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Full Length Article

Cost-effective Biosurfactant Production by *Pseudomonas aeruginosa* Analyzed through Experimental Design Methodology

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Abstract

Pseudomonas aeruginosa strains are well-known for producing rhamnolipid biosurfactants. Their distinct physico-chemical characteristics and diverse biological functions make biosurfactants useful in the detergent, cosmetics, food, pharmaceutical, bioremediation, and agricultural industries. The primary objective of this study was to identify bacteria that could use inexpensive carbon sources to produce rhamnolipid biosurfactants to reduce the production cost of this significant industrial compound. From the oil-contaminated sites along the Karachi coast, a total of 17 bacterial strains were isolated and subjected to biosurfactant screening. Out of these, DGEF106 was chosen as a possible source of rhamnolipid biosurfactant on CTAB agar plates. A partial 16S ribosomal RNA gene sequence revealed that the selected rhamnolipid (Rhl)-producing bacteria were *P. aeruginosa*, accession number OK147928. The selected bacterial strains were used to maximize the production of rhamnolipid biosurfactants on a low-cost carbon substrate with varied quantities of glycerol, brown sugar, coconut pulp, and mango juice. Minitab 19 was used to statistically analyze data, which has been displayed in a box plot. When low-cost carbon and nitrogen sources were evaluated, it was shown that carbon sources significantly affected the formation of biosurfactants. When ammonium nitrate, ammonium chloride, and urea were combined with an inexpensive carbon supply, the yield of rhamnolipids changed significantly. These microbes could exploit a variety of renewable resources, including agro-industrial waste, as possible sources of carbon. This increases the likelihood of producing biosurfactants at a reasonable cost and lessens the pollution produced by synthetic surfactants.

Keywords: Biosurfactants; Carbon source; Low-cost substrate; *Pseudomonas aeruginosa*; Rhamnolipids

Introduction

Different industrial operations in the food, pharmaceutical, cosmetic, detergent and oil sectors frequently use surfactants. These amphiphilic molecules have an annual value of over $10 billion in the global market (Reis *et al.* 2004; Akbari *et al.* 2018). Most of these synthetic surfactants are utilized without taking into consideration their detrimental effects on the ecosystem (Desai and Banat 1997; Matović-Purić *et al.* 2020). Biosurfactants are viable alternatives to these petroleum-derived synthetic products, which are produced by microbial fermentation, mainly *via* bacteria. Numerous bacterial species, such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Acinetobacter venetianus, Burkholderia* sp., *Stenotrophomonas maltophilia* and *Flavobacterium* sp., make biosurfactants (Femi-Ola *et al.* 2015; Shamaa and Bahjat 2019; Alyousif *et al.* 2020).

The treatment and protection of environmental pollutants has been the primary use of biosurfactants in the past few years (Manga *et al.* 2021). Harmful by-products of hydrocarbon-based toxins represent a threat to the environment. Biosurfactants aid in the solubilization of hydrocarbon pollutants, making them more accessible for microbial breakdown and emulsification (Sekhon *et al.* 2017; Sheikh *et al.* 2019). To support the natural remediation of habitats contaminated by hydrocarbons, microbial biosurfactants are essential (Randhawa and Rahman 2014; Perfumo *et al.* 2018). Compared to chemical surfactants, biosurfactants have the advantages of improved oil recovery and environmental preservation (Markande and Nerurkar

