International Journal of Biological and Pharmaceutical Sciences Archive

ISSN: 0799-6616 (Online)

Journal homepage: https://ijbpsa.com/

(RESEARCH ARTICLE)

Isolation of native biosurfactant producers and their sustainable cultivation in a novel cola hispida pod-based medium

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International Journal of Biological and Pharmaceutical Sciences Archive, 2025, 10(02), 196-202

Publication history: Received on 15 November 2025; revised on 24 December 2025; accepted on 26 December 2025

Article DOI: https://doi.org/10.53771/ijbpsa.2025.10.2.0091

Abstract

The search for industrially useful microorganisms often begins in environments where evolutionary pressures have selected for specific metabolic traits. Hydrocarbon-contaminated sites represent rich reservoirs of microorganisms inherently equipped with mechanisms, such as biosurfactant production, to enhance the bioavailability and assimilation of hydrophobic carbon sources. This study aimed to isolate and characterize such indigenous, biosurfactant-proficient bacteria and pioneer their cultivation in a novel, cost-effective fermentation medium formulated with Cola hispida pod, a significant but underutilized agro-waste. Using selective isolation techniques from an engine oil-contaminated soil sample, two distinct bacterial strains were purified. Comprehensive morphological and biochemical profiling identified them as a Bacillus species (Gram-positive, endospore-forming rod) and a Pseudomonas species (Gram-negative, oxidase-positive rod). A defined co-culture consortium combining both isolates was also established. To evaluate the waste substrate, four distinct fermentation media were formulated: a basal mineral salts solution supplemented with (i) Cola hispida pod powder as the sole nutrient source (BMM/Ch), (ii) pod powder and glucose (BMM/Ch/Glu), (iii) glucose only (BMM/Glu), and (iv) commercial nutrient broth (NB) as a reference. Growth kinetics, monitored spectrophotometrically at 600 nm over 12 hours in NB, revealed robust sigmoidal growth curves for all inocula, with the co-culture exhibiting particularly vigorous growth, reaching a maximum optical density (OD_{600}) of approximately 1.0. Critically, all microbial preparations the pure isolates and the consortium demonstrated successful growth and metabolic activity during a 7-day cultivation period in all media, including the BMM/Ch formulation. This finding provides direct, empirical validation that the Cola hispida pod possesses bioavailable nutrients capable of sustaining microbial proliferation.

Keywords: Biosurfactant-producing bacteria; Bacillus; Pseudomonas; Microbial consortium; Agro-waste medium; Selective isolation; Fermentation kinetics; Hydrocarbon-contaminated soil.

1. Introduction

The quest to discover and harness proficient microbial cell factories is a fundamental driver of innovation in industrial biotechnology. A strategically sound approach to this quest involves exploring ecological niches where environmental

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pressures have naturally selected for microorganisms possessing the desired metabolic capabilities. Hydrocarbon-contaminated soils and aquatic systems represent precisely such niches. In these environments, the scarcity of readily available carbon sources, coupled with the presence of complex, water-immiscible hydrocarbons, creates a powerful selective force [1]. Microorganisms that thrive here have often evolved sophisticated mechanisms to access these hydrophobic substrates. A central mechanism is the production of biosurfactants amphiphilic molecules that emulsify hydrocarbons, dramatically increasing their surface area and bioavailability for microbial uptake and catabolism [2,3]. Consequently, native bacterial populations from polluted sites, particularly members of the genera Pseudomonas and Bacillus, are frequently reported as prolific and efficient producers of diverse biosurfactant classes, such as glycolipids (e.g., rhamnolipids) and lipopeptides (e.g., surfactin) [4,5].

While the isolation of metabolically gifted strains is a crucial first step, the path to a viable industrial process is fraught with economic challenges. A predominant hurdle is the formidable cost associated with fermentation media, which can consume 30–50% of total production expenses [6]. Conventional media rely heavily on refined, expensive carbon sources like glucose or glycerol, and complex nitrogen supplements like yeast extract. Therefore, the development of low-cost alternative substrates is not merely beneficial but essential for commercial feasibility. In our preceding research, we characterized the proximate composition of Cola hispida pod, an abundant agro-industrial waste in West Africa, revealing a robust profile rich in carbohydrates, moderate in protein, and containing essential minerals [7]. This composition theoretically positions the pod as an ideal, low-cost fermentation feedstock, aligning with the principles of a circular bioeconomy by valorizing waste into a resource.

