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Optimizing The Physical and Biochemical Factors for Biosurfactant Production Using One Variable at A Time

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ABSTRACT

Biosurfactants are eco-friendly surface-active compounds with broad industrial applications, yet their commercial production is often hindered by low yields and high costs. This study focused on optimizing the physical and biochemical factors affecting biosurfactant production using the One Variable At a Time (OVAT) approach. Two biosurfactant-producing bacterial isolates—*Achromobacter xylosoxidans* (P5c) and *Pseudomonas aeruginosa* (P11)—were subjected to a range of culture conditions to evaluate the impact of different carbon sources, nitrogen sources, temperatures, pH levels, and salt concentrations on biosurfactant yield, emulsification activity (%E24), surface tension (ST), and dry cell biomass (DCBM).

Among the tested parameters, glucose and glycerol emerged as the most favorable carbon sources, while ammonium nitrate supported the highest biosurfactant production among nitrogen sources. Optimal biosurfactant yields were observed at 35 °C and pH 7.0 for both isolates. Salt concentrations between 1% and 2% promoted the best microbial growth and biosurfactant activity, with *P. aeruginosa* showing higher salt tolerance than *A. xylosoxidans*. Emulsification activity and surface tension measurements corroborated the biosurfactant productivity under optimized conditions. This study demonstrated that targeted adjustment of key environmental and nutritional factors can significantly enhance biosurfactant production.

Keywords: *Biosurfactant, Pseudomonas aeruginosa, Achromobacter xylosoxidans, OVAT, Optimization, Emulsification, Surface tension*

1. INTRODUCTION

Biosurfactants are amphiphilic compounds produced by various microorganisms that reduce surface and interfacial tensions between liquids, solids, and gases. Their natural origin, biodegradability, and functional properties make them highly valuable in diverse applications such as bioremediation, enhanced oil recovery, pharmaceuticals, and agriculture [1]. However, despite their advantages, commercial production of biosurfactants remains limited due to high production costs and low yields under non-optimized conditions [2].

To address these limitations, optimizing the culture conditions—particularly the physical (pH, temperature, salinity) and chemical (carbon and nitrogen sources) parameters—has been recognized as a critical strategy. Previous studies have demonstrated that the nature of carbon and nitrogen sources significantly influences biosurfactant yield and activity. For example, glycerol and glucose have been reported to enhance biosurfactant productivity in *Pseudomonas aeruginosa* [3], while nitrogen limitation or specific nitrogen types like ammonium nitrate have also been associated with increased surface-active compound secretion [4].

Existing optimization techniques such as Response Surface Methodology (RSM) and Design of Experiments (DOE) offer higher-order interactions between variables but may overlook individual factor effects if not pre-screened [5]. Therefore, OVAT remains an important preliminary approach to identify key contributors for further optimization [6].

The main limitation of biosurfactant production lies in the complexity and cost of large-scale fermentation and downstream processing [7]. Despite the known benefits of biosurfactants over synthetic surfactants, their commercial viability continues to be challenged by low yield and productivity [8].

This study aims to overcome these bottlenecks by providing an empirical framework for enhancing biosurfactant production via OVAT methodology, targeting critical environmental and nutritional parameters. The ultimate goal is to offer a cost-effective, scalable protocol for biosurfactant production using environmentally robust strains like *P. aeruginosa* and *A. xylosoxidans*, with potential industrial application.

2. MATERIALS AND METHODS

2.1. Selection of Bacterial Isolates

The bacterial strains Pseudomonas aeruginosa (accession no. OP846851.1) and Achromobacter xylosoxidans (accession no. OP060997.1) were selected for their potential in biosurfactant production. These isolates were previously identified and registered for their biosurfactant-producing capabilities.

2.2. Factors Affecting Biosurfactant Production

To optimize biosurfactant production, various physical and chemical parameters were systematically evaluated. Each experiment was conducted in triplicate to ensure reproducibility. Biosurfactant production was assessed by measuring surface tension (ST) reduction, emulsification index (E24%), and dry cell biomass (DCBM).

2.2.1. Effect of Carbon Sources

Mineral Salt Medium (MSM) with a pH of 7.0 was supplemented with 1% (w/v) of different carbon sources: glucose, glycerol, fructose, sodium citrate, mannitol, and starch. Each 250 mL Erlenmeyer flask contained 100 mL of the prepared medium and was inoculated with 5.0 mL of bacterial culture. Additionally, 1.0 mL of crude oil was added as a hydrophobic substrate. The flasks were incubated at 28 ± 2°C on an orbital shaker at 120 rpm for 5 days. Glucose has been previously reported to be an effective carbon source for biosurfactant production in *P. aeruginosa*

2.2.2. Effect of Nitrogen Sources

To determine the optimal nitrogen source, MSM (pH 7.0) was supplemented with 1% (w/v) of various nitrogen sources: yeast extract, peptone, ammonium chloride, ammonium nitrate, and sodium nitrate. Each flask received 1.0 mL of crude oil and 5.0 mL of bacterial inoculum. The cultures were incubated at 28 ± 2°C on an orbital shaker at 120 rpm for 5 days. Previous studies have indicated that nitrate-based nitrogen sources can enhance biosurfactant production in P. aeruginosa [9].

2.2.3. Effect of Temperature

The influence of temperature on biosurfactant production was assessed by incubating the bacterial cultures in MSM (pH 7.0) supplemented with 1.0 mL of crude oil and 5.0 mL of inoculum. The flasks were incubated at temperatures ranging from 25°C to 50°C, in 5°C increments, on an orbital shaker at 120 rpm for 5 days. Optimal biosurfactant production has been reported at 28°C for certain bacterial strains [10].

