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# The Use of Groundnuts Oil as Substrate for Glycoprotein Biosurfactant Production by *Micrococcs* sp. LB11

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**Abstract: Biosurfactants, which are surface-active molecules of microbial origin, are garnering significant attention as sustainable substitutes for synthetic surfactants. Their appeal lies in their inherent biocompatibility, environmental degradability, and stable performance across a range of physicochemical conditions. A primary impediment to their industrial adoption, however, is the substantial expense associated with fermentation feedstocks. The present investigation evaluates the efficacy of groundnut oil, a low-cost agricultural by-product, as an exclusive carbon substrate for the cost-effective microbial synthesis of biosurfactants. A consortium of a bacterial strains, isolated from hydrocarbon-contaminated soil, underwent primary screening for biosurfactant production. This screening employed a tripartite methodological approach: the oil-spreading technique, the drop-collapse assay, and the determination of the emulsification index (E24). Among the evaluated isolates, *Micrococcus* sp. strain LB11 demonstrated superior surface-activity traits, manifesting a 15 mm zone of oil\displacement and a rapid reduction in interfacial tension evidenced by a 45-second drop-collapse duration. While *Alcaligenes faecalis* (IS-7) displayed a considerable capacity for emulsion stabilization, *Micrococcus* sp. LB11 emerged as the most proficient candidate when propagated in a medium formulated with groundnut oil. Cultivation of *Micrococcus* sp. LB11 on this lipid substrate facilitated the generation of a biosurfactant achieving an E24 value of approximately 65% over a 24-hour incubation period. This quantitative measure of emulsion stability indicates the synthesis of a high-quality, robust biosurfactant. The magnitude of the emulsifying activity further suggests the production of a high-molecular-weight compound, potentially possessing a glycoproteinaceous character. These outcomes collectively validate groundnut oil as a proficient and economical substrate for augmenting biosurfactant yield. In summary, these findings position *Micrococcus* sp. LB11 as a highly promising isolate warranting in-depth biochemical analysis and scale-up exploration. The resultant biosurfactant holds considerable promise for deployment in environmentally pertinent applications, including but not limited to soil and water bioremediation, microbial-enhanced hydrocarbon recovery, and various green technological processes.**

***Keywords:*** *Micrococcus sp. LB11, Groundnut Oil, Glycoprotein Biosurfactant, Biosurfactant Production, Sustainable Substrate, Emulsification Index (E24), Stable Emulsion Formation, Low-Cost Fermentation, Agro-Industrial Waste Valorization, Microbial Surface-Active Agents.*

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## I. INTRODUCTION

➢ *Background of the Study*

Biosurfactants represent a distinctive class of surface-active metabolites synthesized by diverse microbial populations, including bacteria, yeasts, and fungi. Their escalating prominence in scientific and industrial domains stems from a suite of advantageous properties, such as inherent biodegradability, reduced ecotoxicity, and sustained functional efficacy under extreme environmental regimes. These attributes collectively designate them as compelling green alternatives to their chemically synthesized counterparts. Surfactants, in a broader context, constitute a foundational pillar of modern industry, with indispensable roles spanning domestic detergents, cosmetic and pharmaceutical formulations, agricultural adjuvants, food

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processing, petroleum recovery, environmental decontamination, and multifaceted manufacturing operations (Varjani & Upasani, 2017).

From a structural perspective, these molecules are amphiphilic, featuring distinct hydrophobic (apolar) and hydrophilic (polar) moieties. This dual nature facilitates their preferential alignment at interfaces between disparate phases such as air-water or oil-water systems thereby effectively lowering interfacial tension and modifying surface properties (Lamichhane et al., 2017). Reflecting their widespread utility, the global surfactant market was valued at approximately USD 25.6 billion in 2014, with forecasts indicating a compound annual growth rate (CAGR) of 4.6% through 2020 (Grand View Research, 2016). Current market dominance is held by petroleum-derived synthetic surfactants, including polysorbates (e.g., Tween series), octylphenol ethoxylates (e.g., Triton X-100), and polyoxyethylene alkyl ethers (e.g., Brij-35) (Lamichhane et al., 2017; Santos et al., 2016).

Notwithstanding their extensive application, conventional synthetic surfactants present significant environmental and economic liabilities. Concerns persist regarding their potential toxicity, environmental persistence, and recalcitrance to biological degradation (Santos et al., 2016). Furthermore, their synthesis is inextricably linked to petrochemical feedstocks, rendering the industry susceptible to volatile crude oil prices and conflicting with global sustainability objectives (Otzen, 2017).

In contrast, microbial biosurfactants have emerged as a sustainable focal point of contemporary research, driven by the demand for renewable, non-toxic, and functionally robust alternatives (Vijayakumar & Saravanan, 2015). This shift is mirrored in market trajectories; the global biosurfactant market was valued at an estimated USD 4.2 billion in 2017, with projections anticipating growth to USD 5.52 billion by 2022 at a CAGR of 5.6% (Markets and Markets, 2017). This growth rate surpasses that of the general surfactant market, underpinned by increasing regulatory and consumer preference for environmentally benign products.

The advantages of biosurfactants extend beyond eco-compatibility. They exhibit remarkable structural diversity, stability across broad pH, temperature, and salinity gradients, and often demonstrate superior surface activity at lower concentrations compared to synthetic analogs (da Rosa et al., 2015; Makkar et al., 2011). A particularly compelling feature is their capacity for synthesis from low-cost, renewable, or waste-derived carbon streams, including industrial by-products and agricultural residues, which enhances their economic and environmental appeal (Li & Yu, 2011; Pacwa-Płociniczak et al., 2011). Their complex molecular architectures, which can include glycolipid, lipopeptide, or glycoprotein structures, enable unique interactions with biological membranes and hydrophobic contaminants, broadening their applicability in sectors such as bioremediation, enhanced oil recovery, pharmaceuticals, and antimicrobial strategies (Otzen, 2017). Consequently, biosurfactants are increasingly hailed as versatile biochemical tools for the 21st century (Santos et al., 2016).

