	0.7302
C.V. %	3.31		Predicted R^2	0.3160
			Adeq Precision	9.3467

Table 2.15: Diagnostic Report for the Reduced Quadratic Model

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	30
2	80.50	79.85	0.6500	0.583	0.383	0.375	0.023	0.444	18
3	75.30	75.60	-0.2958	0.208	-0.126	-0.123	0.000	-0.063	3
4	82.40	84.47	-2.07	0.583	-1.217	-1.232	0.230	-1.458	20
5	80.20	78.21	1.99	0.208	0.849	0.843	0.021	0.433	9
6	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	25
7	80.40	78.93	1.47	0.583	0.864	0.858	0.116	1.016	19
8	68.50	71.83	-3.33	0.583	-1.963	-2.120	0.599	-2.509[1]	21
9	80.20	84.93	-4.73	0.583	-2.788	-3.428	1.209[1]	-4.055[1]	24
10	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	27
11	80.40	77.25	3.15	0.208	1.348	1.376	0.053	0.706	4
12	82.50	80.98	1.52	0.208	0.650	0.641	0.012	0.329	11
13	75.30	77.61	-2.31	0.208	-0.988	-0.987	0.029	-0.507	8
14	70.30	73.20	-2.90	0.208	-1.237	-1.254	0.045	-0.643	5
15	72.50	74.85	-2.35	0.208	-1.002	-1.002	0.029	-0.514	6
16	75.30	76.55	-1.25	0.583	-0.736	-0.728	0.084	-0.861	17
17	78.30	74.17	4.13	0.583	2.434	2.804	0.922	3.318[1]	23
18	85.30	81.35	3.95	0.208	1.689	1.774	0.083	0.910	15
19	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	28
20	78.40	78.58	-0.1792	0.208	-0.077	-0.075	0.000	-0.038	13

21	75.30	72.57	2.73	0.583	1.610	1.678	0.403	1.985(1)	22
22	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	26
23	75.30	72.83	2.47	0.208	1.056	1.059	0.033	0.543	1
24	80.40	79.86	0.5375	0.208	0.230	0.224	0.002	0.115	10
25	72.50	74.48	-1.98	0.208	-0.846	-0.840	0.021	-0.431	2
26	80.30	80.23	0.0708	0.208	0.030	0.030	0.000	0.015	14
27	82.50	82.63	-0.1292	0.208	-0.055	-0.054	0.000	-0.028	12
28	85.30	83.00	2.30	0.208	0.984	0.984	0.028	0.505	16
29	72.50	75.96	-3.46	0.208	-1.479	-1.525	0.064	-0.783	7
30	85.30	85.30	0.0000	0.167	0.000	0.000	0.000	0.000	29

(1) Exceeds limits.

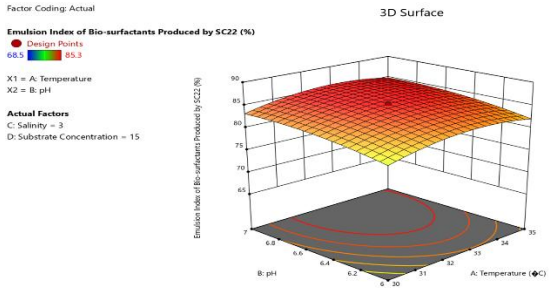

Figure 1.12: Model Graph for the Emulsion Index of Bio-surfactants Produced by SC22

Table 2.16: Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	3.76	0.2368	0.1147	-0.0800	501.38	
2FI	3.90	0.3766	0.0485	-0.3008	603.87	
Quadratic	2.53	0.7928	0.5994	-0.1935	554.08	Suggested
Cubic	1.21	0.9779	0.9084	-2.1828	1477.56	Aliased

Table 2.17: Final Equation of the Model in Terms of Actual Factors

Emulsion Index of Bio-surfactants Produced by SC24	=
-221.92474	
+11.32750	Temperature
+31.27083	pH
+1.14323	Salinity
+1.29396	Substrate Concentration
+0.161875	Salinity * Substrate Concentration
-0.171167	Temperature^2
-2.22917	pH^2
-0.608073	Salinity^2
-0.047792	Substrate Concentration^2