2.2.4. Effect of pH

To evaluate the effect of pH, MSM was adjusted to pH values ranging from 5.0 to 8.0, in 0.5-unit increments. Each flask contained 100 mL of the adjusted medium, 1.0 mL of crude oil, and 5.0 mL of bacterial inoculum. The cultures were incubated at 28°C on an orbital shaker at 120 rpm for 5 days. Biosurfactant production by Bacillus subtilis has been shown to be optimal at pH 6.5 to 7.0 [11].

2.2.5. Effect of Salinity

The impact of salinity on biosurfactant production was studied by supplementing MSM (pH 7.0) with varying concentrations (0–5% w/v) of NaCl and bile salts, in 1% increments. Each flask contained 100 mL of the prepared medium, 1.0 mL of crude oil, and 5.0 mL of bacterial inoculum. The cultures were incubated at 28°C on an orbital shaker at 120 rpm for 5 days. Biosurfactants produced by certain strains have demonstrated stability in high-salinity environments [12].

2.3. Biosurfactant Extraction and Analysis

Post-incubation, biosurfactants were extracted following standard protocols. Surface tension was measured using a tensiometer, emulsification index (E24%) was determined by mixing equal volumes of culture supernatant and hydrocarbon, and DCBM was assessed by drying the cell pellet at 105°C until constant weight [13,14].

2.4. Statistics

The standard error of mean and analysis of variance with Post Hoc Analysis by Tukeys HSD was performed using IBM SPSS ver. 26.

3. RESULTS AND DISCUSSION

3.1 Effect of carbon source on production and activity of biosurfactants

Different carbon sources like glucose, glycerol, fructose, mannitol, and starch were used to evaluate the effect on the

production of biosurfactants from the two bacterial isolates *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11) (Figure 1). Different parameters like dry cell mass, biosurfactant production, % emulsification, and surface tension were found to be significantly different from each other at $p<0.05$ level of significance and are demonstrated in Table 1. Glucose consistently supported the highest dry cell masses, with values of 3.32 ± 0.019 gm/lit and 3.69 ± 0.021 gm/lit in P5c+Glucose and P11+Glucose, respectively. Additionally, glucose-promoted biosurfactant production was substantial, with biosurfactant yields of 1.40 ± 0.008 gm/lit in P5c+Glucose and 0.35 ± 0.002 gm/lit in P11+Glucose. Notably, glucose-derived biosurfactants exhibited significant % emulsification activity, with values of 34.21 ± 0.198 % and 52.77 ± 0.305 % in P5c+Glucose and P11+Glucose, respectively. However, it was observed that with high emulsification activity, the surface tension of the biosurfactant decreased. It was reported to be 26.96 ± 0.156 mN/m for P56+Glucose and 28.07 ± 0.162 mN/m.

Glycerol also demonstrated efficient results in biosurfactant production, particularly in P5c+Glycerol and P11+Glycerol media. Dry cell masses were slightly lower compared to glucose, with values of 2.70 ± 0.016 gm/lit and 1.64 ± 0.009 gm/lit in P5c+Glycerol and P11+Glycerol, respectively. However, glycerol-supported biosurfactant production was considerable, with biosurfactant yields of 1.85 ± 0.011 gm/lit in P5c+Glycerol and 0.80 ± 0.005 gm/lit in P11+Glycerol. The % emulsification activity noted was as efficient as that observed with glucose as a carbon source and reported as 34.20 ± 0.197 % and 58.80 ± 0.315 % in P5c+Glucose and P11+Glucose, respectively.

Fructose and mannitol, while exhibiting moderate effects on biosurfactant production compared to glucose and glycerol, exhibiting distinct characteristics in the results. Fructose-containing media supported relatively higher dry cell masses compared to mannitol, indicating better microbial growth under fructose-rich conditions. The dry cell mass for fructose was recorded at 1.55 ± 0.009 gm/lit for *A. xylosoxidans* (P5c) and 3.12 ± 0.018 gm/lit, while mannitol-supported growth resulted in a slightly lower dry cell mass of 1.49 ± 0.009 gm/lit for *A. xylosoxidans* (P5c) and 2.33 ± 0.013 gm/lit for *P. aeruginosa* (P11). However, both fructose and mannitol demonstrated moderate biosurfactant production, with fructose yielding 1.40 ± 0.008 gm/lit for *A. xylosoxidans* (P5c) and 0.45 ± 0.003 gm/lit for *P. aeruginosa* (P11) and mannitol producing 1.00 ± 0.006 gm/lit for *A. xylosoxidans* (P5c) and 0.30 ± 0.002 gm/lit for *P. aeruginosa* (P11) of biosurfactant. In terms of emulsification efficiency, fructose-containing media displayed moderate performance, with a % Emulsification value of 39.47 ± 0.228 % for *A. xylosoxidans* (P5c) and 54.12 ± 0.312 % for *P. aeruginosa* (P11). Mannitol, on the other hand, exhibited a slightly lower emulsification efficiency of 37.83 ± 0.218 % for *A. xylosoxidans* (P5c) and 36.11 ± 0.208 % for *P. aeruginosa* (P11). Despite their moderate effects on biosurfactant production and emulsification efficiency, both fructose and mannitol supported biosurfactant activity capable of reducing surface tension, with values recorded at 25.85 ± 0.15 mN/m in *A. xylosoxidans* (P5c) and 30.83 ± 0.178 mN/m in *P. aeruginosa* (P11) for fructose and 28.01 ± 0.162 mN/m in *A. xylosoxidans* (P5c) and 30.19 ± 0.174 mN/m in *P. aeruginosa* (P11) for mannitol.