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2021). Six categories of biosurfactants can be distinguished based on their physiological and chemical makeup: polymeric biosurfactants, glycolipids, phospholipids, neutral lipids, lipopeptides and lipoproteins (Zhao *et al.* 2017; Kashif *et al.* 2022). Among the most studied substances produced by bacteria, rhamnolipids (RL) are a significant class of glycolipids that are mostly found to be produced by *P. aeruginosa* (Pathaka and Pranav 2015; Alyousif *et al.* 2020; Araujo *et al.* 2020). RL is composed of β-hydroxyl fatty acids linked to a rhamnose sugar at the carboxyl end and is classified as mono-and di-Rhls based on one or two rhamnose sugar moieties (Rikalović *et al.* 2015). Because of their sugar moiety, these compounds can lessen surface tension, emulsify and solubilize insoluble substrates (Banat *et al.* 2000; Karnwal *et al.* 2023). RL's inherent qualities, such as its emulsion, fungicidal, antimicrobial, and medicinal qualities, make it a popular choice in the industry (Chauhan *et al.* 2023). Food items, pharmaceuticals, oil sludge treatment, petroleum recovery, cosmetics, environmental clean-up, and agriculture as insecticides are just a few of the industries that have used RL (Mulligan 2005; Nguyen *et al.* 2010; Karnwal *et al.* 2023). Applications and characteristics show that RL is one of the most significant commercial and industrial goods that is non-toxic and ecologically benign. Industrial demand for RL increases daily, for which bulk production is required. However, economic barriers, such as high processing costs, relatively expensive raw materials, and low productivity, have prevented the production of RL in bulk (Müller 2011).

To address this, the industrial demand for RL can be fulfilled economically using high-producer microorganisms and inexpensive raw materials (Pandy 2012; Pathaka and Pranav 2015). Dairy waste, oils, bakery waste, and molasses have all been documented by several researchers as inexpensive sources of carbon for RL production (Makkar *et al.* 2011; Henkel *et al.* 2012). In *P. aeruginosa*, the genes *Rhl A* and *Rhl B* are in charge of producing rhamnolipid (Mathiyazhagan *et al.* 2011). The current study's objective was to find a low-priced substrate for rhamnolipid synthesis.

Materials and Methods

Sampling area

To isolate the biosurfactant-producing microorganisms, seawater samples were collected from coastal areas of Karachi in sterilized glass tubes and stored at 4°C before utilization.

Isolation and nutrition of bacterial isolates

After being serially diluted, seawater samples were spread out on R2A (Oxoid) solidified plates and kept for 24 h at 37°C (Anandaraj and Thivakaran 2010). Following incubation, the plates were counted, and around five to six morphologically distinct bacterial colonies were chosen for purification by re-streaking twice on Luria Bertani (LB) agar plates (Sambrook 1989). For subsequent screening, the chosen bacterial isolates were kept in LB agar slants in a refrigerator.

Cellular morphology

Under a 100x oil immersion microscope, the pure strains' cells were examined, and their shape, organization, and Gram reactions were noted (Shoeb *et al.* 2015).

GSP agar plates

Glutamate starch phenol-red (GSP) agar plates were streaked with an overnight bacterial culture. Plates were incubated for 24 h at 37°C. GSP agar contains starch and glutamate nutrients and RL-producing *Pseudomonas* species can grow and degrade these nutrients (Stanier *et al.* 1966).

Techniques for Screening Biosurfactant Production

Hemolytic activity: Hemolytic studies were conducted using blood agar plates. 50 μL of broth cultures was used as a spot inoculant on blood agar plates and the plates were then incubated for 48 h at 37°C. On the plates, the colony's surrounding zone of clearing (haemolysis) was visually inspected. The diameter of the clearance zone is one qualitative method for figuring out biosurfactant production (Mulligan 2005).

Oil spreading method: The technology described by Youssef *et al.* (2004) was used to disperse the oil. 100 μL of crude oil was applied to the surface of each petri dish with 50 mL of distilled water. Ten microlitters of cell-free culture broth was applied to the crude oil surface and the formation of a clear zone indicated the synthesis of biosurfactants. Since the diameter of the clear zone was directly connected to the amount of biosurfactant, it was quantified and compared with a negative control of 10 μL of distilled water (Zhao *et al.* 2017).

Bacterial adhesion to hydrocarbons (bath) assay: As previously described by Rosenberg and Rosenberg (1985), the BATH assay was conducted to measure the hydrophobicity of bacterial cells. Bacterial cells were suspended in a buffer salt solution, then vortexed and agitated with crude oil. After shaking, the crude oil and aqueous phases separated for one h. The optical density of the aqueous phase was measured at 600 nm to determine the hydrophobicity of the cells. The following formula was used to assess hydrophobicity, which is expressed as the proportion of cell adherence to crude oil:

$$1 - (aqueous\ phase OD / initial\ cell\ suspension\ OD) \times 100$$

CTAB agar plate: The bacterial strains were grown on mineral salt agar plates, supplemented with cetyltrimethylammonium bromide and methylene blue (0.2 and 5 mg mL^{-1}). To identify the formation of extracellular glycolipids, CTAB agar plates were employed (Siegmund and Wagner 1991). Dark blue haloes formed surrounding the colonies, indicating the presence of biosurfactants.