However, theoretical nutritional potential must be validated through practical microbiological application. It is imperative to demonstrate that target microorganisms can not only tolerate but also actively grow, divide, and maintain their metabolic integrity in a medium where this novel waste product serves as the primary, if not sole, source of nutrition. Furthermore, contemporary bioprocess research is increasingly investigating the potential of microbial consortia. Defined co-cultures can sometimes outperform pure cultures due to synergistic interactions, such as cross-feeding, division of metabolic labor, or the mitigation of end-product inhibition, potentially leading to enhanced growth rates, substrate utilization, or product titers [8,9].

To bridge the gap between substrate characterization and biosurfactant production, this study was designed with three sequential and interdependent objectives. First, to selectively isolate and phenotypically characterize indigenous bacteria with biosurfactant-producing potential from a local hydrocarbon-contaminated environment. Second, to formulate and prepare a series of fermentation media utilizing Cola hispida pod powder as a central, sustainable ingredient. Third, to comprehensively evaluate and compare the growth kinetics and cultivation success of the isolated pure cultures, as well as a deliberately constructed consortium of both, across these novel media formulations. This work thus serves as the critical linchpin, establishing a functional and sustainable microbial cultivation system that directly utilizes agro-waste, setting the stage for subsequent biosurfactant production and characterization.

2. Materials and Methods

2.1. Source and Collection of Environmental Sample

Soil samples were collected for microbial isolation from a chronically hydrocarbon-impacted site: the automobile service center within the Faculty of Engineering, University of Nigeria, Nsukka. This site was selected due to the persistent presence of spent engine oils, diesel, and petrol, creating a long-term selective pressure for hydrocarbon-tolerant microbiota. Surface soil (approximately 5–10 cm depth) was aseptically collected using a sterile spatula into pre-sterilized glass containers. Samples were transported immediately to the laboratory and processed within two hours of collection to preserve microbial community integrity.

2.2. Selective Isolation and Purification of Bacterial Strains

A two-pronged selective strategy was employed to target the desired bacterial genera. For the isolation of Pseudomonas species, 1 gram of soil was suspended in 9 mL of sterile 0.85% (w/v) physiological saline and subjected to serial decimal dilutions up to 10^{-7}. An aliquot (0.1 mL) from appropriate dilutions (10^{-5} to 10^{-7}) was spread-plated onto the surface of pre-poured Cetrimide Agar plates. Cetrimide Agar is a selective and differential medium; cetrimide (cetyltrimethylammonium bromide) inhibits most Gram-positive and many Gram-negative bacteria while allowing Pseudomonas, particularly P. aeruginosa, to grow, often producing distinctive pyocyanin pigment [10]. For the isolation of Bacillus species, which form resilient endospores, a selective enrichment was performed. One gram of soil was added to 10 mL of sterile saline and subjected to a heat shock treatment at 80°C in a water bath for 10 minutes to eliminate

vegetative cells while selecting for heat-resistant Bacillus endospores [11]. After cooling, serial dilutions of this heat-treated suspension were spread-plated onto general-purpose Nutrient Agar.

All plates were incubated in an inverted position at 30°C for 24–48 hours. Following incubation, morphologically distinct colonies (varying in size, shape, color, elevation, and margin) were carefully picked from each selective medium. These colonies were subjected to repeated streaking on fresh Nutrient Agar plates until pure, axenic cultures were obtained, as evidenced by uniform colony morphology. Pure isolates were maintained on Nutrient Agar slants at 4°C for short-term working stocks and preserved long-term in cryovials containing 20% (v/v) sterile glycerol at -20°C.

2.3. Morphological and Biochemical Characterization

The purified isolates were subjected to a standardized battery of tests for phenotypic identification to the genus level, following established microbiological manuals [12].

Microscopic Morphology: Gram staining was performed using the Hucker's method to differentiate cell wall structure. Endospore staining was conducted using the Schaeffer-Fulton method to confirm the presence of endospores in the Gram-positive isolate.

Colony Morphology: Characteristics such as form, elevation, margin, surface texture, optical property, and pigmentation were observed on Nutrient Agar after 24-hour incubation.

