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SYSTEMATIC OPTIMIZATION OF CULTURE CONDITIONS FOR HIGH-YIELD BIOSURFACTANT SYNTHESIS BY *STUTZERIMONAS STUTZERI* PS1

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ABSTRACT

Biosurfactants are versatile compounds produced by bacteria that have several industrial and biomedical applications. In the present study, 12 bacterial strains were isolated from oil-contaminated soil and subjected to primary screening for biosurfactant activity. Initial screening on a CTAB-MB agar plate showed blue halos, confirming the glycolipid nature of the produced biosurfactant. The most potent isolate, identified as *Stutzerimonas stutzeri* PS1 using 16S rRNA sequencing. Growth dynamics were monitored through growth curve analysis, revealing a maximum biomass of 8.87±0.02 g/L and peak biosurfactant yield of 4.66 ± 0.57 g/L. Under these conditions, the *Stutzerimonas stutzeri* PS1 demonstrated significant surface activity, reducing surface tension to 43.33 ± 0.57 mN/m with an emulsification index (E_{24}) of 47.43 ± 2.22 %. Systemic optimization of nutritional parameters identified glycerol and urea as the ideal carbon and nitrogen sources, respectively. The peak production was achieved at a physiological pH of 7 and an incubation temperature of 37°C. These findings highlight *Stutzerimonas stutzeri* PS1 as a significant and efficient candidate for scalable, sustainable production of biosurfactant for biotechnological and biomedical applications.

KEYWORDS: Biosurfactant, Glycerol, *Stutzerimonas stutzeri* PS1, Emulsification index, Optimization.

1. INTRODUCTION

Over the past few decades, the search for novel, biocompatible therapeutic agents has become essential for modern medical biotechnology. While surfactants are crucial in pharmaceuticals, serving as drug delivery agents, antimicrobial agents, and stabilizers. (Bjerk et al., 2021) However, these methods often rely on the synthetic surfactants such as Sodium Lauryl Sulfate (SLS) or Linear Alkylbenzene Sulfonates (LAS). They act as secondary pollutants due to their high toxicity and poor biodegradability (Bondi et al., 2015; Bradai et al., 2016). Beyond environmental remediation, the use of synthetic surfactants has increased across various agricultural and cosmetic sectors (Castro et al., 2013; Wirtu, 2024). Similarly, in the medical field, chemically synthesized surfactants are routinely utilized in drug delivery and as stabilizers in pharmaceutical formulations (Nagaraj and Kamalesu, 2025). Despite their use, chemically synthesized surfactants often carry serious side effects, including skin irritation, cellular toxicity, and long-term environmental persistence (Ying, 2006; Nagaraj and Arunachalam, 2013; Collivignarelli et al., 2019).

In response to these challenges, biosurfactants have emerged as a sustainable and eco-friendly alternative (Nagaraj and Kamalesu, 2025). The microbial-derived molecules offer several advantages over their chemical counterparts, including lower toxicity, greater biocompatibility, biodegradability, and enhanced thermal tolerance (Zalic et al., 1983; Cameotra and Makkar, 1998). Moreover, their amphiphilic nature enables them to reduce surface tension more effectively than synthetic agents (Abbot et al., 2022). Biosurfactants are broadly classified based on their chemical structures into glycolipids, lipopeptides, fatty acids, and polymeric surfactants. Among these classes, rhamnolipids are a type of glycolipid that consists of rhamnose sugar molecules bound with β-hydroxyl fatty acid chains (Shu et al., 2021).

Numerous studies have highlighted that oil-contaminated soils are unique ecological niches. These niches are used to isolate novel microbial strains capable of high-yield glycolipid production (Nkosi et al., 2024). *Pseudomonas aeruginosa* is a well-documented glycolipid producer (Rikalović et al., 2012). However, its opportunistic pathogenic nature (classified as a Biosafety Level 2 organism) has become a significant barrier to its application in the pharmaceutical, food industries, and various biomedical sectors (Azam 2019). Consequently, *Stutzerimonas stutzeri* has gained attention for its glycolipid production (Sony et al., 2025). Recent genomic and taxonomic reassessments have moved this species from the genus *Pseudomonas* to *Stutzerimonas* due to its distinct metabolic versatility (Gomila et al., 2022). It is well known for its superior emulsifying properties, stability in extreme pH and Temperature, and high biodegradability (Sony et al., 2025).