Emulsification index (E24): The emulsification index was calculated by adding xylene to cell-free extract, vortexed for 2 min and measured after 24 h (Ilori *et al.* 2005). The following formula was used to calculate the emulsification activity:

$$E24 = \text{height of emulsion/height of liquid column} \times 100$$

Drop-collapse test: The drop-collapse test, was first described by Jain *et al*. (1991) and modified by Bodour and Miller-Maier (1998), was used to screen for biosurfactant production. After applying crude oil to a 96-well microplate cover five microliters of 48-h cell-free broth were introduced. Drop size was measured and compared to a negative control of deionised water.

Amplification of partial *16s rRNA* gene by PCR

DNA was extracted from DGEF106 using the Gene Jet DNA extraction kit from Thermos Scientific. A gradient thermal cycler was used to amplify the 16S rRNA gene using universal primers 27F and U1492R (Santos *et al.* 2019). The purified product was sent to Macrogen for Sanger sequencing. The sequence was compared with the NCBI Nucleotide Database using BLAST and submitted to the NCBI GenBank database (Altschul *et al.* 1990).

Optimization of the low-cost substrate

Four inexpensive carbon sources were used to maximize RL production: commercial glycerol, brown sugar, mango juice and coconut pulp. Ammonium chloride, ammonium nitrate and urea were the three nitrogen sources that were utilized. Four carbon sources were examined, both with and without these three nitrogen sources. To put it briefly, the mineral medium was added with 1% of each of the carbon and nitrogen supplies. All sixteen treatment combinations were administered with fresh overnight cultures. The procedure was conducted in three separate trials, and the Emulsification Index (E24) was used to measure the amount of RL produced after 48, 72 and 96 h of incubation.

Statistical analysis

Three independent variables and one dependent variable were used to calculate the results. The response variable output was quantitative in nature, whereas the independent variables C, N and D were treated with four, four, and three levels, respectively. Each treatment combination was applied to three sampling units (three replicates for each combination). A single run of experimentation had 4 × 4 × 3 = 48 sampling units and for the three completers, the total number of instances was 144 (Table 1). The mean ± standard error of the mean was used to represent three separate experiments. For the analysis statistical program, Minitab 19 was used.

Table 1: Summary of the experimental variables

Factor	Levels	Interpretation of the levels
C	4	1 = Coconut pulp, 2 = Brown sugar, 3 = Mango juice, 4 = Glycerol
N	4	1 = No nitrogen source, 2 = Ammonium nitrate, 3 = Ammonium chloride, 4 = Urea
D	3	1 = 48 h after incubation, 2 = 72 h after incubation, 3 = 96 h after incubation

Results

Isolation of bacterial isolates

Seventeen distinct biosurfactant-producing microbial strains were identified and assigned the codes DGEF104-120.

Cellular morphology

The results of examining the cellular morphology of the 17 purified strains are displayed in Table S1.

GSP agar plates

Pink colonies were observed when the cultures were grown on GSP agar (Table S1).

Screening for Biosurfactant Production

Biosurfactant activity was evaluated for each isolate. All 17 isolates tested positive for beta-hemolysis. The oil spreading technique indicated 0.2–2.5 cm of diameter produced by different isolates. The BATH assay revealed positive adherence of all 17 isolates to the crude oil. Eight of the seventeen bacterial isolates produced dark blue halos surrounding the colonies, according to the CTAB agar plate assay. All isolates confirmed a positive emulsification index ranging from 26–35% with xylene. In the drop collapse test, all the isolates showed a collapsed drop. Table S2 displays all the screening test findings. The best biosurfactant production method's outcomes led to the selection of DGEF106 for 16S rRNA gene sequence analysis and the development of an inexpensive substrate for improved biosurfactant synthesis.