Starch-containing media demonstrated the efficient emulsification efficiency among the tested carbon sources, with values of 48.71% in P5c+Starch and 37.14% in P11+Starch. Although starch-supported dry cell masses were moderate. However, the biosurfactant production was found to be high in *A. xylosoxidans* (P5c) when supplemented with starch but was lowest in *P. aeruginosa* (P11).

Table 1 Effect of carbon source on production and activity of biosurfactants

Sr. No	Carbon source	Dry cell mass g/L.	Biosurfactant g/L.	% Emulsification	Surface tension (mN/m)
1	*A. xylosoxidans* (P5c)+Glucose	3.32 ± 0.019	1.40 ± 0.008^{ac}	34.21 ± 0.198^{c}	26.96 ± 0.156^{de}
2	*A. xylosoxidans* (P5c)+Glycerol	2.70 ± 0.016	1.85 ± 0.011	34.20 ± 0.197^{c}	26.43 ± 0.153^{cdf}
3	*A. xylosoxidans* (P5c)+Fructose	1.55 ± 0.009^{b}	1.40 ± 0.008^{ab}	39.47 ± 0.228	25.85 ± 0.15^{c}
4	*A. xylosoxidans* (P5c)+Mannitol	1.49 ± 0.009^{b}	1.00 ± 0.006	37.83 ± 0.218^{b}	28.01 ± 0.162^{b}
5	*A. xylosoxidans* (P5c)+Starch	2.31 ± 0.013^{a}	1.40 ± 0.008^{bc}	48.71 ± 0.281	26.82 ± 0.155^{ef}
6	*P. aeruginosa* (P11)+Glucose	3.69 ± 0.021	0.35 ± 0.002	52.77 ± 0.305	28.07 ± 0.162^{b}
7	*P. aeruginosa* (P11)+Glycerol	1.64 ± 0.009	0.80 ± 0.005	58.80 ± 0.315	46.13 ± 0.266
8	*P. aeruginosa* (P11)+Fructose	3.12 ± 0.018	0.45 ± 0.003	54.12 ± 0.312	30.83 ± 0.178^{a}
9	*P. aeruginosa* (P11)+Mannitol	2.33 ± 0.013^{a}	0.30 ± 0.002	36.11 ± 0.208^{a}	30.19 ± 0.174^{a}

10	*P. aeruginosa* (P11)+Starch	1.94 ± 0.011	0.25 ± 0.001	37.14 ± 0.214^{ab}	45.02 ± 0.260

Any value followed by ± is the standard error of the mean. The values with common superscripts denote similar means at $p<0.05$ level of significance.

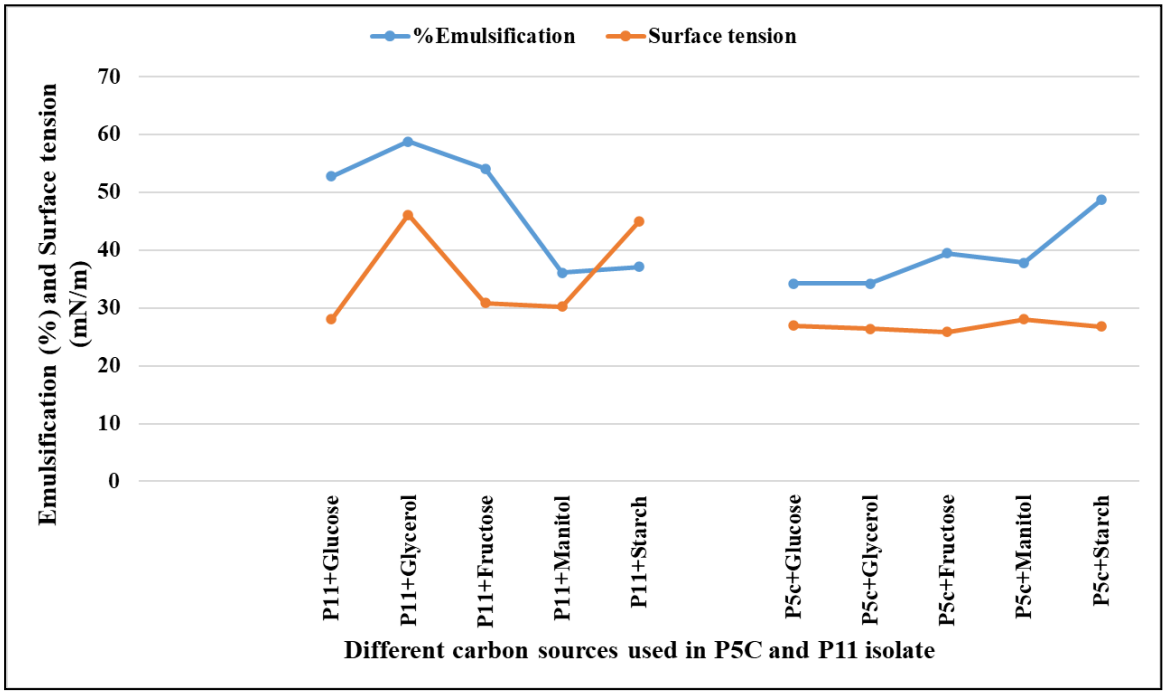

Figure 1 Effect of different carbon sources on emulsification and surface tension activity of *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11)

3.2 Effect of nitrogen source on production and activity of biosurfactants

The effect of nitrogen sources on the production and activity of biosurfactants was investigated, exhibiting variations in microbial growth, biosurfactant production, emulsification efficiency, and surface tension. Across different nitrogen sources tested from bacterial isolates *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11), the isolates showed significant production of the biosurfactant at $p<0.05$ level of significance and is exhibited in Table 2 and Figure 2. Among both the bacterial isolates, *P. aeruginosa* (P11) showed efficient growth and biosurfactant production compared to *A. xylosoxidans* (P5c), however, the overall emulsification activity was high in *A. xylosoxidans* (P5c) s. Among the nitrogen sources, ammonium nitrate was the most efficient in helping to increase biosurfactant production, emulsification activity, however, the overall microbial biomass was high in ammonium chloride. The lowest biosurfactant production was in peptone extract while the low emulsification activity was observed in ammonium chloride media.