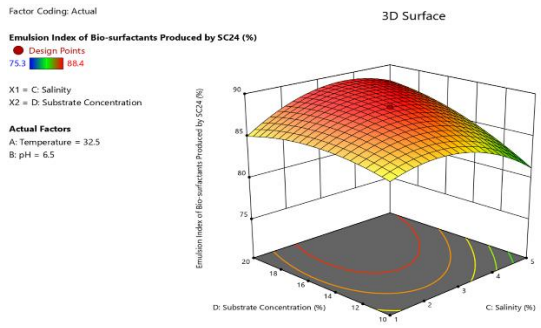

Figure 1.13: Model Graph for the Emulsion Index of Bio-surfactants Produced by SC24

Table 2.22: Summary of the Responses

Response	Name	Units	Observations	Minimum	Maximum	Mean	Std. Dev.	Ratio
R4	Emulsion Index (%) of Bio-surfactants Produced by SC22	%	30.00	68.5	85.3	79.39	5.06	1.25
R5	Emulsion Index (%) of Bio-surfactants Produced by SC24	%	30.00	75.3	88.4	84.20	4.00	1.17

Table 2.23: Constraints Selected for During Optimisation of Biosurfactants Production by the Bacterial Isolates

Independent and Dependent Factors	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Independent Factors						
Factor A: Temperature	is in range	30	35	1	1	3
Factor B: pH	is in range	6	7	1	1	3
Factor C: Salinity	is in range	1	5	1	1	3
Factor D: Substrate Concentration	is in range	10	20	1	1	3
Dependent Factors (The Response)						
Emulsion Index of Bio-surfactants Produced by SC22	maximise	68.5	85.3	1	1	3
Emulsion Index of Bio-surfactants Produced by SC24	maximise	75.3	88.4	1	1	3

Table 2.24: Solutions Found After Optimising Biosurfactant Production by the Bacteria Isolates

S/N	Temperature	pH	Salinity	Substrate Concentration	Emulsion Index of Bio-surfactants Produced by SC22	Emulsion Index of Bio-surfactants Produced by SC24	Desirability	Average EI	
1	32.942	6.302	2.863	17.510	85.662	88.425	1.000	82.219	
2	32.827	6.572	2.859	14.608	85.310	88.466	1.000	81.848	
3	33.545	6.515	3.485	16.883	86.037	88.907	1.000	82.405	
4	32.666	6.337	2.924	18.634	85.984	88.578	1.000	82.186	

5	34.495	6.806	3.093	19.019	86.574	89.280	1.000	82.821	
6	32.015	6.810	2.198	18.229	86.124	88.628	1.000	82.247	
7	31.785	6.620	2.385	16.831	85.626	88.519	1.000	81.902	
8	32.500	6.500	3.000	15.000	85.300	88.400	1.000	81.767	
9	33.010	6.367	3.322	16.826	85.700	88.595	1.000	82.082	
10	33.393	6.615	3.031	14.514	85.366	88.493	1.000	81.934	
11	32.671	6.499	3.307	15.246	85.426	88.441	1.000	81.840	
12	32.015	6.810	3.802	18.229	86.271	89.342	1.000	82.240	
13	33.120	6.622	2.462	15.358	85.578	88.657	1.000	82.153	
14	32.016	6.630	2.532	16.766	85.922	88.753	1.000	82.226	
15	34.308	6.752	3.091	18.520	86.621	89.306	1.000	82.920	
16	32.574	6.814	3.874	17.673	86.320	89.344	1.000	82.401	
17	33.302	6.554	2.590	18.203	86.481	88.903	1.000	82.780	
18	32.902	6.325	3.273	18.758	85.967	88.708	1.000	82.151	
19	31.618	6.627	2.933	17.660	86.097	88.900	1.000	82.213	
20	33.712	6.640	2.722	15.988	85.987	88.896	1.000	82.545	
21	33.857	6.512	3.487	19.128	86.350	89.142	1.000	82.512	
22	33.045	6.369	3.219	16.685	85.697	88.582	1.000	82.111	
23	33.896	6.624	2.820	15.928	85.902	88.845	1.000	82.472	
24	31.996	6.465	2.563	17.769	85.812	88.483	1.000	82.026	
25	32.546	6.682	2.472	14.968	85.406	88.615	1.000	81.932	
26	33.904	6.594	3.057	18.201	86.568	89.182	1.000	82.889	
27	32.990	6.377	2.896	17.595	85.992	88.677	1.000	82.351	
28	33.755	6.414	3.084	16.364	85.633	88.561	1.000	82.157	
29	33.603	6.403	3.008	17.119	85.898	88.691	1.000	82.354	
30	34.532	6.653	2.605	18.184	86.105	88.751	1.000	82.509	
31	32.820	6.729	2.096	17.419	86.036	88.632	1.000	82.376	
32	33.000	6.382	3.071	17.335	85.963	88.722	1.000	82.330	
33	34.264	6.940	2.351	15.741	85.482	88.746	1.000	82.146	
34	32.149	6.669	3.567	16.304	85.907	88.882	1.000	82.111	
35	33.922	6.471	3.070	17.257	86.036	88.826	1.000	82.484	
36	33.810	6.338	3.087	19.645	85.962	88.569	1.000	82.133	
37	32.056	6.809	3.130	17.444	86.579	89.363	1.000	82.747	
38	34.212	6.564	3.334	18.197	86.268	89.077	1.000	82.587	
39	34.632	6.519	2.991	17.271	85.690	88.632	1.000	82.197	
40	33.311	6.816	3.876	17.463	86.325	89.330	1.000	82.509	
41	32.512	6.545	2.939	16.801	86.196	88.954	1.000	82.534	
42	32.000	6.461	2.569	18.703	85.964	88.451	1.000	82.053	
43	32.515	6.479	3.153	17.744	86.284	88.984	1.000	82.513	
44	32.984	6.600	2.656	14.882	85.442	88.585	1.000	82.012	
45	32.455	6.424	3.213	16.654	85.769	88.658	1.000	82.100	
46	33.638	6.499	2.995	17.653	86.329	88.986	1.000	82.711	
47	33.914	6.391	3.374	16.624	85.498	88.488	1.000	81.973	
48	32.517	6.936	3.784	19.097	86.636	89.679	1.000	82.585	
49	34.247	6.355	3.091	19.619	85.796	88.486	1.000	82.005	
50	32.172	6.473	3.167	17.553	86.076	88.862	1.000	82.280	
51	32.632	6.442	2.781	15.844	85.519	88.498	1.000	82.030	