2.4. Biochemical Profiling: Key tests included:

- Catalase Test: A loopful of culture was mixed with a drop of 3% hydrogen peroxide (H_2O_2); effervescence indicated a positive result.
- Oxidase Test: Using commercially available oxidase test strips, a colony was smeared on the strip; development of a deep purple color within 10-30 seconds indicated a positive reaction.
- Urease Test: Isolates were streaked on Christensen's Urea Agar slants; a color change from yellow to bright pink indicated hydrolysis of urea.
- Citrate Utilization: Isolates were streaked on Simmons Citrate Agar slants; a color change from green to blue indicated the ability to utilize citrate as a sole carbon source.
- Motility-Indole-Lysine (MIL) Decarboxylation Test: Isolates were stab-inoculated into MIL medium; observations were made for motility (cloudy growth away from the stab line), indole production (detected by adding Kovac's reagent), and lysine decarboxylation (purple color).
- Carbohydrate Fermentation: Acid and gas production from sugars like glucose, lactose, and sucrose were tested in phenol red broth with Durham tubes.

Identification was concluded by comparing the observed phenotypic profile with standard descriptions in Bergey's Manual of Determinative Bacteriology.

2.5. Preparation of Inoculum and Establishment of a Defined Diculture

To generate active, standardized inocula for fermentation experiments, single, well-isolated colonies of each pure isolate (Bacillus sp. and Pseudomonas sp.) were transferred into separate 250 mL Erlenmeyer flasks, each containing 100 mL of sterile Nutrient Broth. These pre-cultures were incubated at 30°C with continuous agitation at 150 rpm on an orbital shaker (GFL, Germany) for approximately 18 hours, allowing the cells to reach the late exponential growth phase. The optical density (OD_{600}) of each pre-culture was adjusted to approximately 0.8–1.0 using sterile broth to standardize cell density. For the co-culture (consortium) inoculum, equal volumes (1 mL each) of the standardized Bacillus sp. and Pseudomonas sp. pre-cultures were aseptically combined in a sterile container, creating a 1:1 (v/v) mixed population.

2.6. Formulation and Preparation of Fermentation Media

To rigorously test the efficacy of the Cola hispida pod as a substrate, four distinct liquid fermentation media were formulated, each prepared in triplicate with a 100 mL working volume in 250 mL Erlenmeyer flasks.

- BMM/Ch (Pod-based medium): This was the test medium of primary interest. It consisted of a defined Basal Mineral Medium (BMM) supplemented with 14% (w/v) sterile Cola hispida pod powder as the sole source of carbon, nitrogen, and minerals. The BMM composition per liter was: K_2HPO_4, 12.5 g; KH_2PO_4, 3.8 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and 1 mL of a trace element solution (containing $FeSO_4$, $MnCl_2$, $ZnSO_4$, etc.) [13].

- BMM/Ch/Glu (Pod-Glucose medium): This medium contained BMM supplemented with both 14% (w/v) pod powder and 14% (w/v) glucose. It served to evaluate if the pod and a pure sugar source had an additive or synergistic effect on growth.
- BMM/Glu (Glucose control): This medium contained BMM with 14% (w/v) glucose as the sole carbon source. It acted as a positive control to benchmark growth on a conventional, readily metabolizable substrate within the same mineral background.
- NB (Nutrient Broth control): This was a commercial, complex medium (13 g/L, Oxoid) used as a second positive control, representing a nutritionally rich environment ideal for vigorous growth.

All media were sterilized by autoclaving at 121°C for 15 minutes. For media containing glucose and pod powder (BMM/Ch/Glu), glucose was filter-sterilized (0.22 µm pore size) and added aseptically to the autoclaved basal medium to avoid Maillard reaction products.

2.7. Cultivation Procedure and Growth Kinetics Monitoring

Each of the four media types was inoculated with a 2% (v/v) inoculum of either the pure Bacillus sp. culture, the pure Pseudomonas sp. culture, or the prepared co-culture. The inoculated flasks were incubated at 30°C with shaking at 150 rpm for a total fermentation period of 7 days. To specifically capture and compare the early-stage growth dynamics of the different microbial preparations, a separate, dedicated set of cultures was established. These cultures, using only the rich NB medium, were inoculated as described and then sampled destructively every 2 hours over a 12-hour period. At each time point, 3 mL of culture broth was aseptically withdrawn, and its optical density was measured at 600 nm (OD_{600}) using a spectrophotometer (Spectronic 20D, Milton Roy, England) against a blank of sterile NB. This provided a detailed growth curve for each inoculum type under optimal nutritional conditions.