In this work, we identified the isolate as *Stutzerimonas stutzeri* PS1 and provide a detailed analysis of its growth kinetics and surface-active properties. Furthermore, we optimized the growth conditions to achieve peak biosurfactant yields, assessing the resulting compounds ability to reduce surface tension and stabilize emulsion. These findings establish *Stutzerimonas stutzeri* PS1 as a notable candidate for the scalable production of versatile glycolipids.

2. MATERIALS AND METHODS

2.1 Isolation of microorganism

Soil samples were collected from oil-contaminated sites in the Kanchanwadi and Ghanwade villages of Radhanagari Tehsil, Kolhapur District. Samples were obtained at a depth of 12 cm from the surface. A total of five soil samples were randomly collected in sterile containers, transported to the laboratory, and stored at 4°C until further use. To enrich for biosurfactant-producing microorganisms, 1g of each soil sample was inoculated into separate flasks containing 100 mL of sterile Bushnell-Haas (BH) broth. The flasks were incubated at 37°C in a shaking incubator (120 rpm) for 7 days. Following enrichment, the culture suspensions were serially diluted in sterile saline from 10^{-1} to 10^{-9}. A 0.1 mL aliquot of the 10^{-7} to 10^{-9} dilutions was spread onto sterile nutrient agar plates and incubated at 37°C for 48 hours. After incubation, twelve colonies exhibiting distinct morphologies were selected and purified. The isolated strains were maintained on sterile nutrient agar slants at 4°C and subsequently screened for biosurfactant production.

2.2 Screening of biosurfactant producers

The screening and selection of biosurfactant-producing bacteria were conducted using the following tests:

2.2.1 Hemolytic activity

Hemolytic activity was evaluated using sterile blood agar plates supplemented with 5% (v/v) sheep blood. The isolates were inoculated onto the plates and incubated at 37°C for 24-48 hours. *Lactobacillus* sp. served as the negative control. The formation of a clear zone around the colonies (indicating β-hemolysis) confirmed biosurfactant production, as these molecules cause the lysis of red blood cells (Vigneshwaran et al., 2018).

2.2.2 CTAB-MB agar method

CTAB-MB agar plates were prepared using 15 g/L bacteriological agar, 0.005 g/L methylene blue (MB), and 0.2 g/L N-Cetyl-N,N,N-trimethylammonium bromide (CTAB). CTAB is a cationic surfactant, while methylene blue is a positively charged dye. The anionic nature of the produced biosurfactants allows them to react with the cationic CTAB and MB dye, forming an insoluble ion pair. This complex precipitates to form a distinct dark blue halo around the well, indicating a positive result. Distilled water was used as the negative control, while Triton X-100 served as the positive control (Siegmund and Wagner, 1991).

2.2.3 Oil displacement test

20 mL of distilled water was added to a clean petri dish, followed by 100 µL of Sudan black-stained sunflower oil placed at the centre to form a uniform layer. Subsequently, 10 µL of cell-free supernatant was added onto the oil layer, and the resulting zone of displacement was observed. Distilled water and Triton X-100 were used as negative and positive controls, respectively (Sharma and Pandey, 2020).

2.2.4 Emulsification index

The cell-free supernatant was tested for emulsification activity by measuring the emulsification index (E24%) using xylene as a substrate. Specifically, 1.5 mL of xylene was mixed with an equal volume of cell-free supernatant. The mixture was vortexed for 2 minutes to ensure thorough mixing and then left undisturbed for 24 hours. The emulsification index (E24%) was calculated using the following equation (Lai et al., 2009).

$$(E_{24}\%) = \frac{\text{Total height of emulsified layer (mm)}}{\text{Total height of liquid layer (mm)}} \quad X\ 100$$

2.2.5 Biuret test

2mL of cell-free supernatant was first heated at 70°C and then mixed with 1 M NaOH solution. A 1% $CuSO_4$ solution was added dropwise, and the color change was monitored. The reaction between the copper ions and the peptide bonds in proteins resulted in the formation of a pink or violet-colored ring, signifying a positive result. This test was used for the detection of lipopeptide biosurfactants (Feignier et al., 1995).