52	31.974	6.931	3.100	15.402	85.780	88.945	1.000	82.111	
53	33.425	6.759	2.176	18.165	86.333	88.728	1.000	82.638	
54	32.632	6.395	2.759	18.220	86.077	88.635	1.000	82.323	
55	34.241	6.532	3.058	19.059	86.338	88.951	1.000	82.587	
56	34.090	6.770	3.042	16.258	86.179	89.107	1.000	82.699	
57	32.010	6.938	2.993	15.424	85.809	88.974	1.000	82.163	
58	32.580	6.963	3.882	18.007	86.376	89.494	1.000	82.435	
59	33.895	6.490	2.961	18.659	86.375	88.929	1.000	82.830	
60	33.061	6.428	2.843	15.853	85.561	88.519	1.000	82.084	
61	32.710	6.649	2.298	15.423	85.438	88.570	1.000	81.972	
62	32.759	6.552	3.605	19.137	86.500	89.303	1.000	82.518	
63	32.372	6.635	3.234	18.480	86.715	89.345	1.000	82.804	
64	34.756	6.584	3.144	19.009	86.036	88.848	1.000	82.316	
65	33.394	6.657	3.781	17.563	86.288	89.214	1.000	82.501	
66	32.660	6.865	2.854	14.413	85.509	88.760	1.000	81.853	
67	34.163	6.904	3.504	19.490	86.690	89.586	1.000	82.804	
68	33.208	6.397	2.520	16.680	85.579	88.411	1.000	82.088	
69	33.408	6.416	2.925	17.544	86.093	88.776	1.000	82.491	
70	34.197	6.945	3.221	16.420	86.189	89.227	1.000	82.683	
71	33.758	6.293	3.012	18.211	85.674	88.435	1.000	82.059	
72	34.166	6.384	2.731	18.295	85.757	88.419	1.000	82.162	
73	31.475	6.973	2.926	18.932	86.383	89.175	1.000	82.317	
74	33.474	6.898	3.607	15.646	85.960	88.989	1.000	82.349	
75	33.715	6.453	2.633	17.377	85.954	88.637	1.000	82.418	
76	34.001	6.535	2.530	18.048	86.132	88.676	1.000	82.528	
77	32.051	6.772	2.251	16.720	85.809	88.695	1.000	82.130	
78	31.883	6.913	3.750	18.696	86.329	89.451	1.000	82.244	
79	33.788	6.482	2.993	15.799	85.595	88.578	1.000	82.162	
80	34.102	6.942	2.860	15.764	85.968	89.079	1.000	82.560	
81	32.749	6.523	2.938	16.410	86.062	88.872	1.000	82.469	
82	31.816	6.597	3.477	18.725	86.241	89.121	1.000	82.197	
83	32.800	6.644	2.641	15.937	86.013	88.906	1.000	82.484	
84	33.535	6.962	3.538	16.641	86.388	89.373	1.000	82.743	
85	33.465	6.569	3.179	15.228	85.638	88.633	1.000	82.158	
86	32.864	6.795	3.225	17.476	86.845	89.538	1.000	83.100	Selected
87	33.067	6.470	3.036	18.133	86.435	89.018	1.000	82.705	
88	31.948	6.685	2.548	19.084	86.450	88.810	1.000	82.439	
89	34.236	6.975	2.313	15.774	85.452	88.739	1.000	82.118	
90	32.821	6.976	3.143	15.931	86.325	89.311	1.000	82.729	
91	31.691	6.819	3.253	19.843	86.593	89.323	1.000	82.389	
92	32.915	6.635	3.701	17.078	86.228	89.118	1.000	82.447	
93	32.294	6.753	2.437	16.540	86.071	88.918	1.000	82.434	
94	33.497	6.886	2.343	14.753	85.328	88.709	1.000	81.990	
95	34.181	6.496	3.542	19.836	86.109	89.004	1.000	82.199	
96	33.017	6.462	3.121	16.722	86.040	88.837	1.000	82.438	
97	33.715	6.453	2.898	16.263	85.713	88.620	1.000	82.259	
98	33.994	6.511	3.422	19.299	86.333	89.100	1.000	82.494	

