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Functional superiority and robust stability of biosurfactants from a *bacillus-pseudomonas* consortium cultivated on Cola hispida pod Agro-waste

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Abstract

For biosurfactants to transition from laboratory discoveries to industrially viable products, two critical factors must be addressed: cost-effective production and demonstrably superior, stable performance. This study aimed to produce, quantify, and characterize the functional performance and stability of biosurfactants synthesized by a Bacillus sp., a Pseudomonas sp., and a defined consortium of both, using Cola hispida pod a validated agro-waste substrate in submerged fermentation. Following acid precipitation and extraction, biosurfactant activity was rigorously assessed through standard assays. The consortium-produced biosurfactant demonstrated exceptional efficacy, achieving the highest emulsification index (E_{24}) of 73.33% with kerosene, the largest oil displacement diameter (3.23 cm), and the most significant reduction in surface tension (to 38.0 mN/m). A pivotal finding was that the medium containing solely Cola hispida pod as the nutrient source consistently yielded biosurfactants with superior emulsifying activity compared to media supplemented with pure glucose or commercial nutrients. All biosurfactants exhibited remarkable resilience, retaining over 50% emulsification capacity across a wide pH spectrum (2–10), at elevated temperatures up to 90°C, and in the presence of high NaCl concentrations (up to 10% w/v). These results highlight two transformative insights: firstly, the engineered microbial consortium exhibits clear synergistic interaction, producing a biosurfactant with enhanced functional properties compared to its constituent monocultures. Secondly, Cola hispida pod is not merely an alternative but a preferable, low-cost substrate for generating high-performance, industrially robust biosurfactants. This work validates an integrated sustainable platform for producing potent, stable surfactants suitable for demanding applications in environmental remediation, enhanced hydrocarbon recovery, and formulation chemistry.

Keywords: Biosurfactant activity; Emulsification index; Oil displacement assay; Surface tension reduction; pH and thermal stability; Salinity tolerance; Microbial synergy; Cola hispida pod

1. Introduction

The compelling case for microbial biosurfactants as sustainable alternatives to petrochemical-derived counterparts rests on their intrinsic environmental compatibility high biodegradability, low toxicity, and renewable origins [1,2]. However, for these advantages to translate into widespread market adoption, the produced biomolecules must

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consistently demonstrate functional performance that meets stringent industrial benchmarks. This performance is quantified by key interfacial properties: the capacity to significantly lower surface and interfacial tension, effectively emulsify and disperse hydrophobic compounds, and maintain these activities under the physically and chemically challenging conditions often encountered in real-world applications [3,4]. Parameters such as the emulsification index (E_{24}), oil displacement area, and stability across pH, temperature, and salinity gradients are therefore critical metrics of biosurfactant quality and applicability [5].

The functional profile of a biosurfactant is not an intrinsic, fixed property but is profoundly shaped by the interplay between the producing microorganism(s) and the fermentation environment. While pure cultures of genera like Bacillus and Pseudomonas are renowned for producing potent surfactants like lipopeptides and rhamnolipids [6,7], the strategic use of defined microbial consortia is gaining traction. Synergistic interactions within such consortia can lead to enhanced product yields or the synthesis of surfactant blends with complementary or superior functional characteristics compared to those from individual strains [8,9]. Equally influential is the fermentation substrate. The carbon and nutrient source can direct microbial metabolism toward specific biosynthetic pathways, ultimately affecting the structural class, congener composition, and thus, the functional efficacy of the resulting biosurfactant [10].

In our foundational studies, we established the nutritional viability of Cola hispida pod agro-waste [11] and demonstrated its successful use in cultivating biosurfactant-proficient Bacillus and Pseudomonas isolates, both individually and in consortium [12]. Building upon this established bioprocess platform, the present study aimed to critically evaluate and compare the functional characteristics of the biosurfactants produced. Our specific objectives were to: (i) quantify and compare the emulsification capacity, surface activity, and oil displacement ability of biosurfactants from monocultures versus a defined co-culture; (ii) assess the influence of the Cola hispida pod substrate on biosurfactant functionality relative to conventional substrates; and (iii) determine the operational stability of these biosurfactants under extremes of pH, temperature, and salinity. We hypothesized that the consortium would leverage microbial synergy to produce a biosurfactant with enhanced activity, and that the complex nutrient matrix of the pod would induce the synthesis of biosurfactants with robust stability profiles. By providing this comprehensive functional characterization, this work directly addresses the performance validation essential for positioning these sustainably produced biosurfactants as credible candidates for industrial deployment.