Amplification of partial *16S rRNA* and *rhlA* genes by PCR

Using the BLAST (www.ncbi.nlm.nih.gov) search program, the sequences derived from the PCR product were examined and found to be 99% similar to the 16S rRNA gene of *P. aeruginosa*. Under accession number OK147928, the acquired sequence was added to the NCBI GenBank database.

Statistical analysis for low-cost substrate optimization

Table 2 presents the outcomes of the statistical analysis conducted for the low-cost substrate optimization. The

Table 2: Statistical analysis of the low-cost substrate

Variable	N	Mean	SE Mean	St. Dev	Min	Median	Maximum
Output	144	44.193	0.759	9.109	14.200	45.450	59.500

Table 3: The analysis of variance (ANOVA) results for the components and their interactions

Source	DF	Adj SS	Adj MS	F-value	*P*-value
C	3	1819.5	606.51	28.09	0.000
N	3	230.2	76.74	3.55	0.017
D	2	185.4	92.71	4.29	0.016
C*N	9	3130.6	347.84	16.11	0.000
C*D	6	696.6	116.10	5.38	0.000
N*D	6	255.9	42.66	1.98	0.077
C*N*D	18	3473.3	192.96	8.94	0.000
Error	96	2072.7	21.59		
Total	143	11864.4			

Table 4: Model summary of Table 3

S	R-sq	R-sq(adj)	R-sq(pred)
4.64658	82.53%	73.98%	60.69%

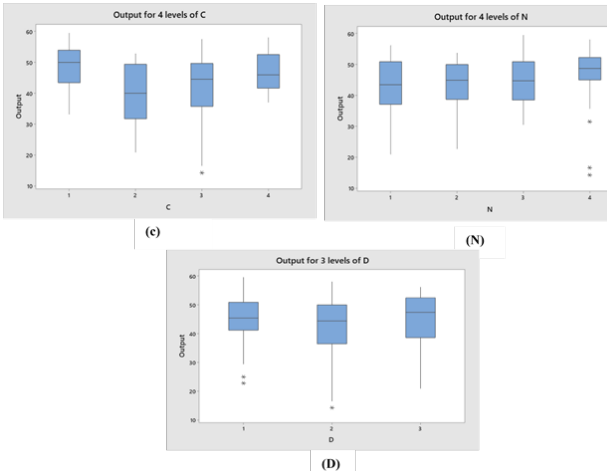

Fig. 1: The average response for each treatment at its various levels is shown in the box plots (top) C, middle) N and bottom) D

average of the 144 occurrences' response variable, which ranged from 14.2 to 59.5, was 44.193. Box plots show the average response for treatments at various levels (Fig. 1). At the first level (coconut pulp), Treatment C had the highest average (higher than the overall average). The average then fell to the second level and then increased once again to reach higher levels. For the fourth level, however, incredibly tiny values were noted. As the nitrogen sources shifted from left to right, the average output steadily rose. The longer the incubation period, the lower the output. In total, these factors accounted for over 74% of the variation. Treatment C and its two- and three-factor interactions were likewise highly significant (P = *0.000*), even though all treatments and their interactions were significant (Table 3 and Table 4).

Plots illustrating the primary effects of these factors are displayed in Fig. 2. Like how box plots evaluate the behaviour of treatments at their levels, the plot does the same. As their levels varied, the primary effect curves, however, did not alter systematically. This demonstrated how important the interaction impact was. It was referred to as their interaction effect when the two factors' combined effect was both different and significant. The interaction graphs for the factor pairs are provided below.

Fig. 3 illustrates the relationship between C and N. There was a noticeable shift in Factor C's performance at various levels as N varied its levels. This suggests that C and N have an interaction effect on the synthesis of biosurfactants. Fig. 4 illustrates the interaction impact of factors D and C. While the second and third levels of C responded similarly but showed distinct output variations, the first and fourth levels of C appeared to behave in the other way.

As Fig. 5 illustrates, factors D and N interacted. While the first level of N had a negative effect on output, the second level of N had a positive and almost uniform shift throughout the levels of D. Although the modifications from the first to the second level of D were different, the third and fourth levels displayed remarkably similar trends.