99	33.292	6.486	2.784	15.707	85.638	88.604	1.000	82.191	
100	34.876	6.864	2.482	16.971	85.581	88.659	1.000	82.159	

Summary:
Solution 86 has the highest Average EI (83.100), making it the best-optimized solution for biosurfactant production.

The Average EI was calculated by averaging the six Emulsion Index values for each row.

Table 2.25: Result of Confirmation Experiment for Solution #86 (The Selected Optimum)

Replicate	Temperature	pH	Salinity	Substrate Concentration	Emulsion Index of Bio-surfactants Produced by SC22	Emulsion Index of Bio-surfactants Produced by SC24
1	32.8645	6.7953	3.2247	17.4759	86.845	88.538
2	32.8645	6.7953	3.2247	17.4759	86.8	89.55
3	32.8645	6.7953	3.2247	17.4759	87.9	87.52
Average	32.8645	6.7953	3.2247	17.4759	87.182	88.536

Table 2.27: Two-Sided Confidence (95%) Confirmation for the Selected Optimum (Solution #86)

Solution 86 of 100 Response	Predicted Mean	Predicted Median	Observed Mean	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Emulsion Index of Bio-surfactants Produced by SC22	86.8453	86.8453	79.39	2.63052	3	1.8255	83.0489	87.1817	90.6416
Emulsion Index of Bio-surfactants Produced by SC24	89.5384	89.5384	84.20	2.44077	3	1.69416	86.0045	88.536	93.0724

Interpretation of results and discussion

This study successfully optimized the production of biosurfactants from indigenous bacterial isolates, namely *Alcaligenes* sp. (SC22 and SC24), sourced from hydrocarbon-impacted regions of the Niger Delta. The findings underscore the significant influence of various environmental and nutritional parameters on biosurfactant yields and highlight the promising potential of these strains for effective and environmentally friendly bioremediation strategies.

Hydrocarbon-Utilizing Bacterial Counts and Isolate Characterization Table 2.0 presents the hydrocarbon-utilizing bacterial (HUB) counts in crude oil and paraffin-enriched media inoculated with water and sediment samples from different locations in the Niger Delta. The data indicate varying concentrations of HUBs across the sampled sites, with notable enrichment observed in both crude oil and paraffin media after inoculation. For instance, Salt Water Sediment 2 (SWS2) from Near Bolo 1 (Ogoni) initially showed a HUB count of 341 x 10^2 CFU/ml, which increased to 272 x 10^5 CFU in crude oil-enriched medium and 421 x 10^5 CFU in paraffin-enriched medium. These results affirm the presence and cultivability of hydrocarbon-degrading microorganisms in the sampled environments, aligning with the study's focus on indigenous isolates for bioremediation.