2. Materials and Methods

2.1. Microbial Strains, Cultivation, and Biosurfactant Production

The bacterial isolates used were a Bacillus sp. and a Pseudomonas sp., previously isolated from hydrocarbon-contaminated soil and characterized [12]. A defined co-culture consortium was prepared by combining equal volumes (1:1 v/v) of standardized 18-hour Nutrient Broth pre-cultures of both isolates. Biosurfactant production was carried out via submerged fermentation. The primary production medium was the Basal Mineral Medium (BMM) supplemented with 14% (w/v) sterile Cola hispida pod powder (BMM/Ch), prepared as described previously [11,12]. For comparative substrate evaluation, parallel fermentations were conducted in BMM supplemented with 14% (w/v) glucose (BMM/Glu) and in commercial Nutrient Broth (NB). Fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of medium, inoculated with 2% (v/v) of the respective pre-culture (Bacillus sp., Pseudomonas sp., or co-culture). Flasks were incubated at 30°C with orbital shaking at 150 rpm for 7 days. Post-fermentation, the entire culture broth was centrifuged at 8,000 × g for 30 minutes at 4°C (Centurion Scientific K3 Series, UK). The resulting cell-free supernatant (CFS) was carefully decanted and used directly for initial qualitative and quantitative activity screening.

2.2. Biosurfactant Recovery and Concentration

For assays requiring concentrated or solvent-extracted material, biosurfactants were recovered from the CFS. The pH of the CFS was adjusted to 2.0 using 6 N HCl under constant stirring and then held at 4°C for 24 hours to facilitate complete precipitation of acidic biosurfactants (common to Bacillus and Pseudomonas). The precipitate was collected by centrifugation (8,000 × g, 30 min, 4°C). The pellet was then dissolved in a minimal volume of a 2:1 (v/v) chloroform-methanol mixture. The organic solvent was evaporated to dryness under a gentle stream of nitrogen gas in a fume hood. The resultant crude, dried biosurfactant was weighed to determine yield and subsequently re-dissolved in distilled water, phosphate buffer (pH 7.0), or appropriate solvents for specific analyses [13].

2.3. Functional Activity Screening and Assays

2.3.1. Emulsification Index (E_{24})

The emulsifying capacity of the CFS was determined using the method described by Cooper and Goldenberg [14]. Briefly, 2 mL of a hydrocarbon substrate (kerosene, diesel, used engine oil, petrol) or vegetable oil (olive oil) was added to 2 mL of CFS in a graduated glass test tube. The mixture was vortexed vigorously at high speed for 2 minutes to form an emulsion and then allowed to stand undisturbed at room temperature for 24 hours. The height of the stable emulsion layer was measured. The emulsification index (E_{24}) was calculated as:

$$E_{24}\ (\%) = (\text{Height of the emulsion layer} / \text{Total height of the liquid column}) \times 100$$

Assays were performed in triplicate for each hydrocarbon and each microbial culture.

2.3.2. Oil Displacement Test

This test assesses the wetting and spreading capability of the biosurfactant [15]. Fifty milliliters of distilled water was placed in a large Petri dish (15 cm diameter). Twenty microliters of crude oil (Bonny Light) was gently added to the center of the water surface using a micropipette, forming a thin oil slick. Subsequently, 10 µL of the CFS was carefully dropped onto the center of the oil slick. The diameter of the clear zone formed due to the displacement of oil by the biosurfactant was measured in centimeters after 30 seconds. Distilled water served as a negative control.

2.3.3. Hemolytic Activity

As a rapid, qualitative indicator of surface-active compound production, hemolytic activity was tested [16]. Aliquots (10 µL) of the CFS were spotted onto the surface of blood agar plates (containing 5% defibrinated human blood). The plates were incubated at 37°C for 48 hours. The presence of a clear, transparent zone (β-hemolysis) around the spot after incubation indicated positive hemolytic (and by proxy, surface-active) activity. The diameter of the hemolytic zone was measured and scored qualitatively (+, ++, +++).