Discussion

Biosurfactant-producing bacteria were isolated and screened through conventional methods using the R2A medium developed by Reasoner and Geldreich (1985) as a nutritionally reduced medium (Trudgeon *et al.* 2020). CTAB agar plating distinguished RL-producing colonies with dark blue halos. These halos were formed as a result of the binding of the anionic secreted RL biosurfactant and the cationic CTAB surfactant with methylene blue (Shamaa and Bahjat 2019). The selective medium known as glutamate starch phenol-red (GSP) agar is used to identify *Pseudomonas* species (Flint and Hartley 1996). For the statistical analysis of the low-cost RL production strain, DGEF106 was selected because it showed RL production on CTAB and GSP agar plates, indicating RL-producing *Pseudomonas* sp., which was further verified through partial 16S rRNA gene identification as *P. aeruginosa*.

Carbon sources are crucial to the production of RL. Agro-industrial by-products and inexpensive renewable substrates can be cheaper and practical for RL production than carbon substrates, which make up a significant amount of the total production cost during RL fermentation (Kosaric and Sukan 2014). In this study, various low-cost carbon sources were examined to enhance RL production. Commercial glycerol, brown sugar, mango juice, and coconut pulp were employed as low-cost substrates. In addition to being inexpensive and plentiful, these carbon sources are environmentally safe (Silva *et al.* 2009). The provision of nitrogen is thought to lead to a larger yield of RL production in addition to carbon sources (Reis *et al.* 2010; Onwosi and Odibo 2012). Adding a nitrogen supply can assist in increasing the output even if nitrogen limitation is not a

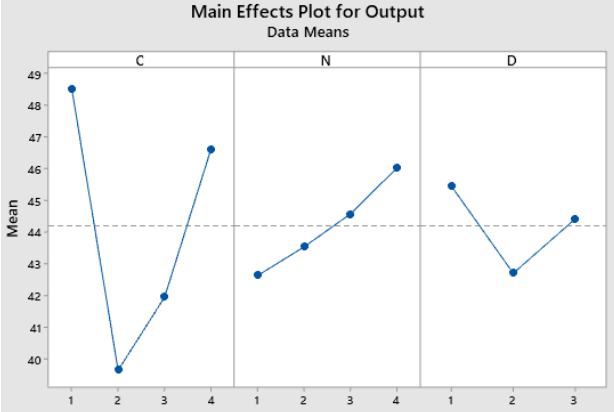

Fig. 2: The three factors' primary effect graphs are C, N and D. As their levels fluctuate, the main effect curves do not alter in a systematic manner, which suggests the interaction effect

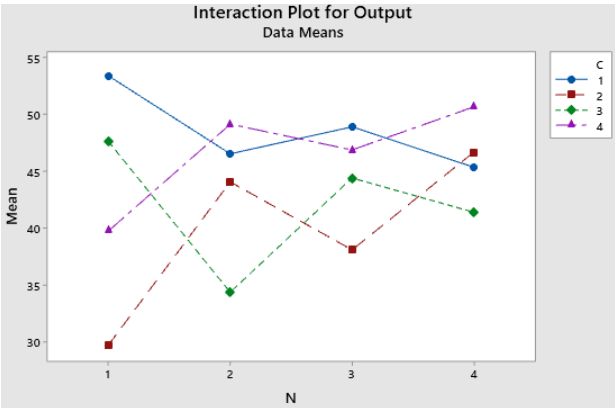

Fig. 3: The interaction between C and N. The first level (blue curve) has a tiny and negative change, whereas the second level (red curve) has a huge and positive change. This is an indication of the influence of interaction