2.8. Data Presentation and Analysis

Growth kinetic data from the 12-hour monitoring experiment are presented as the mean OD_{600} value ± standard deviation derived from three independent replicate flasks (n=3). These data were plotted to generate comparative growth curves. The success of cultivation in the various novel media over 7 days was assessed through visual observation of turbidity, pellet formation, and subsequent biochemical activity assays.

3. Results and Discussion

3.1. Isolation and Phenotypic Characterization of Bacterial Isolates

The selective isolation strategies yielded two morphologically and biochemically distinct bacterial strains, designated for this study as Isolate B and Isolate P. The results of their comprehensive phenotypic characterization are consolidated in Table 1. Isolate B presented as Gram-positive, rod-shaped cells capable of forming central, oval endospores that did not swell the sporangium a classic feature of the Bacillus genus. Its colonies were large, flat, and off-white with irregular edges. Biochemically, it was catalase and oxidase positive, could utilize citrate, was motile, but was urease and indole negative. This profile is strongly indicative of a member of the Bacillus subtilis group.

Isolate P was identified as a Gram-negative rod, non-spore-forming, and exhibited a positive oxidase reaction a cardinal test for the genus Pseudomonas. Colonies were smooth, convex, and displayed a cream-to-light yellow pigmentation. Like Isolate B, it was catalase positive, citrate positive, motile, urease negative, and indole negative. The isolation of these particular genera from a hydrocarbon-polluted site is not coincidental but ecologically logical. Both Bacillus and Pseudomonas are renowned for their metabolic versatility and are frequently dominant members of the biodegradative consortia in contaminated environments, with biosurfactant production being a common adaptive trait to improve hydrocarbon access [14,15]. Their presence in our sample strongly suggests native biosurfactant-producing capability, a premise to be tested in subsequent work.

Table 1 Morphological and Biochemical Characteristics of the Bacterial Isolates

Isolates Colony and Morphology Gram Stain Shape Endospore Urease Citrate Oxidase Motility Indole Catalase

Isolates	C and M	Gs	Shape	Es	Urease	Citrate	Oxidase	Mot	Idl	CAT
Pseudomonas sp.	Creamy raised colony, smooth edge	–	Rod	–	–	+	+	+	–	+
Bacillus sp.	Colourless flat colony, smooth edge	+	Rod	+	–	+	+	+	–	+

Key: – = Negative, + = Positive

3.2. Comparative Growth Kinetics in a Standard Rich Medium

The growth profiles of the two pure isolates and their intentionally combined co-culture in Nutrient Broth over the first 12 hours of incubation are depicted in Figure 1. All three microbial preparations exhibited characteristic sigmoidal bacterial growth curves, confirming their viability and active metabolism. A brief lag phase of approximately 2 hours was observed, followed by a steep exponential (logarithmic) growth phase spanning from hour 2 to hour 8–10, after which the cultures began to enter the stationary phase.

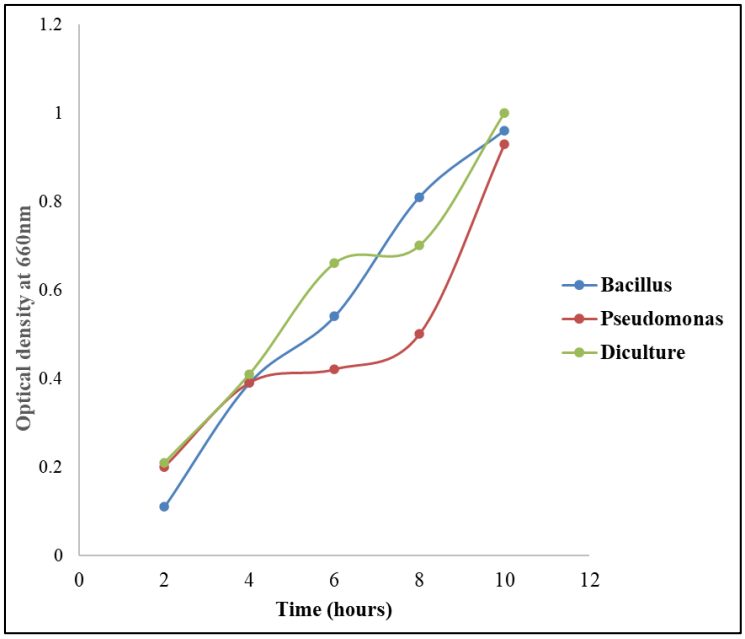

Figure 1 Growth kinetics of Bacillus sp., Pseudomonas sp., and their diculture consortium in Nutrient Broth over 12 hours of incubation at 30°C. Values represent mean OD_{600} ± standard deviation (error bars) from triplicate cultures.