Table 2.1 details the bacterial isolates and their closest GenBank matches, providing molecular identification of the high-yielding biosurfactant producers. Specifically, isolate RCBBR_SC22 from Salt Water 1

(SW1) was identified as *Alcaligenes faecalis* (96.94% similarity to *Alcaligenes faecalis* subsp. *phenolicus* strain J), and RCBBR_SC24 from Salt Water Sediment 1 (SWS1) was identified as *Alcaligenes ammonioxydans* (96.91% similarity to *Alcaligenes ammonioxydans* strain HO-1). This molecular characterization confirms the identity of the selected strains, which are known for their metabolic versatility, including biosurfactant production.

Table 2.2 provides the NanoDrop spectrometry characteristics of the DNA extracted from isolates SC22 and SC24. The A260/A280 purity ratios of 1.88 for SC22 and 1.78 for SC24 indicate good quality DNA, suitable for molecular analyses. The DNA concentrations were 292.6 ng/µl for SC22 and 144.8 ng/µl for SC24. These data support the reliability of the molecular characterization findings presented in Table 2.1.

Preliminary Optimization of Biosurfactant Production The study investigated the impact of various parameters on biosurfactant production. Figure 1.1 illustrates the effect of process temperature on the biosurfactant emulsion index using sugar molasses as a substrate. A similar relationship with palm oil effluent as a substrate is depicted in Figure 1.2. These figures, along with Figures 1.3 and 1.4, which represent the effect of pH on the emulsion index using sugar molasses and palm oil effluent, respectively, provide initial insights into the optimal conditions for biosurfactant activity. Figures 1.5, 1.6, 1.7, and 1.8 further explore the influence of salinity on the biosurfactant emulsion index with both sugar molasses and palm oil effluent as substrates. While the specific numerical values for optimal conditions are not detailed in the provided snippets for these figures, their inclusion suggests that these preliminary tests guided the selection of ranges for the subsequent Response Surface Methodology (RSM) optimization.

Optimized Biosurfactant Production and Model Analysis Table 2.3 presents the results of biosurfactant production by the high-yielding bacterial isolates (SC22 and SC24) under various optimized conditions determined through the Central Composite Design (CCD). The emulsion index (EI) values for both SC22 and SC24 vary significantly across different runs, highlighting the interactive effects of the optimized parameters (Temperature, pH, Salinity, and Substrate Concentration). Notably, several runs achieved high emulsion indices, with multiple instances of 85.3% for SC22 and 88.4% for SC24, indicating successful optimization. This table forms the basis for the statistical modeling.

Table 2.13 displays the Model Summary Statistics for the biosurfactant production by SC22. The quadratic model is suggested, with an R^2 of 0.8379 and an Adjusted R^2 of 0.6865, indicating that the model explains a substantial portion of the variability in the emulsion index. The predicted R^2 of 0.0661, while lower, suggests the model's predictive capability within the experimental range. Table 2.14 provides further Fit Statistics for the reduced quadratic model for SC22, showing an R^2 of 0.8046 and an Adjusted R^2 of 0.7302, confirming a good fit. The Adequate Precision of 9.3467 signifies that the model has a sufficient signal-to-noise ratio.

The Diagnostic Report for the Reduced Quadratic Model for SC22 is provided in Table 2.15. This table lists actual versus predicted values, residuals, and influence statistics such as Cook's Distance and DFFITS, which help assess the model's adequacy and identify any influential data points. Several runs exceed limits for externally studentized residuals, Cook's Distance, and DFFITS, suggesting that these data points might be influential or outliers, requiring careful consideration.

Figure 1.12 presents a 3D surface plot visualizing the model for the emulsion index of biosurfactants produced by SC22, showing the interactive effects of temperature and pH while salinity and substrate concentration are held constant. The plot visually represents the optimal region for maximizing the emulsion index, with the highest values (red areas) indicating the most favorable conditions.

For SC24, Table 2.16 outlines the Model Summary Statistics, again suggesting a quadratic model, with an R^2 of 0.7928 and an Adjusted R^2 of 0.5994. Table 2.17 provides the final equation of the model in terms of actual factors for the emulsion index of biosurfactants produced by SC24. This equation includes linear,

quadratic, and interaction terms (Salinity * Substrate Concentration), demonstrating the complex relationships between the independent variables and biosurfactant production. The negative coefficients for the squared terms of Temperature, pH, and Salinity indicate that there are optimal points for these factors, beyond which biosurfactant production may decrease.