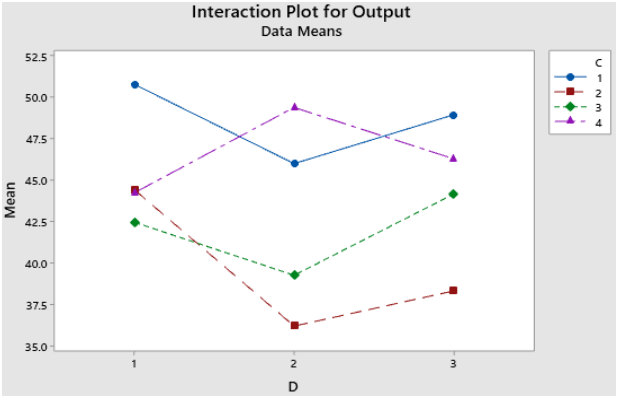

Fig. 4: An interaction effect is also shown in factors D and C. There is an inverse link between the first and fourth levels of C. Although the outputs from the second and third layers of C differ, they function similarly

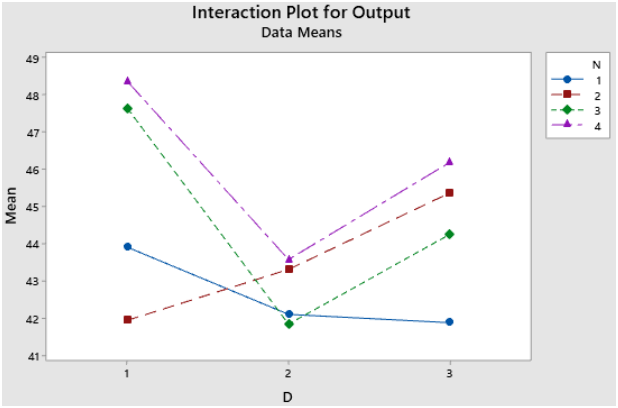

Fig. 5: There is also an interaction between factors D and N. While the first level of N had a negative effect on the output, the second level of N had a positive and uniform change throughout the levels of D. Though they differed from the first and second levels of D, the third and fourth levels displayed a similar trend

necessary condition for the formation of biosurfactants (Long 2013). We examined the growth and synthesis of biosurfactants both with and without a nitrogen supply considering nitrogen's limitations. Based on the degree of emulsification, the emulsification index (E24) is a technique for verifying the synthesis of biosurfactants. Xylene was employed as a hydrophobic substrate in this investigation (Nayarisseri *et al.* 2018). Optimization of the culture medium was carried out because it is the most cost-effective and practical approach for boosting RL synthesis (Luo *et al.* 2013). The importance of both carbon and nitrogen supplies for the synthesis of biosurfactants was taken into consideration during arithmetical analysis, and the effects of both sources under consistent physical conditions were enhanced (Trujillo *et al.* 2015).

Carbon sources interacted with nitrogen sources, as well as incubation time. Among the variables tested, the effect on biosurfactant production was shown by carbon sources, while nitrogen sources were found to interact with these carbon sources. Interestingly, different carbon sources had a significant impact on biosurfactant production, with coconut pulp showing the highest average. Worldwide, coconut water is widely consumed, and the pulp and husk from its production are byproducts that produce a lot of garbage (Santana *et al.* 2011). When we used this pulp to produce biosurfactants, we discovered that it had the best emulsification index when there was no nitrogen source present in the medium. Sinaga *et al.* (2015) reported sufficient amounts of nitrogen in different forms of coconut pulp. After 48 h, the highest amount of biosurfactant was produced; prolonged incubation times had detrimental effects. ANOVA was employed to verify the significance of the experiment.

Conclusion

The results of this study demonstrate the encouraging

possibility of generating rhamnolipid biosurfactants from inexpensive and sustainable substrates, such as coconut pulp. The strain DGEF106 of *P. aeruginosa* showed notable biosurfactant activity when coconut pulp was utilized as the only carbon source, obviating the necessity for extra nitrogen. This approach not only reduces production costs but also aligns with environmentally friendly practices by utilizing agricultural by-products. The findings point to a practical solution for making rhamnolipid production more economically viable, paving the way for wider industrial use. Moving forward, refining the production process and exploring larger-scale applications will be crucial steps in bringing this method to the market.

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Author Contributions

ES, JA, UB and KS conceptualized and supervised the original research. FAA, BA and AN worked on methodology and formatting. BA worked on statistical analysis.

Conflicts of Interest

There is no conflict of interest.