2.2.6 Phosphate test

2 mL of supernatant was mixed with 10 drops of 6M HNO_3 solution and heated at 70°C. Subsequently, a 5% ammonium molybdate solution was added dropwise. The formation of a yellow color, followed by a fine yellow

precipitate, indicated the presence of phospholipid biosurfactants (Okpokwasili and Ibiene, 2006).

2.2.7 Benedict's test

Benedict's test was employed for the qualitative detection of reducing sugar moieties in the compound. This assay is based on the color change resulting from the reaction with free carbonyl groups. Briefly, 1 mL of cell-free supernatant was mixed with 2 mL of Benedict's reagent and heated in a boiling water bath for 3 to 5 minutes. The subsequent color change –ranging from green to brick-red-was observed as an indicator of reducing sugar presence (Hernández-López et al., 2020).

2.2.8 Measurement of surface tension

An isolated bacterial strain was cultured in Bushnell-Haas medium for 144 h at 37 °C and 180 rpm. Following incubation, the culture was centrifuged at 5000 rpm for 20 min, and the supernatant was used to measure surface tension at 25°C by the Du Nouy ring method using a tensiometer (CSC Scientific Company). To ensure the precision of the measurements, the results were reported as the average of three independent readings. (Gudina et al., 2010).

2.3 Identification of bacterial isolate

Genomic DNA was isolated from the selected strain for identification. A fragment of the 16S rDNA gene was amplified using the universal primers 1492R and 27F (Eurofins, India). Forward and reverse DNA sequencing reactions were performed using the BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer. A consensus sequence was generated, and multiple sequence alignment was conducted using ClustalW. A phylogenetic tree was subsequently constructed via the maximum likelihood method using MEGA version 7 (Dastager et al., 2009).

2.4 Growth curve

The growth curve of *Stutzerimonas stutzeri* PS1 was monitored to understand the growth dynamics of a microbial culture over time. The experiment was conducted in 500 mL of sterile Bushnell-Haas (BH) medium supplemented with 1% sucrose as the carbon source, under orbital shaking at 180 rpm and a temperature of 37°C. Samples were collected at 24-hour intervals for up to 168 hrs. Each collected sample was centrifuged at 5000 rpm for 30 min. The resulting pellet was used for biomass estimation, while the supernatant was used to measure pH, emulsification index, surface tension, and biosurfactant yield (Lima et al., 2024).

2.5 Optimization of Carbon and Nitrogen Sources

To investigate the effects of various carbon and nitrogen sources on biosurfactant production, an optimization study was conducted. Different carbon sources (1%, w/v), such as sucrose, glucose, starch, glycerol, and sunflower oil, were used as the sole carbon source in Bushnell-Haas medium with basal NH_4Cl as the nitrogen source. Subsequently, five different nitrogen sources (1% w/v)-yeast extract, ammonium sulfate, ammonium chloride, urea, and peptone- were evaluated alongside the optimized carbon source. The experiments were performed over 7 days at 37°C and 180 rpm. The emulsification index was measured daily to identify the most suitable carbon and nitrogen sources, which were employed for further studies.

2.6 Effect of physiological conditions on growth and biosurfactant production

The optimized medium was used to determine the ideal physiological conditions for biosurfactant production by *Stutzerimonas stutzeri* PS1. Various pH levels (4.0, 5.0, 6.0, 7.0, and 8.0) and temperature ranges (25°C, 37°C, 40°C, 45°C, and 50°C) were evaluated. For each physiological factor, the emulsification index was monitored daily from the 1st to the 7th day of incubation to assess its impact on biosurfactant production.

2.7 Statistical analysis

Data were analyzed using GraphPad Prism software. All experiments were performed in triplicate, and results are presented as mean ± standard deviation (SD).

3. RESULT AND DISCUSSION

3.1 Screening and isolation of biosurfactant producers

A total of 12 bacterial strains were isolated from oil-contaminated soil samples using the enrichment method in sterile BH broth. Among these, five isolates demonstrated biosurfactant production, as indicated by the formation of dark blue halos around the wells on CTAB-methylene blue (CTAB-MB) agar plates. These isolates were further characterized using several screening assays, including hemolytic activity, oil displacement, and emulsification index (E_{24}). Additionally, the cell-free supernatant was subjected to the Biuret, Benedict's, and phosphate tests to determine the chemical nature of the biosurfactant **(Fig. 1).** The resulting data are compiled in (**Table 1)**.