In *A. xylosoxidans* (P5c), ammonium nitrate and ammonium chloride supported relatively low dry cell masses (DCM) compared to other nitrogen sources, with values of 0.24 ± 0.001 gm/lit and 0.26 ± 0.002 gm/lit, respectively. However, these nitrogen sources promoted biosurfactant production, with ammonium nitrate yielding 0.84 ± 0.005 gm/lit of biosurfactant and ammonium chloride producing 0.71 ± 0.004 gm/lit. Despite their moderate dry cell masses, both ammonium nitrate and ammonium chloride exhibited significant emulsification efficiency, with ammonium nitrate resulting in 61.29 ± 0.354% emulsification and ammonium chloride showing 33.33 ± 0.192% emulsification. However, surface tension was more pronounced in ammonium nitrate, with a surface tension value of 34.17 ± 0.197 mN/m, compared to 43.75 ± 0.253 mN/m in ammonium chloride.

Ammonium sulfate, peptone, and yeast extract in *A. xylosoxidans* (P5c) also influenced biosurfactant production and activity. Ammonium sulfate supported a higher dry cell mass of 0.30 ± 0.002 gm/lit compared to other nitrogen sources. However, biosurfactant production was relatively lower, with a yield of 0.42 ± 0.002 gm/lit. Peptone and yeast extract exhibited varying effects on microbial growth and biosurfactant production, with peptone resulting in a dry cell mass of 0.21 ± 0.001 gm/lit and yeast extract yielding 0.26 ± 0.002 gm/lit. Despite their lower dry cell masses, both peptone and yeast extract supported biosurfactant production, with values of 0.23 ± 0.001 gm/lit and 0.36 ± 0.002 gm/lit, respectively. Notably, peptone and yeast extract demonstrated comparable emulsification efficiencies, with values of 60.60 ± 0.35% and 54.83 ± 0.317%, respectively.

However, yeast extract exhibited a slightly higher surface tension compared to peptone, with values of 29.87 ± 0.172 mN/m and 31.56 ± 0.182 mN/m, respectively.

In *P. aeruginosa* (P11), ammonium nitrate supported moderate dry cell masses, with a value of 1.44 ± 0.008 gm/lit, and substantial biosurfactant production, yielding 0.88 ± 0.005 gm/lit. Ammonium chloride and ammonium sulfate resulted in higher dry cell masses compared to ammonium nitrate, with values of 2.03 ± 0.012 gm/lit and 1.84 ± 0.011 gm/lit, respectively. However, biosurfactant production was relatively lower for these nitrogen sources. Peptone and yeast extract supported moderate dry cell masses and biosurfactant production, with values ranging from 1.00 ± 0.006 gm/lit to 1.03 ± 0.006 gm/lit and 0.47 ± 0.003 gm/lit to 0.51 ± 0.003 gm/lit, respectively. Notably, peptone and yeast extract exhibited comparable emulsification efficiencies, with values ranging from 30.00 ± 0.173% to 33.33 ± 0.192%, respectively. However, yeast extract demonstrated higher surface tension compared to peptone, with values of 43.67 ± 0.252 mN/m and 35.00 ± 0.202 mN/m, respectively.

Table 2 Effect of nitrogen source on production and activity of biosurfactants

Sr. No	Nitrogen Source	DCM g/L.	Biosurfactant g/L	% Emulsification	Surface tension (mN/m)
1	*A. xylosoxidans* (P5c)+Ammonium nitrate	0.24 ± 0.001^{bde}	0.84 ± 0.005	61.29 ± 0.354^{c}	34.17 ± 0.197b
2	*A. xylosoxidans* (P5c)+Ammonium chloride	0.26 ± 0.002^{bc}	0.71 ± 0.004	33.33 ± 0.192^{a}	43.75 ± 0.253^{c}
3	*A. xylosoxidans* (P5c)+Ammonium sulphate	0.30 ± 0.002	0.42 ± 0.002	54.50 ± 0.315^{b}	50.98 ± 0.294
4	*A. xylosoxidans* (P5c)+Peptone	0.21 ± 0.001^{d}	0.23 ± 0.001	60.60 ± 0.35^{c}	31.56 ± 0.182
5	*A. xylosoxidans* (P5c)+Yeast extract	0.26 ± 0.002^{ce}	0.36 ± 0.002	54.83 ± 0.317^{b}	29.87 ± 0.172^{a}
6	*P. aeruginosa* (P11)+Ammonium nitrate	1.44 ± 0.008	0.88 ± 0.005	58.06 ± 0.335	29.1 ± 0.168^{a}
7	*P. aeruginosa* (P11)+Ammonium chloride	2.03 ± 0.012	0.60 ± 0.003	18.75 ± 0.108	41.85 ± 0.242
8	*P. aeruginosa* (P11)+Ammonium sulphate	1.84 ± 0.011	0.65 ± 0.004	21.87 ± 0.126	38.33 ± 0.221
9	*P. aeruginosa* (P11)+Peptone	1.00 ± 0.006^{a}	0.51 ± 0.003	30.00 ± 0.173	35.00 ± 0.202^{b}
10	*P. aeruginosa* (P11)+Yeast extract	1.03 ± 0.006^{a}	0.47 ± 0.003	33.33 ± 0.192^{a}	43.67 ± 0.252^{c}

Any value followed by ± is the standard error of the mean. The values with common superscripts denote similar means at $p<0.05$ level of significance.