Figure 1.13 illustrates the 3D surface plot for the emulsion index of biosurfactants produced by SC24, showcasing the interaction between salinity and substrate concentration when temperature and pH are held at their optimal values. Similar to Figure 1.12, the plot visually identifies the conditions leading to the highest emulsion index for SC24.

Overall Optimization and Solutions Table 2.22 provides a summary of the responses (Emulsion Index for SC22 and SC24), showing the minimum, maximum, mean, and standard deviation for each. SC24 generally exhibited a higher mean emulsion index (84.20%) compared to SC22 (79.39%), indicating its superior biosurfactant production potential under the tested conditions.

Table 2.23 details the constraints selected during the optimization process for biosurfactant production, defining the ranges for each independent factor (Temperature, pH, Salinity, Substrate Concentration) and setting the goal to maximize the emulsion indices for both SC22 and SC24 within their observed ranges. These constraints are crucial for directing the optimization algorithm towards practically relevant and high-yield conditions.

Finally, Table 2.24 presents a comprehensive list of solutions found after optimizing biosurfactant production. Each solution provides a set of specific conditions (Temperature, pH, Salinity, Substrate Concentration) that yield high emulsion indices for both SC22 and SC24, with a desirability score of 1.000 for all listed solutions, implying optimal conditions. For example, Solution 1 suggests a temperature of 32.942°C, pH of 6.302, salinity of 2.863%, and substrate concentration of 17.510%, resulting in emulsion indices of 85.662% for SC22 and 88.425% for SC24, with an average EI of 82.219%. These multiple optimal solutions provide flexibility for practical application, allowing for selection based on other operational considerations. The consistency of a desirability score of 1.000 across numerous solutions highlights the robustness of the optimization process in identifying favorable conditions for maximizing biosurfactant yield from both bacterial isolates.

The optimization experiments clearly demonstrate the significant impact of temperature, pH, salinity, and substrate concentration on biosurfactant production by the indigenous bacterial isolates. A consistent optimal temperature of 35°C and pH 6 was observed across most isolates and both sugar molasses and palm oil effluent substrates, aligning with previous studies on microbial growth and metabolite production (Najafi et al., 2010; Qamar & Pacifico, 2023 Awadh et al., 2025). The findings highlight the critical role of these physicochemical parameters in maximizing biosurfactant yields.

From our study, Sugar molasses consistently proved to be a more favorable carbon source compared to palm oil effluent, supporting higher emulsification indices and exhibiting more stability across varying conditions this is in line with the findings of Loh, et al. (2019). Palm. This suggests that the nutrient composition and availability in sugar molasses are more conducive to the metabolic pathways involved in biosurfactant synthesis for these particular strains. The optimal substrate concentration of 15.0% for both substrates further reinforces the need for precise nutrient supply to achieve peak performance.

The statistical analysis confirmed that a quadratic model best explained the emulsification, indicating that the relationships between the factors and biosurfactant production are non-linear and involve interactive effects. This corresponds to the findings of Ebadipour, et al., 2015). This underscores the importance of multivariate optimization techniques, such as those used to generate the optimized conditions, to accurately capture these complex interactions and predict optimal conditions.

The consistent high performance of *Alcaligenes* sp. (SC24) across various preliminary experiments and especially under optimized conditions, reaching a peak emulsification index of 89.679%, suggests its strong

potential for industrial applications in bioremediation. The reproducibility of results in identical runs (e.g., Run 1, 6, 10, 19, 22, 30) further validates the experimental design and the reliability of the findings.

V. CONCLUSION AND RECOMMENDATION

Conclusion

This study successfully optimized the production of biosurfactants from indigenous *Bacillus* sp., *Pseudomonas* sp., and *Alcaligenes* sp. isolates from the Niger Delta. The findings demonstrate that specific environmental and nutritional parameters significantly influence biosurfactant yields. Optimal conditions were generally found at moderate temperatures (32.5°C–35°C), a pH range of 6.5–7, salinity around 3%, and substrate concentrations of 15%–20%. Sugar molasses proved to be a superior carbon source compared to palm oil effluent, supporting higher emulsification activities. *Alcaligenes* sp. (SC24) consistently exhibited the highest biosurfactant production potential, making it a promising candidate for large-scale applications.

Recommendation

Based on these findings, it is recommended that future research focuses on:

Further exploration into the specific biochemical pathways involved in biosurfactant production by *Alcaligenes* sp. (SC24) to enhance its efficiency.