2.3.4. Surface Tension Measurement

The surface tension of the CFS was measured using the capillary rise method, a reliable and accessible technique [17]. A clean, calibrated glass capillary tube of known internal radius (r) was vertically immersed in a sample vial containing the CFS. The height (h) to which the liquid rose inside the capillary, above the external liquid level, was measured using a travelling microscope. The surface tension (γ) was calculated using Jurin's law:

$$\gamma = (½)\ \rho\ g\ h\ r$$

where ρ is the density of the CFS (approximated as 1000 kg/m^3), g is the acceleration due to gravity (9.81 m/s^2), and r is the capillary radius in meters. The surface tension of distilled water ($\gamma \approx 72$ mN/m at 25°C) was measured daily to verify the calibration. All measurements were conducted in triplicate at a constant temperature of 25°C.

2.4. Stability Studies of the Biosurfactant

The stability of the biosurfactant present in the CFS from the BMM/Ch medium was evaluated by subjecting it to various environmental stresses and then measuring the residual emulsification activity (E_{24} with kerosene).

pH Stability: The pH of the CFS was adjusted to values ranging from 2 to 10 using either 1 N HCl or 1 N NaOH. The samples were incubated at room temperature for 1 hour, after which the pH was readjusted to 7.0 to ensure consistent measurement conditions before determining the E_{24}.

Thermal Stability: Aliquots of CFS were heated in sealed tubes at temperatures ranging from 30°C to 110°C (in increments of 20°C) in a thermostatically controlled water bath for 15 minutes. The samples were then cooled to room temperature before the E_{24} was measured.

NaCl (Salinity) Tolerance: Sodium chloride was added to the CFS to achieve final concentrations ranging from 0% to 30% (w/v). The mixtures were incubated at room temperature for 1 hour with gentle mixing before the E_{24} was determined.

2.5. Statistical Analysis

All experiments were conducted with three independent biological replicates, and analytical measurements were performed in triplicate. Data are presented as mean ± standard deviation (SD). To determine the statistical significance of differences between means (e.g., E_{24} values across different cultures or substrates), one-way Analysis of Variance (ANOVA) was performed. Where ANOVA indicated significant differences ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) post-hoc test was applied to identify which specific means differed. All statistical analyses were conducted using SPSS software (Version 25.0, IBM Corp., USA).

3. Results and Discussion

3.1. Emulsification Capacity and Hydrocarbon Substrate Preference

The biosurfactants produced by all three microbial systems demonstrated significant capacity to form and stabilize oil-in-water emulsions. A pivotal observation was that the Cola hispida pod-based medium (BMM/Ch) consistently yielded biosurfactants with superior emulsifying activity compared to those produced in media containing pure glucose (BMM/Glu) or the complex Nutrient Broth (NB) (Figure 1A). This is a crucial finding that elevates the pod from a simple growth substrate to a performance-enhancing feedstock. The complex mixture of carbohydrates, proteins, and possibly trace elements in the pod likely directs a more favorable metabolic flux toward the synthesis of effective emulsifying agents, or induces the production of a more complex and functionally robust mixture of surfactant congeners [18].

Among the various hydrophobic substrates tested, kerosene consistently elicited the highest E_{24} values from all biosurfactants (Figure 1B). The biosurfactant produced by the co-culture (SD) exhibited the highest E_{24} of 73.33 ± 1.53%, which was statistically significantly greater ($p < 0.05$, Tukey's test) than those from the Bacillus monoculture (SB, 64.62 ± 1.61%) and the Pseudomonas monoculture (SP, 59.33 ± 2.52%). This superior performance of the consortium underscores a synergistic interaction. The co-culture may produce a complementary blend of biosurfactants (e.g., lipopeptides from Bacillus and glycolipids from Pseudomonas) that act in concert to more effectively lower the interfacial tension and form a more cohesive film at the kerosene-water interface, leading to a more stable emulsion [9,19].