Data Availability

The corresponding author would be able to share the data on request.

Ethics Approval

No ethical issues applicable to this paper.

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Table S1. Cellular morphology and GSP agar identification of selected strains

Strain no.	Strain code	Cellular morphology				GSP agar identification
		Shape	Alignment	Structure	Gram reaction	
1	DGEF104	Short rod	Chain	Strepto bacilli	+ve	*P. aeruginosa*
2	DGEF105	Short rod	Scattered	Coccobacillus	-ve	-ve
3	DGEF106	Short rod	Chain	Coccobacillus	-ve	*P. aeruginosa*
4	DGEF107	Rods	Scattered	Strepto bacilli	+ve	-ve
5	DGEF108	Short rod	Chain	Diplobacilli	+ve	*P. aeruginosa*
6	DGEF109	Short rod	Bunches	Diplobacilli	-ve	*P. aeruginosa*
7	DGEF110	Short rod	Bunches	Diplobacilli	+ve	-ve
8	DGEF111	Rounds	Bunches	Coccobacillus	-ve	-ve
9	DGEF112	Rod	Bunches	Coccobacillus	+ve	-ve
10	DGEF113	Short rod	Scattered	Strepto bacilli	+ve	-ve
11	DGEF114	Short rod	Scattered	Diplobacilli	-ve	-ve
12	DGEF115	Short rod	Scattered	Cocci	+ve	-ve
13	DGEF116	Short rod	Scattered	Cocci	+ve	-ve
14	DGEF117	Round	Chain	Bacilli	-ve	*P. aeruginosa*
15	DGEF118	Rods	Scattered	Diplobacilli	+ve	*P. aeruginosa*
16	DGEF119	Round	Scattered	Bacilli	-ve	*P. aeruginosa*
17	DGEF120	Round	Scattered	Bacilli	-ve	*P. aeruginosa*

Gram staining of the isolates revealed nine gram-positive and eight gram-negative bacteria. The cellular structures were short rods, round, and scattered or bunched. Coccobacillus and Diplobacilli are common strains. GSP agar identification revealed eight strains of *Pseudomonas aeruginosa*.

Table S2. Screening results of the selected strains

Strain no.	Code no.	Hemolytic activity	CTAB agar test	Emulsification activity (E24%)	Drop collapse test	Oil spreading test (cm)	Bath assay (%)
1	DGEF104	α	No zone	33	Collapse	2.4	40
2	DGEF105	β	No zone	33	Collapse	2.4	45

3	DGEF106	α	Blue halo	35	Collapse	2.5	50
4	DGEF107	β	Blue halo	33	Collapse	1.0	30
5	DGEF108	β	Blue halo	31	Collapse	1.0	33
6	DGEF109	α	Blue halo	31	Collapse	1.3	31
7	DGEF110	β	No zone	31	Collapse	0.2	33
8	DGEF111	β	No zone	30	Collapse	0.3	30
9	DGEF112	β	No zone	30	Collapse	0.2	30
10	DGEF113	β	No zone	26	Collapse	1.5	20
11	DGEF114	β	No zone	26	Collapse	0.5	16
12	DGEF115	β	Blue halo	31	Collapse	0.3	16
13	DGEF116	β	No zone	30	Collapse	0.5	20
14	DGEF117	β	No zone	28	Collapse	1.2	34
15	DGEF118	α	Blue halo	35	Collapse	1.2	28
16	DGEF119	α	Blue halo	33	Collapse	2.5	25
17	DGEF120	β	Blue halo	30	Collapse	1.2	33

Selected rhamnolipid-producing strains were screened for hemolysis, CTAB, E24, drop collapse, oil spreading, and the BATH assay. For hemolysis, α indicates complete lysis of red blood cells and β indicates partial lysis. For CTAB, some strains produced blue halos around the colonies, indicating a positive biosurfactant activity. E24 activity was expressed as a percentage, showing a maximum emulsion of 33–35%. In the drop collapse assay, all the strains showed positive biosurfactant activity. In the oil spreading test strains displaced oil in the range of 1.5–2.5 cm and in BATH assay all strains showed adherence to hydrocarbon from 20% to 45%