A notable observation was the performance of the co-culture. It consistently demonstrated a slightly shorter apparent lag phase and achieved a higher final optical density (OD_{600} ~1.0) compared to the individual pure cultures by the 12-hour mark. This enhanced growth vigor in a consortium format is intriguing and aligns with emerging literature on microbial synergies. Potential explanations could include cross-feeding, where one organism metabolizes a waste product of the other, or complementary enzymatic activities that collectively create a more favorable microenvironment [16]. For instance, the Bacillus strain might secrete proteases or amylases that break down complex components in the medium, releasing peptides or sugars that benefit both itself and the Pseudomonas strain. This synergistic interaction, even in a rich medium, suggests potential for improved substrate utilization when grown on more complex waste materials like the Cola hispida pod.

3.3. Validation of Agro-Waste Medium through Successful Cultivation

The most significant practical finding of this study was the unequivocal demonstration of successful microbial growth in the novel Cola hispida pod-based media. Over the 7-day fermentation period, visible turbidity, indicative of microbial proliferation, was observed in all flasks inoculated with Bacillus sp., Pseudomonas sp., and their co-culture, across all

four media formulations (BMM/Ch, BMM/Ch/Glu, BMM/Glu, and NB). This visual evidence was later corroborated by downstream analyses confirming metabolic activity.

The growth in the BMM/Ch medium is of paramount importance. In this formulation, the processed pod powder was the sole intentional nutrient addition to the basal mineral salts. The fact that both Gram-positive (Bacillus) and Gram-negative (Pseudomonas) bacteria, with their different membrane structures and metabolic preferences, could proliferate using this substrate provides robust validation of the compositional analysis presented in our companion paper [7]. It confirms that the carbohydrates, proteins, and minerals within the pod are not only present in sufficient quantities but are also in a bioavailable form. Microorganisms, likely through the secretion of extracellular hydrolytic enzymes (e.g., cellulases, hemicellulases, proteases), were able to break down the complex pod matrix into assimilable monomers to fuel their growth. This transforms the pod from a theoretical nutrient source into an empirically verified fermentation feedstock.

The comparable growth in BMM/Glu confirms that the isolates can utilize simple sugars, while growth in the mixed BMM/Ch/Glu medium suggests no major inhibitory compounds are present in the pod that prevent growth in the presence of glucose. The vigorous growth in NB serves as a baseline for optimal growth potential. The successful cultivation of the co-culture in the pod-based medium is particularly promising, as the potential synergistic interaction observed in NB could be even more beneficial for degrading the complex lignocellulosic structure of the pod, leading to more efficient substrate utilization in future biosurfactant production runs [17].

4. Conclusion

This study successfully bridges the gap between agro-waste characterization and applied microbial biotechnology. We isolated and identified a Bacillus sp. and a Pseudomonas sp. from a hydrocarbon-polluted environment, both of which are strong candidates for biosurfactant production based on their ecological origin. More importantly, we pioneered and validated a sustainable cultivation platform by demonstrating that these isolates, both individually and as a defined consortium, can robustly grow in fermentation media where Cola hispida pod agro-waste serves as the primary nutrient source. The successful growth in the BMM/Ch medium is a critical proof-of-concept, confirming the bioavailability and sufficiency of the pod's nutrients for supporting microbial metabolism. The enhanced growth kinetics observed with the co-culture consortium further opens an interesting avenue for process optimization. This work effectively establishes a functional, low-cost, and environmentally aligned bioprocess foundation. It sets the stage for the next critical phase: harnessing this very system to produce, quantify, and characterize the biosurfactants synthesized by these microbes when cultivated on this sustainable substrate, thereby completing the valorization cycle from waste to high-value product.

Compliance with ethical standards

Acknowledgments

The authors acknowledge the technical support provided by the Department of Microbiology, University of Nigeria, Nsukka, for access to microbiological media and incubation facilities.

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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