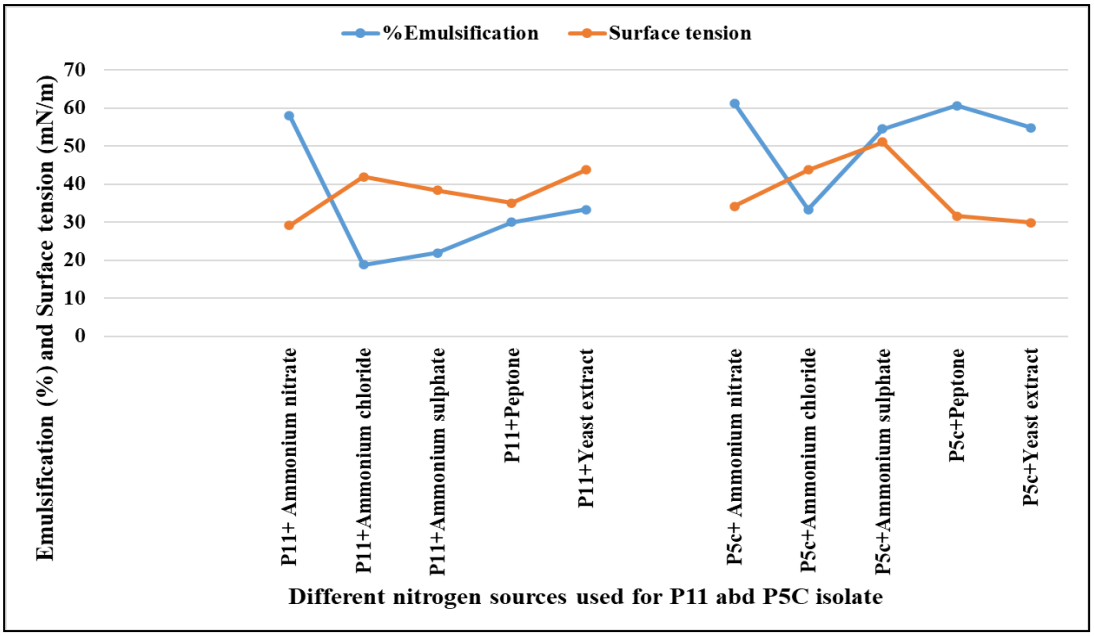

Figure 2 Effect of different nitrogen sources on emulsification and surface tension activity of *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11) s

3.3 Effect of temperature on production and activity of biosurfactants

The effect of temperature on biosurfactant production and activity was investigated, demonstrating significant variations in microbial growth, biosurfactant yield, emulsification efficiency, and surface tension at $p<0.05$ level of significance (Figure 3). The optimization of biosurfactants was conducted using two different bacterial isolates, *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11), at temperatures ranging from 25°C to 40°C and is given in Table 3. The most efficient temperature for the biosurfactant production, microbial growth, and emulsification activity was 35°C and the lowest biosurfactant production was obtained at 25°C for *A. xylosoxidans* (P5c) and 40°C for *P. aeruginosa* (P11).

In *A. xylosoxidans* (P5c), varying temperatures influenced microbial growth and biosurfactant production differently. At 25°C, a dry cell mass of 0.28 ± 0.002 gm/100ml was recorded, with a corresponding biosurfactant yield of 0.32 ± 0.002 gm/lit. Despite the low dry cell mass, the temperature exhibited a remarkable 14.70 ± 0.085% emulsification and 43.25 ± 0.25 mN/m surface tension reduction. As the temperature increased to 35°C, microbial growth improved significantly, resulting in a dry cell mass of 1.02 ± 0.006 Gm/100ml and a higher biosurfactant yield of 0.88 ± 0.005 Gm/lit. Consequently, emulsification efficiency increased to 67.70 ± 0.391%, accompanied by a reduction in surface tension to 36.21 ± 0.209 mN/m. However, at 40°C, both dry cell mass and biosurfactant yield decreased, although emulsification efficiency and surface tension reduction remained relatively high. Similarly, in *P. aeruginosa* (P11), temperature variations exerted differential effects on microbial growth and biosurfactant production. At 25°C, a dry cell mass of 0.36 ± 0.002 gm/100ml and a biosurfactant yield of 0.59 ± 0.003 gm/lit were observed, resulting in an emulsification efficiency of 48.64 ± 0.281% and a surface tension reduction of 42.56 ± 0.246 mN/m. At 35°C, microbial growth improved significantly, with a dry cell mass of 1.06 ± 0.006 gm/100ml and a biosurfactant yield of 0.95 ± 0.005 gm/lit. Consequently, emulsification efficiency increased to 57.57 ± 0.332%, accompanied by a reduction in surface tension to 29.71 ± 0.172 mN/m. However, at 40°C, both dry cell mass and biosurfactant yield decreased compared to the optimal temperature of 35°C.