The most potent isolate exhibited superior surface-active properties, characterized by a significant oil displacement, the presence of reducing sugars, and a high emulsification index. Consequently, this strain was identified via 16S rRNA gene sequencing and deposited in GenBank under the accession number PQ274421.

3.2 Identification of biosurfactant producer

Isolate PS1 was identified as *Stutzerimonas stutzeri* strain PS1 based on 16s rRNA gene sequencing and phylogenetic analysis. The 1474 bp nucleotide sequence was obtained from forward and reverse sequencing of the isolate. The sequence was submitted to the NCBI GenBank database, where it was assigned the accession number PQ274421. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. Nucleotide homology and phylogenetic analysis revealed that the isolate (PQ274421) exhibited high similarity with other

Stutzerimonas stutzeri strains, as illustrated in the phylogenetic tree (**fig 2).**

3.3 Growth curve analysis

The growth curve illustrated the relationship between incubation time and biosurfactant production over 168 h at 24 h intervals. Biosurfactant production increased steadily from 24 h to 144 h, reaching a maximum yield of 4.66± 0.57 g/L, before declining at 168 h. In contrast, biomass production increased linearly from 24 h to 96 h (2.21±0.01 to 8.87± 0.02 g/L) and then declined at 120 h (7.60 ± 0.01g/L), after which it reached a steady state. These results indicate that maximum biosurfactant production occurred during the stationary phase (**Fig 3).** Furthermore, the emulsification index (E_{24}) increased from 3.84± 0.00% to 37.17± 2.22% between 24 h and 96 h, peaking at 47.43± 2.22 % at 144 h. Simultaneously, the surface tension was reduced from 75.33 ± 0.57 mN/m to 43.33 ± 0.57 mN/m. A slight initial increase in medium pH was observed, likely due to metabolic reactions during microbial growth (Lima et al., 2024). Similarly, da silva et al. reported efficient biosurfactant production from *C. mogii* using mineral-based medium, reducing surface tension from 71.042 ± 0.021 to 28.662 ± 0.563 mN/m (da Silva et al., 2024). (Albasri et al., 2024) also observed a maximum biomass of 4.256 g/L and a biosurfactant yield of 0.574 g/L after 5 days of cultivation using *Geobacillus stearothermophilus* BGSC 9A20.

3.4 Effect of different carbon and nitrogen sources

Optimizing carbon and nitrogen sources is essential for achieving maximum growth and efficient biosurfactant production. In this study, various sources (previously mentioned) were evaluated to determine their impact on yield. The emulsification index (E_{24}) served as a reliable quantitative measure to assess and compare biosurfactant production and the emulsifying efficiency of the culture conditions. Among the tested substrates, glycerol and urea yielded the highest emulsification indices of 48.71±2.22% and 51.28±2.21%, respectively. Consequently, glycerol and urea were identified as the most suitable carbon and nitrogen sources for biosurfactant production by this strain (**fig 4).**

The ability of biosurfactants to emulsify hydrophobic substances is a critical characteristic for assessing their potential industrial significance in remediation technologies, medicine, and cosmetics (Lovaglio et al., 2011). Maťátková et al. investigated rhamnolipid production by *Pseudomonas aeruginosa* DBM 3374 using an RSM-predicted medium; their findings showed emulsification activity against hexane (54.9%) and crude oil (54.5%) (Maťátková et al., 2022). Similarly, (Chaida et al., 2021) evaluated the emulsification index of rhamnolipids isolated from *Pseudomonas sp*. LGMS7, using a mineral salts medium with 2% glycerol as a carbon source, and reported 66.66% activity. In addition to carbon sources, nitrogen sources are vital for optimization. For instance, *Pseudomonas sp*. S2WE synthesizes rhamnolipids using a combination of glycerol and urea, achieving an emulsification index of 60.65±0.64% (Phulpoto et al., 2020a).