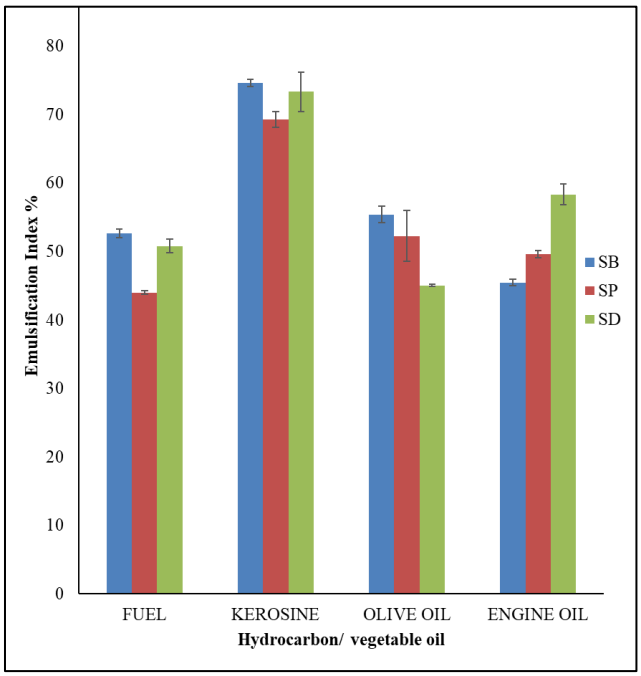


Figure 1 Emulsification potential of biosurfactants. (A) Comparison of the emulsification index (E_{24} with kerosene) of biosurfactants produced by the diculture (SD) in different nutrient media: Cola hispida pod-based medium (BMM/Ch), glucose-based medium (BMM/Glu), and Nutrient Broth (NB). (B) Substrate specificity of emulsification, showing E_{24} values of biosurfactants from Bacillus sp. (SB), Pseudomonas sp. (SP), and their diculture (SD) grown in BMM/Ch medium, tested against different hydrocarbons and olive oil. Values are mean ± SD (n=3). Bars with different lowercase letters within a panel indicate statistically significant differences ($p < 0.05$, one-way ANOVA with Tukey's HSD test)

3.2. Quantitative Surface Activity and Oil Displacement Efficacy

The high emulsification capacity was directly correlated with potent surface activity. As summarized in Table 1, all biosurfactant-containing supernatants dramatically reduced the surface tension of water from approximately 72.0 mN/m to below 40.0 mN/m. The co-culture supernatant (SD) achieved the lowest surface tension of 38.0 ± 0.9 mN/m, indicating the highest concentration and/or efficacy of surface-active molecules. This result aligns perfectly with the principle that effective emulsifiers are also potent reducers of surface tension [20].

The oil displacement test provided a visual and quantitative measure of this activity in an interfacial context. The co-culture biosurfactant again outperformed the others, producing the largest clear zone diameter of 3.23 ± 0.05 cm (Table 1). A larger displacement diameter reflects a lower interfacial tension between oil and water, allowing the biosurfactant solution to spread more effectively, thereby "pushing" the oil aside. This property is directly relevant to applications like oil spill dispersion, where rapid spreading and penetration of the surfactant are critical for breaking up oil slicks [21].

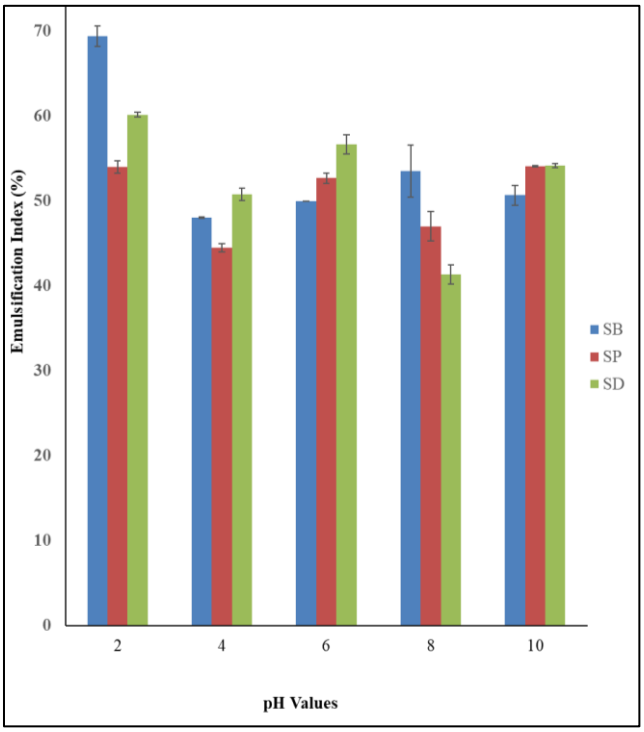
Table 1 Surface Activity and Oil Displacement Capacity of Biosurfactants from Cola hispida Pod Medium