Table 3 Effect of temperature on production and activity of biosurfactants

Sr. No	Temperature (°C)	DCBM g/100mL.	Biosurfactant g/L.	% Emulsification	Surface tension (mN/m)
1	*A. xylosoxidans* (P5c)+25°C	0.28 ± 0.002[a]	0.32 ± 0.002	14.70 ± 0.085	43.25 ± 0.25[a]
2	*A. xylosoxidans* (P5c)+30°C	0.30 ± 0.002	0.55 ± 0.003	41.17 ± 0.238	38.49 ± 0.222
3	*A. xylosoxidans*	1.02 ± 0.006	0.88 ± 0.005	67.70 ± 0.391	36.21 ± 0.209

	(P5c)+35°C				
4	*A. xylosoxidans* (P5c)+40°C	0.34 ± 0.002	0.36 ± 0.002[a]	27.27 ± 0.157	41.86 ± 0.242[b]
5	*P. aeruginosa* (P11)+25°C	0.36 ± 0.002	0.59 ± 0.003	48.64 ± 0.281	42.56 ± 0.246[ab]
6	*P. aeruginosa* (P11)+30°C	0.27 ± 0.002[a]	0.42 ± 0.002	44.44 ± 0.257	33.21 ± 0.192
7	*P. aeruginosa* (P11)+35°C	1.06 ± 0.006	0.95 ± 0.005	57.57 ± 0.332	29.71 ± 0.172
8	*P. aeruginosa* (P11)+40°C	0.13 ± 0.001	0.36 ± 0.002[a]	37.50 ± 0.217	31.25 ± 0.18

Any value followed by ± is the standard error of the mean. The values with common superscripts denote similar means at $p<0.05$ level of significance.

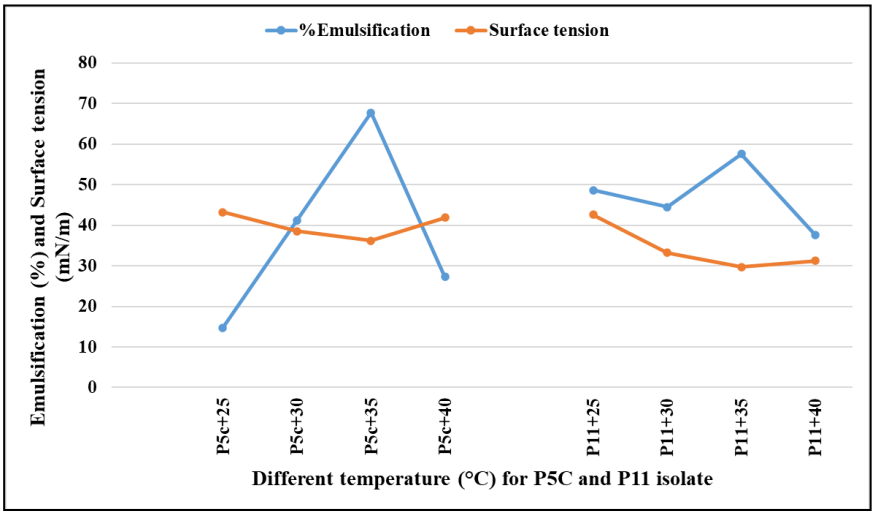

Figure 3 Effect of different temperatures (°C) on emulsification and surface tension activity of *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11)

3.4 Effect of pH on production and activity of biosurfactants

The effect of pH on biosurfactant production and activity was thoroughly examined, demonstrating significant variations in microbial growth, biosurfactant yield, emulsification efficiency, and surface tension (Figure 4). The study involved optimizing biosurfactant production using two distinct bacterial isolates, namely *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11), across a temperature ranging from pH 5 to pH 8, with statistical significance set at $p < 0.05$ and is exhibited in Table 4. The maximum biosurfactant production and emulsification activity of both the isolates was found at neutral pH (7.0), but as the pH decreased or increased, the biosurfactant production also decreased. Additionally, the microbial growth was also maximum at pH 7.0.

In *A. xylosoxidans* (P5c), pH 7.0 supported the highest dry cell masses, with a value of 1.32 ± 0.008 g/100mL, indicating favorable conditions for microbial growth. Biosurfactant production was also substantial at pH 7.0, yielding 1.44 ± 0.008 Gm/lit. Additionally, biosurfactants produced at pH 7.0 exhibited significant emulsification efficiency, with a % Emulsification value of 46.45 ± 0.268% and surface tension reduction to 31.23 ± 0.18 mN/m. Conversely, pH 8.0 resulted in a lower dry cell mass of 0.87 ± 0.005 Gm/100ml, but biosurfactant production was relatively higher, with a yield of 1.29 ± 0.007 gm/lit. However, biosurfactants produced at pH 8.0 showed slightly lower emulsification efficiency and surface tension reduction compared to pH 7.0.

In *P. aeruginosa* (P11), pH 7.0 and pH 8.0 exhibited similar trends, with pH 7.0 supporting the highest dry cell mass of 0.38 ± 0.002 Gm/100ml and pH 8.0 yielding the highest biosurfactant production of 2.11 ± 0.012 Gm/lit. Biosurfactants produced at pH 8.0 demonstrated significant emulsification efficiency, with a % Emulsification value of 47.21 ± 0.273%, and surface

tension reduction to 28.55 ± 0.165 mN/m. pH 5.0 and pH 6.0 showed lower dry cell masses and biosurfactant production compared to pH 7.0 and pH 8.0.

Table 4 Effect of pH on production and activity of biosurfactants

Sr. No	pH	DCM g/100mL	Biosurfactant g/L	% Emulsification	Surface tension
1	*A. xylosoxidans* (P5c) + pH 5.0	0.96 ± 0.006	0.87 ± 0.005	26.31 ± 0.152[a]	41.12 ± 0.237
2	*A. xylosoxidans* (P5c) + pH 6.0	1.23 ± 0.007	0.98 ± 0.006	32.15 ± 0.186	37.86 ± 0.219
3	*A. xylosoxidans* (P5c) + pH 7.0	1.32 ± 0.008	1.44 ± 0.008	46.45 ± 0.268[b]	31.23 ± 0.18[b]
4	*A. xylosoxidans* (P5c) + pH 8.0	0.87 ± 0.005	1.29 ± 0.007[a]	39.78 ± 0.23	34.96 ± 0.202[a]
5	*P. aeruginosa* (P11) + pH 5.0	0.29 ± 0.002[a]	0.81 ± 0.005	25.64 ± 0.148[a]	34.89 ± 0.201[a]
6	*P. aeruginosa* (P11) + pH 6.0	0.31 ± 0.002[a]	1.29 ± 0.007[a]	34.78 ± 0.201	30.33 ± 0.175[b]
7	*P. aeruginosa* (P11) + pH 7.0	0.38 ± 0.002	2.74 ± 0.016	56.69 ± 0.327	26.12 ± 0.151
8	*P. aeruginosa* (P11) + pH 8.0	0.15 ± 0.001	2.11 ± 0.012	47.21 ± 0.273[b]	28.55 ± 0.165

Any value followed by ± is the standard error of the mean. The values with common superscripts denote similar means at $p<0.05$ level of significance.