3.5 Effect of different pH and temperature

Temperature and pH are two critical factors that regulate microbial growth, metabolism, and enzymatic activity. The biosurfactant produced by *Stutzerimonas stutzeri* PS1 exhibited stable emulsification across a wide pH range. Under acidic conditions, the emulsification activity was lower, peaking at pH 7.0 (52.56±2.21%). Furthermore, 37°C was found to be the optimal temperature for biosurfactant production; After 6 days of incubation, the biosurfactant reached its highest emulsification index of 55.12±2.22% (**fig 5).** These findings align with studies by Ikhwani et al., who examined a pH range of 1-10 and recorded the highest emulsification value at pH 7 against diesel (27.35%) (Ikhwani et al., 2017). Similarly, *Pseudomonas aeruginosa* PBSC1-derived biosurfactant and Pseudomonas sp. CQ2 exhibited maximum emulsification activities of 75.12% and 72%, respectively, at pH 7.0 (Nagar 2014; Sun et al., 2020). Conversely, (Marchut-Mikolajczyk et al., 2021) found that 30°C was the optimal temperature for biosurfactant production by *Bacillus pumilus* 2A, achieving 69.11% emulsification activity. Our study confirms that a temperature of 37°C and a pH of 7.0 are the ideal conditions for maximizing biosurfactant yield, consistent with previous reports (Asgher et al., 2020a; Asgher et al., 2020b).

Figures

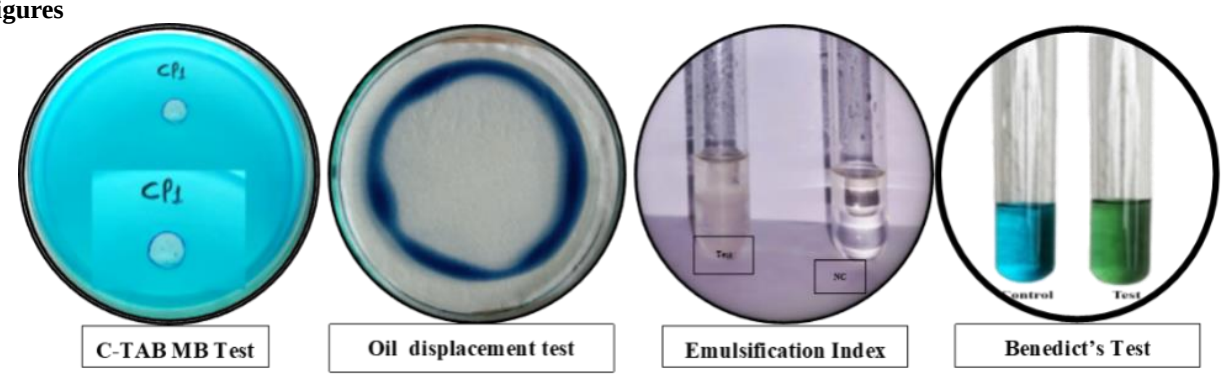


Fig 1: Screening of biosurfactant producer.