Sample	Surface Tension (mN/m) Mean ± SD	Oil Displacement Diameter (cm) Mean ± SD
Bacillus sp. (SB) CFS	39.2 ± 0.37	2.90 ± 0.07
Pseudomonas sp. (SP) CFS	38.4 ± 0.23	2.90 ± 0.01
Diculture (SD) CFS	38.0 ± 0.09	3.23 ± 0.05
Distilled Water (Control)	72.8 ± 0.50*	0.00

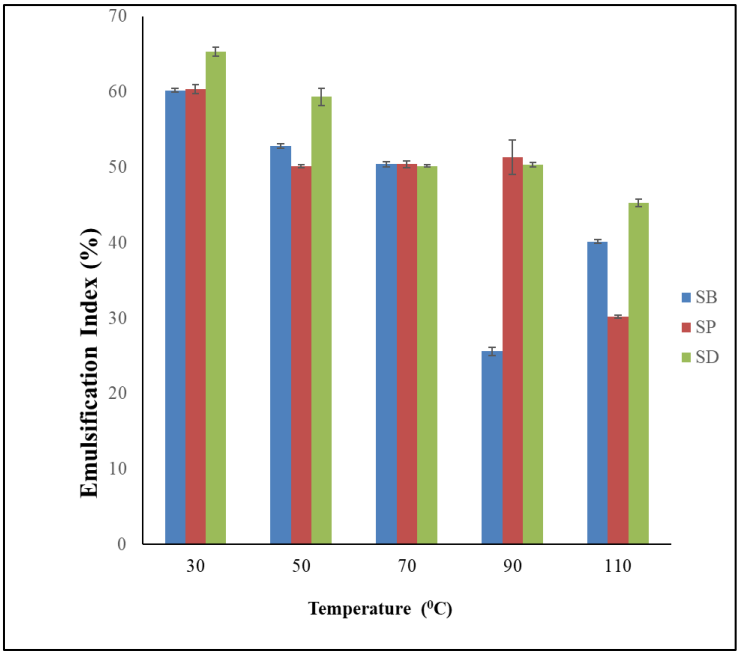
The measured value for distilled water at 25°C aligns with the accepted literature value.

3.3. Resilience Under Physicochemical Stress: pH, Thermal, and Salinity Stability

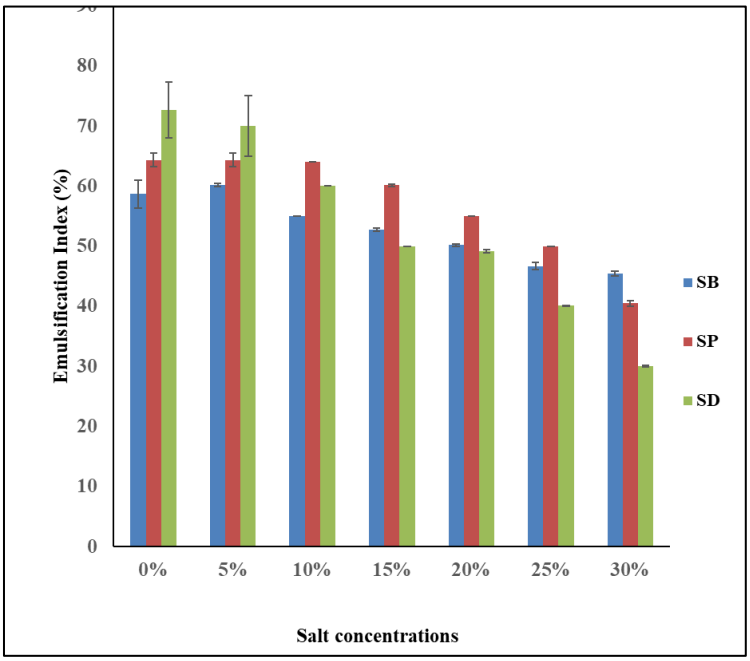
A defining advantage of many biosurfactants is their robustness under extreme conditions, a feature clearly demonstrated here (Figure 2).