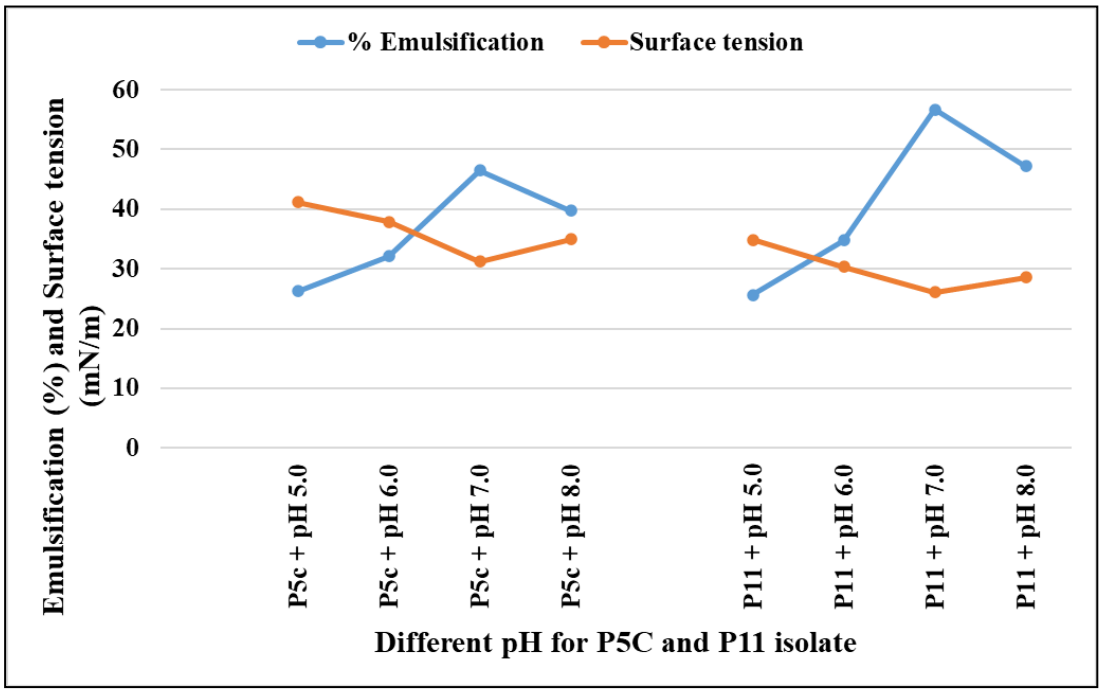

Figure 4 Effect of different pH on emulsification and surface tension activity of *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11) s

3.5 Effect of salt on production and activity of biosurfactants

The effect of different salt (sodium chloride) concentrations on biosurfactant production and activity was investigated, showing significant variations in microbial growth, biosurfactant production, emulsification efficiency, and surface tension at $p<0.05$ level of significance (Figure 5). Across different salt concentrations tested in both *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11), the microbial growth and biosurfactant production were maximum between 1 to 2 % salt concentration, however no growth at 4 % and 5 % salt concentrations was reported. It was also observed that *P. aeruginosa* (P11) tolerated higher salt concentration compared to *A. xylosoxidans* (P5c). The results are given in Table 5.

In *A. xylosoxidans* (P5c), the absence of salt treated as control (0%) supported moderate dry cell masses and biosurfactant production, with values of 1.12 ± 0.006 gm/100ml and 1.23 ± 0.007 gm/lit, respectively. Biosurfactants produced under these conditions exhibited moderate emulsification efficiency (30.58 ± 0.177%) and surface tension reduction (41.33 ± 0.239 mN/m). As salt concentration increased, microbial growth and biosurfactant production decreased. At 1% salt concentration, dry cell mass and biosurfactant production increased significantly, with values of 1.87 ± 0.011 gm/100ml and 1.94 ± 0.011 gm/lit, respectively, resulting in higher emulsification efficiency (42.69 ± 0.246%) and surface tension reduction (27.45 ± 0.158 mN/m). However, beyond 1% salt concentration, biosurfactant production decreased gradually, with no detectable biosurfactant production observed at 4% and 5% salt concentrations.

Similarly, in *P. aeruginosa* (P11), the absence of salt (0%) supported moderate dry cell masses and biosurfactant production, with values of 0.78 ± 0.005 Gm/100ml and 0.91 ± 0.005 Gm/lit, respectively. Biosurfactants produced under these conditions exhibited moderate emulsification efficiency (25.12 ± 0.145%) and surface tension reduction (56.98 ± 0.329 mN/m). As salt concentration increased, microbial growth and biosurfactant production initially increased. At 2% salt concentration, the highest dry cell mass (0.95 ± 0.005 gm/100ml) and biosurfactant production (2.06 ± 0.012 Gm/lit) were observed, resulting in higher emulsification efficiency (44.81 ± 0.259%) and surface tension reduction (25.45 ± 0.147 mN/m). However, beyond 2% salt concentration, biosurfactant production decreased gradually, with no detectable biosurfactant production observed at 5% salt concentration.