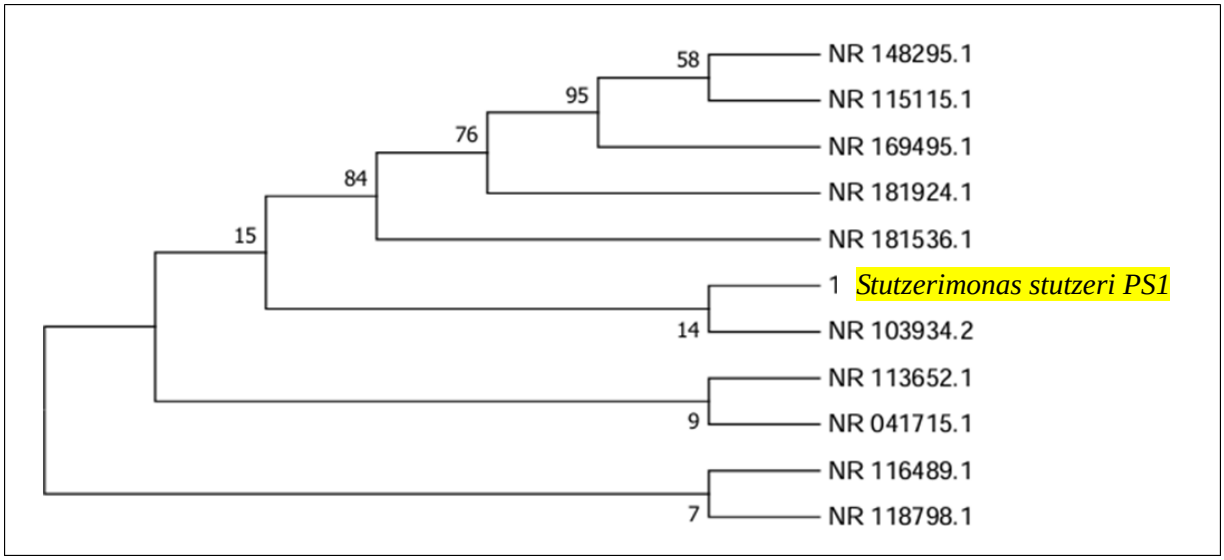


Fig. 2: Maximum likelihood phylogenetic tree of *Stutzerimonas stutzeri* PS1.

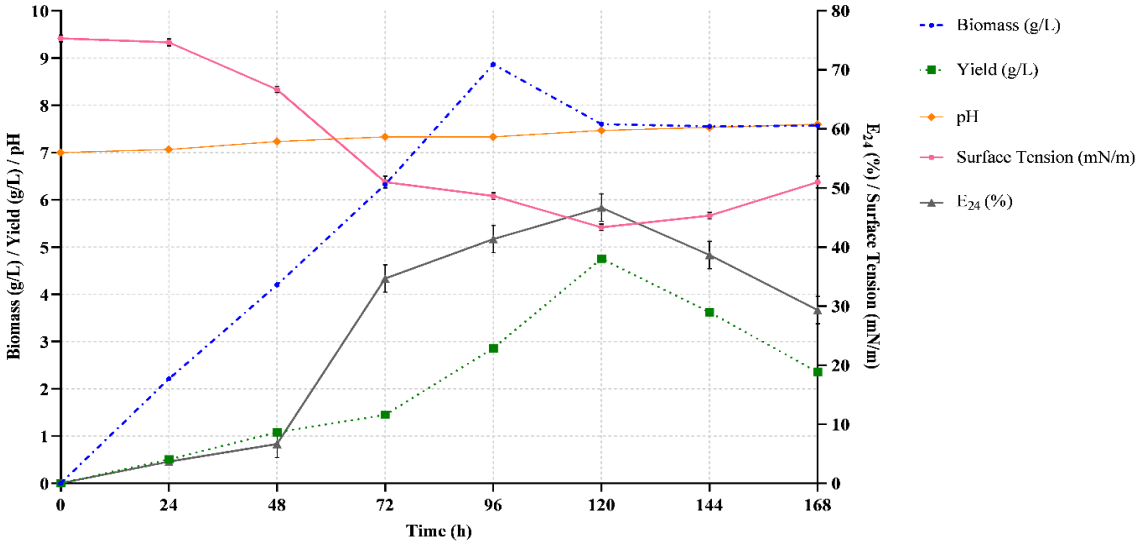


Fig. 3: Growth curve of *Stutzerimonas stutzeri* PS1 grown for 168 h at 180 rpm and 37°C in Bushnell and Haas (BH) medium supplemented with 1% sucrose.

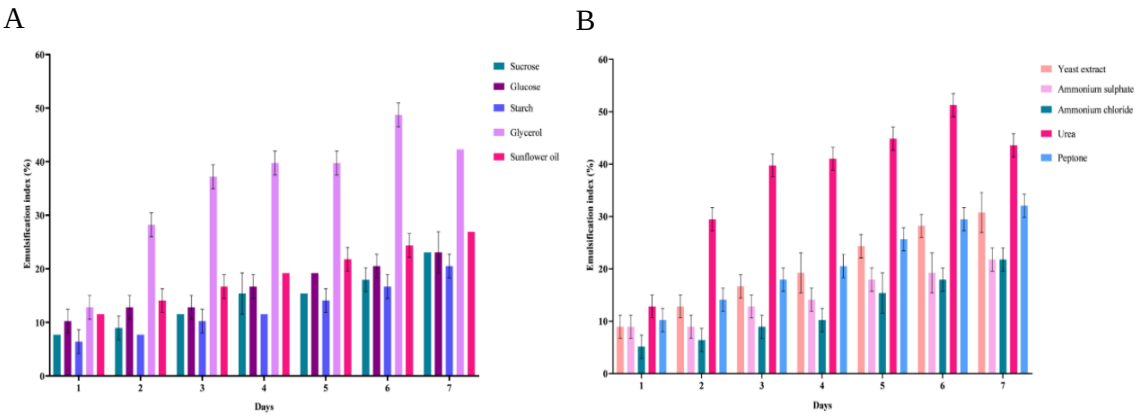


Fig. 4: Effect of carbon (A) and nitrogen (B) source on activity of biosurfactant produced by *Stutzerimonas stutzeri* PS1 strain.