pH Stability (Figure 2A): The biosurfactants from all three cultures maintained high emulsification activity (E_{24} > 50%) across an exceptionally broad pH range from 2 to 10. Activity was largely unimpaired under strongly acidic (pH 2–4) and alkaline (pH 9–10) conditions. This pH resilience is invaluable for applications in diverse industrial effluents, acid mine drainage remediation, or alkaline cleaning formulations [22]



Thermal Stability (Figure 2B) The biosurfactants exhibited notable thermostability, retaining over 80% of their emulsification capacity after exposure to temperatures up to 90°C for 15 minutes. A marked decline was observed only at 110°C, likely due to thermal denaturation of any proteinaceous components or structural degradation of the surfactant molecules themselves. This stability at elevated temperatures supports potential use in microbial enhanced oil recovery (MEOR) in subterranean reservoirs or in hot-process industries [23]



NaCl (Salinity) Tolerance (Figure 2C) The biosurfactants were not only tolerant to but in some cases slightly stimulated by the presence of salt. Optimal activity was often observed at NaCl concentrations of 5–10% (w/v), with significant emulsification retained even at 20–30% salinity. This halotolerance is a critical attribute for applications in marine environments, oil reservoir brines, or in the formulation of personal care products containing electrolytes [24]

Figure 2 Stability profiles of biosurfactants from Cola hispida pod medium under physicochemical stress. Residual emulsification index (E_{24} with kerosene) of biosurfactants from Bacillus sp. (SB), Pseudomonas sp. (SP), and their diculture (SD) after exposure to: (A) varying pH conditions, (B) different temperatures, and (C) increasing concentrations of sodium chloride (NaCl). Data points represent mean ± SD (n=3)

3.4. Hemolytic Activity: A Qualitative Corroboration

All culture supernatants produced distinct zones of β-hemolysis on blood agar plates, with the co-culture CFS generating the largest zone (scored as +++). Hemolysis occurs due to the surfactant's interaction with and disruption of the erythrocyte membrane, a phenomenon directly linked to its surface-active properties [25]. This qualitative result provides supportive, albeit non-specific, evidence consistent with the quantitative data on biosurfactant production and activity.

4. Conclusion

This study delivers a comprehensive functional validation of a sustainable biosurfactant production platform. The key outcomes are threefold. First, the defined Bacillus-Pseudomonas consortium consistently outperformed its constituent monocultures, synthesizing a biosurfactant with superior emulsification capacity (73.33% E_{24} with kerosene), enhanced surface tension reduction (to 38.0 mN/m), and greater oil displacement power (3.23 cm diameter), demonstrating clear microbial synergy. Second, the Cola hispida pod agro-waste was confirmed not only as a viable but as a preferable substrate, eliciting biosurfactants with higher functional activity compared to conventional glucose or complex media. Third, the produced biosurfactants exhibit exceptional operational resilience, maintaining high activity across a wide pH range (2–10), at elevated temperatures (up to 90°C), and in high-salinity environments (up to 10% NaCl). These combined attributes high performance, substrate-driven sustainability, and remarkable stability collectively position these Cola hispida-derived biosurfactants as highly promising candidates for demanding industrial applications. They are particularly suited for environmental biotechnology (e.g., hydrocarbon spill remediation, soil washing), enhanced oil recovery processes, and as stable bioactive ingredients in formulations requiring robustness under variable conditions. This work successfully bridges the gap between sustainable production and proven, high-quality functionality.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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