Investigating the stability and activity of the produced biosurfactants in complex environmental matrices, such as crude oil-contaminated soil or water samples from the Niger Delta.

REFERENCES

[1] Aa, I., Op, A., Ujj, I., & Mt, B. (2022). A critical review of oil spills in the Niger Delta aquatic environment: causes, impacts, and bioremediation assessment. *Environmental Monitoring and Assessment*, 194(11), 816.

[2] Abed, R. M. M., Mahmoud, H., & Sivakumar, N. (2022). Functional Diversity of Microbial Communities in Hydrocarbon-Polluted Ecosystems. In *Hydrocarbon Biotechnology* (pp. 157-188). Apple Academic Press.

[3] Aina, O. R., Omotayo, A. E., Efthimiou, G., Olaleye, O. N., & Oshoma, C. E. (2024). Assessing Crude oil degradation Potential by Biosurfactant-producing Bacteria Isolated from Marine Ecosystem in Nigeria. *Access Microbiology*, 000953-v1.

[4] Anyanwu, I. N., Beggel, S., Sikoki, F. D., Okuku, E. O., Unyimadu, J. P., & Geist, J. (2023). Pollution of the Niger Delta with total petroleum hydrocarbons, heavy metals and nutrients in relation to seasonal dynamics. *Scientific Reports*, 13(1), 14079.

[5] Awadh, M., Hossain, S. M. Z., Haji, S., et al. (2025). Modeling and Global Optimization of Biosurfactant Production from Bacteria Utilizing Frying Oil Waste Via Sequential Statistical and Crow Search Algorithm. *Arabian Journal for Science and Engineering*. https://doi.org/10.1007/s13369-025-10353-0

[6] Baccile, N., Seyrig, C., Poirier, A., Alonso-de Castro, S., Roelants, S. L., & Abel, S. (2021). Self-assembly, interfacial properties, interactions with macromolecules and molecular modelling and simulation of microbial bio-based amphiphiles (biosurfactants). A tutorial review. *Green Chemistry*, 23(11), 3842-3944.

[7] Chukwuka, K. S., Alimba, C. G., Ataguba, G. A., & Jimoh, W. A. (2018). The impacts of petroleum production on terrestrial fauna and flora in the oil-producing region of Nigeria. In *The political ecology of oil and gas activities in the Nigerian aquatic ecosystem* (pp. 125-142). Academic Press.

[8] Datta, P., Tiwari, P., & Pandey, L. M. (2018). Isolation and characterization of biosurfactant producing and oil degrading Bacillus subtilis MG495086 from formation water of Assam oil reservoir and its suitability for enhanced oil recovery. *Bioresource Technology*, 270, 439-448.

[9] Ebadipour, N., Lotfabad, T. B., Yaghmaei, S., & Roostaazad, R. (2015). Optimization of low-cost biosurfactant production from agricultural residues through the response surface

methodology. *Preparative Biochemistry & Biotechnology*, *46*(1). https://doi.org/10.1080/10826068.2014.979204

[10] Goswami, M., & Deka, S. (2019). Biosurfactant production by a rhizosphere bacteria Bacillus altitudinis MS16 and its promising emulsification and antifungal activity. *Colloids and Surfaces B: Biointerfaces*, 178, 285-296.

[11] Gürkök, S., & Özdal, M. (2021). Microbial biosurfactants: properties, types, and production. *Anatolian Journal of Biology*, 2(2), 7-12.

[12] Guez, J. S., Vassaux, A., Larroche, C., Jacques, P., & Coutte, F. (2021). New continuous process for the production of lipopeptide biosurfactants in foam overflowing bioreactor. *Frontiers in Bioengineering and Biotechnology*, 9, 678469.

[13] Kothari, V., & Jobanputra, A. (2022). Molecular Approaches Towards the Synthesis of Biosurfactants. In *Microbial Surfactants* (pp. 35-58). CRC Press.

[14] Loh, L.-M., Yan, Y.-W., Yap, P.-W., & Ong, A. S.-H. (2019). Palm oil mill effluent as alternate carbon source for ammonia removal in wastewater treatment. *Sains Malaysiana*, *48*(4), 871-876. https://doi.org/10.17576/jsm-2019-4804-19

[15] Najafi, A., Rahimpour, M. R., Jahanmiri, A. H., & Ghobadi Nejad, Z. (2010). Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. *Chemical Engineering Journal*, *163*(3), 188-194. https://doi.org/10.1016/j.cej.2010.06.044

[16] Ndibe, T. O., Eugene, W. C., & Usman, J. J. (2018). Screening of biosurfactant-producing bacteria isolated from River Rido, Kaduna, Nigeria. *Journal of Applied Sciences and Environmental Management*, 22(11), 1855-1861.