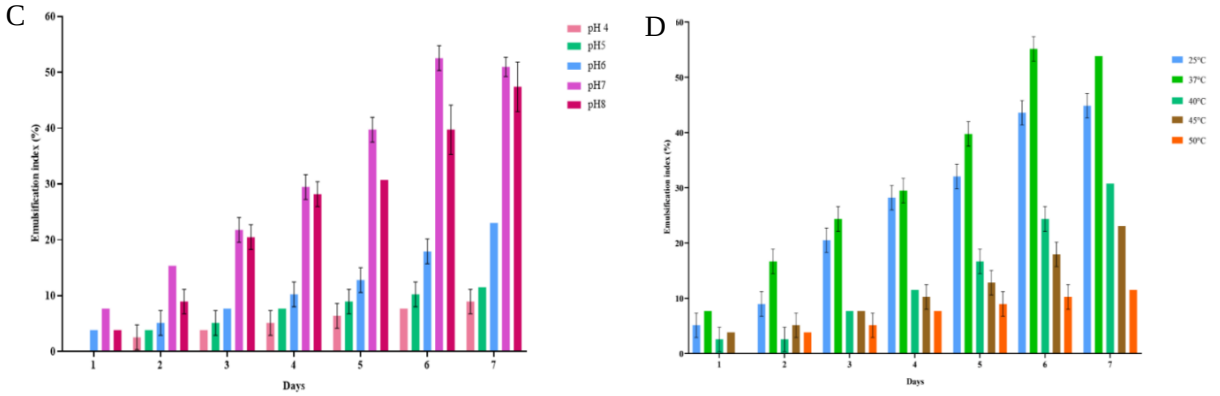


Fig. 5: Effect of pH (C) and temperature (D) on activity of biosurfactant produced by *Stutzerimonas stutzeri* PS1 strain.

Table 1: Screening tests for biosurfactant producing strains.

Isolate	Hemolytic activity	CTAB-MB agar	Emulsification index	Oil displacement	Biuret test	Phosphate test	Benedict's test
CP1	NIL	++	44%	++	NIL	NIL	++
MG1	+	+	32%	+	+	NIL	+
BH1	NIL	NIL	NIL	NIL	+	+	NIL
BH2	NIL	NIL	NIL	NIL	+	NIL	NIL
BH3	NIL	+	20%	+	NIL	NIL	NIL
BH4	NIL	NIL	NIL	NIL	NIL	NIL	NIL
BH5	NIL	NIL	NIL	NIL	NIL	NIL	NIL
BH6	NIL	+	24%	+	NIL	NIL	+
BH7	NIL	NIL	NIL	NIL	NIL	NIL	+
BH8	+	+	24%	+	NIL	+	NIL
BH9	+	+	28%	+	+	+	NIL
BH10	++	++	28%	++	+	+	+

Abbreviations- CTAB-MB- Cetyl trimethylammonium bromide methylene blue, (++) – Good activity, (+) – poor activity, NIL- No result.

4. CONCLUSION

Overall, the systematic optimization of *Stutzerimonas stutzeri* PS1 revealed that glycerol and urea are the most effective substrates for maximizing glycolipid output. The resulting biosurfactant's high emulsification index (47.43%) and significant surface activity under physiological conditions suggest that this strain is an ideal candidate for industrial-scale fermentation processes where cost-efficient carbon sources are prioritized.

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Author contributions: CRediT

Suvarna Umape: Original draft, Validation, Methodology, Conceptualization; Nisha Nerlekar: Writing – Original draft, Validation, Methodology, Conceptualization; Padma B. Dandge: Supervision, Methodology, Conceptualization.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY

The data are available from the corresponding author upon request.

FUNDING

The authors reported there is no funding associated with the work featured in this article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol does not include animals or humans for the experiments.

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