Table 5 Effect of salt concentrations on production and activity of biosurfactants

Sr. No	Salt	DCM (g/100ml)	Biosurfactant (g/lit)	% Emulsification	Surface tension
1	*A. xylosoxidans* (P5c) + 0 %	1.12 ± 0.006	1.23 ± 0.007	30.58 ± 0.177	41.33 ± 0.239[a]
2	*A. xylosoxidans* (P5c) +1 %	1.87 ± 0.011	1.94 ± 0.011	42.69 ± 0.246	27.45 ± 0.158
3	*A. xylosoxidans* (P5c) + 2 %	1.33 ± 0.008	1.05 ± 0.006	28.74 ± 0.166	33.61 ± 0.194
4	*A. xylosoxidans* (P5c) + 3 %	0.71 ± 0.004	0.42 ± 0.002	24.22 ± 0.14[a]	39.08 ± 0.226
5	*A. xylosoxidans* (P5c) + 4 %	0	0	0	0
6	*A. xylosoxidans* (P5c) + 5 %	0	0	0	0
7	*P. aeruginosa* (P11) + 0 %	0.78 ± 0.005	0.91 ± 0.005	25.12 ± 0.145[a]	56.98 ± 0.329
8	*P. aeruginosa* (P11) +1 %	0.82 ± 0.005	1.75 ± 0.01	31.97 ± 0.185	42.35 ± 0.245[a]
9	*P. aeruginosa* (P11) + 2 %	0.95 ± 0.005	2.06 ± 0.012	44.81 ± 0.259	25.45 ± 0.147
10	*P. aeruginosa* (P11) + 3 %	0.64 ± 0.004	1.36 ± 0.008	27.29 ± 0.158	27.91 ± 0.161
11	*P. aeruginosa* (P11) + 4 %	0.29 ± 0.002	0.13 ± 0.001	21.74 ± 0.126	35.2 ± 0.203
12	*P. aeruginosa* (P11) + 5 %	0	0	0	0

Any value followed by ± is the standard error of the mean. The values with common superscripts denote similar means at $p<0.05$ level of significance.

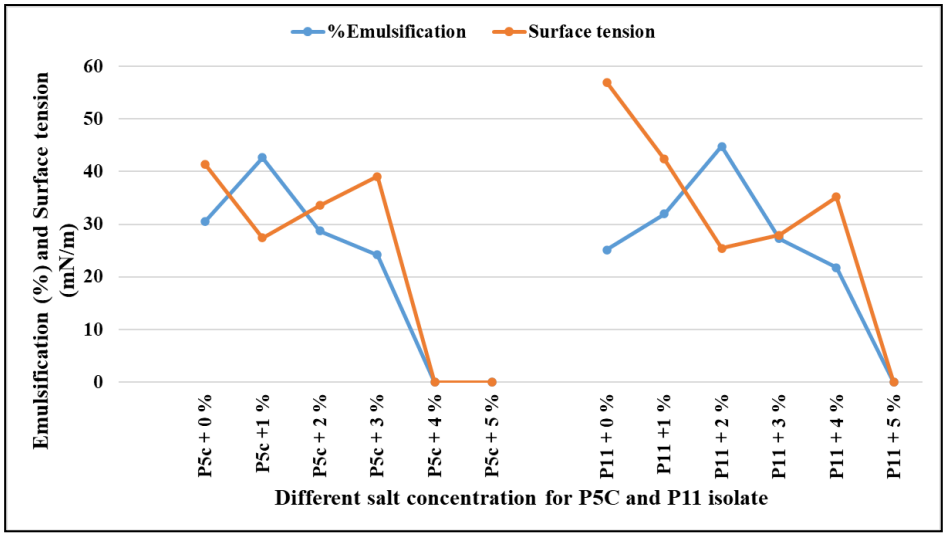

Figure 5 Effect of different salt (NaCl) concentration on emulsification and surface tension activity of *A. xylosoxidans* (P5c) and *P. aeruginosa* (P11) s

4. CONCLUSION

The present study successfully identified and optimized key physical and biochemical parameters influencing biosurfactant production by two potent bacterial isolates, *Achromobacter xylosoxidans* (P5c) and *Pseudomonas aeruginosa* (P11), using the One Variable At a Time (OVAT) method. Among the tested carbon sources, glucose and glycerol significantly enhanced biosurfactant yield, emulsification index, and biomass production, while ammonium nitrate proved to be the most effective nitrogen source. Optimal production was achieved at 35 °C and neutral pH (7.0), conditions that supported both microbial growth and metabolic activity. Moreover, moderate salt concentrations (1–2%) positively influenced biosurfactant synthesis, with P. aeruginosa displaying a higher tolerance to saline stress compared to *A. xylosoxidans*.

These findings underscore the critical role of environmental and nutritional conditions in optimizing biosurfactant production and highlight the value of empirical approaches like OVAT as a preliminary step toward process development. While the study provides a foundational framework for improving biosurfactant yields, further work involving advanced statistical optimization (e.g., Response Surface Methodology) and scale-up studies is warranted to enhance industrial applicability. The isolates used in this study, particularly *P. aeruginosa* (P11), show promising potential for cost-effective and sustainable biosurfactant production in diverse biotechnological applications.

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CONFLICT OF INTEREST

The author declares that there is no conflict of intreat.

AUTHORS CONTRIBUTION

All author s has made a substantial direct and intellectual contribution to work, and approved it for publication

Data Availability

All data set analysed during this study are included in the manuscript

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