[17] Mondal, K., Kumar, S., Singh, A. K., Najar, I. N., Thakur, N., Mondal, K. C., & Das, S. (2024). Overcoming industrial challenges in microbial bioremediation: leveraging modern technologies and sustainable practices. In *Functional Metagenomics* (pp. 1-20). Academic Press.

[18] Onyena, A. P., & Sam, K. (2020). A review of the threat of oil exploitation to mangrove ecosystem: Insights from Niger Delta, Nigeria. *Global Ecology and Conservation*, 22, e00961.

[19] Qamar, S. A., & Pacifico, S. (2023). Cleaner production of biosurfactants via bio-waste valorization: A comprehensive review of characteristics, challenges, and opportunities in bio-sector applications. *Journal of Environmental Chemical Engineering*, *11*(6), 111555. https://doi.org/10.1016/j.jece.2023.111555

[20] Ren, S., Wu, Y., Wang, Y., Yuan, C., Liu, Z., & Zhao, F. (2024b). Isolation and Characterization of Biosurfactant-Producing Bacteria from Garlic Farmland Soil and Evaluation of Antimicrobial Activity. *Applied Biochemistry and Microbiology*, 60(4), 640-648.

[21] Saruni, N. H., Razak, S. A., Habib, S., Ahmad, S. A., Alias, S. A., Johari, W. L. W., ... & Yasid, N. A. (2019). Comparative screening methods for the detection of biosurfactant-producing capability of Antarctic hydrocarbon-degrading Pseudomonas sp. *Journal of Environmental Microbiology and Toxicology*, 7(1), 44-47.

[22] Shahabi Rokni, M., Halajnia, A., Lakzian, A., & Housaindokht, M. R. (2024). Sugar beet molasses bioconversion into biosurfactant: optimization and comparison with other carbon sources. *Biomass Conversion and Biorefinery*, 14(21), 27293-27305.

[23] Sharon, A. B., Ahuekwe, E. F., Nzubechi, E. G., Oziegbe, O., & Oniha, M. (2023, June). Statistical optimization strategies on waste substrates for solving high-cost challenges in biosurfactants production: a review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1197, No. 1, p. 012004). IOP Publishing.

[24] Soltanighias, T., Singh, A. E., Satpute, S. K., Banpurkar, A. G., Koolivand, A., & Rahi, P. (2019). Assessment of biosurfactant-producing bacteria from oil contaminated soils and their hydrocarbon degradation potential. *Environmental Sustainability*, 2, 285-296.

[25] Somoza-Coutiño, G., Wong-Villarreal, A., Blanco-González, C., Pérez-Sariñana, B., Mora-

Herrera, M., Mora-Herrera, S. I., & Yañez-Ocampo, G. (2020). A bacterial strain of Pseudomonas aeruginosa B0406 pathogen opportunistic produces a biosurfactant with tolerance to changes of pH, salinity and temperature. *Microbial Pathogenesis*, 139, 103869.

[26] Suhandono, S., Kusuma, S. H., & Meitha, K. (2021). Characterization and production of rhamnolipid biosurfactant in recombinant escherichia coli using autoinduction medium and palm oil mill effluent. *Brazilian Archives of Biology and Technology*, 64, e21200301.

[27] Ukhurebor, K. E., Athar, H., Adetunji, C. O., Aigbe, U. O., Onyancha, R. B., & Abifarin, O. (2021). Environmental implications of petroleum spillages in the Niger Delta region of Nigeria: a review. *Journal of Environmental Management*, 293, 112872.

[28] Vigneshwaran, C., Sivasubramanian, V., Vasantharaj, K., Krishnanand, N., & Jerold, M. (2018). Potential of *Brevibacillus sp.* AVN 13 isolated from crude oil contaminated soil for biosurfactant production and its optimization studies. *Journal of Environmental Chemical Engineering*, 6(4), 4347-4356.

[29] Zompra, A. A., Chasapi, S. A., Twigg, M. S., Salek, K., Anestopoulos, I., Galanis, A., & Spyroulias, G. A. (2022). Multi-method biophysical analysis in discovery, identification, and in-depth characterization of surface-active compounds. *Frontiers in Marine Science*, 9, 1023287.