*Micrococcus* sp. LB11 has been identified as a promising microbial platform for biosurfactant biosynthesis. Preliminary studies indicate its metabolic versatility and ability to produce extracellular surface-active agents under various cultivation conditions. However, a critical and underexplored avenue is its potential to synthesize glycoprotein-type biosurfactants a class noted for its high molecular weight and potent emulsifying activity using inexpensive lipid substrates. The specific physiological and metabolic pathways enabling this production, particularly when utilizing groundnut oil as the primary carbon source, remain poorly elucidated, representing a significant knowledge gap.

- *Statement of the Problem*

While *Micrococcus* sp. LB11 demonstrates preliminary promise for biosurfactant production, substantial scientific lacunae hinder its commercial exploitation. Existing literature provides a generalized understanding of biosurfactant production but offers scant insight into the specific synthesis of glycoprotein biosurfactants by this strain. Glycoprotein biosurfactants possess distinct structural and functional merits, including high emulsification power and stability, yet their production dynamics remain obscure.

Moreover, the economic feasibility of biosurfactant processes is critically dependent on substrate cost. The utilization of low-value agro-industrial by-products, such as groundnut oil, represents a strategic approach to cost reduction. Nevertheless, the efficacy of groundnut oil as a dedicated substrate for glycoprotein biosurfactant synthesis by *Micrococcus* sp. LB11 has not been systematically investigated. The absence of definitive data on optimal fermentation parameters, substrate metabolism, and the subsequent characterization of the biosurfactant formed under these conditions constrains the development of optimized, scalable, and economically viable bioprocesses.

Therefore, a targeted investigation is imperative to delineate the capacity of *Micrococcus* sp. LB11 to produce a glycoprotein biosurfactant from groundnut oil, to identify the key variables influencing its yield and quality, and to comprehensively characterize the resultant biomolecule for its potential in industrial and environmental applications.

- *Justification of the Study*

This research is justified by its concurrent pursuit of environmental sustainability and bioprocess economics. By employing groundnut oil an affordable and readily available agro-industrial residue as the principal carbon source, this study aligns with circular bioeconomy principles, adding value to a by-product stream while reducing dependency on expensive, purified substrates.

The selection of *Micrococcus* sp. LB11, a non-pathogenic bacterium, addresses inherent biosafety concerns associated with large-scale fermentation, facilitating safer downstream handling and application. The persistent barrier

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to the widespread adoption of biosurfactants is their high production cost relative to synthetic surfactants. This work directly tackles this impediment by validating a cost-effective substrate strategy, thereby enhancing the commercial viability of biosurfactant technology.

From an environmental perspective, the study advances green biotechnology. The glycoprotein biosurfactant produced holds significant potential for use in bioremediation of hydrocarbon-contaminated sites, microbial enhanced oil recovery (MEOR), and other pollution mitigation strategies. Consequently, the findings of this study are poised to contribute substantively to the foundational knowledge required for developing efficient, sustainable, and application-ready biosurfactant production platforms.

➢ *Aim of the Study*

The principal aim of this study is to evaluate the feasibility and efficacy of utilizing groundnut oil as a substrate for the production of a glycoprotein biosurfactant by the bacterium *Micrococcus* sp. LB11.

➢ *Objectives of the Study*

To achieve the stated aim, the following specific objectives were delineated:

- To evaluate the biosurfactant-producing potential of *Micrococcus* sp. LB11 using groundnut oil as the sole carbon source in a defined cultivation medium.
- To extract, purify, and characterize the chemical and functional properties of the biosurfactant produced, with emphasis on confirming its glycoproteinaceous nature.

## II. LITERATURE REVIEW

➢ *Introduction*

The escalating global imperative for sustainable industrial solutions has catalyzed significant scientific exploration into microbial biotechnology, with biosurfactants occupying a prominent position. Biosurfactants are amphiphilic secondary metabolites synthesized by diverse microorganisms, including bacteria, yeasts, and fungi. Their molecular architecture, comprising a hydrophilic moiety (such as a carbohydrate, peptide, or phosphate group) and a hydrophobic domain (typically a long-chain fatty acid), enables them to preferentially adsorb at interfaces of differing polarities. This adsorption effectively lowers interfacial and surface tensions, a fundamental property that underpins their multifunctional utility.

Unlike conventional surfactants derived from petrochemical feedstocks, biosurfactants offer compelling advantages: they are inherently biodegradable, exhibit low ecotoxicity, and frequently maintain functional stability under extreme conditions of temperature, pH, and salinity. These characteristics have amplified their relevance across a spectrum of industries, from environmental remediation and enhanced hydrocarbon recovery to advanced applications in pharmaceuticals, cosmetics, and food processing.

➢ *Classification and Chemical Nature of Biosurfactants*

Biosurfactants are categorized primarily based on their biochemical composition and microbial origin. This classification reflects a broad structural diversity, which directly dictates their physicochemical behaviors and application-specific efficacy. The principal classes include:

- Glycolipids
- Lipopeptides and lipoproteins
- Phospholipids and fatty acids
- Polymeric biosurfactants
- Particulate biosurfactants

Each class encompasses distinct molecular families with unique properties, enabling tailored use in various technological domains.

➢ *Glycolipids*

Glycolipids represent one of the most extensively researched and commercially promising classes. They consist of a carbohydrate head group linked to a hydrophobic tail of long-chain aliphatic acids or hydroxy fatty acids.

- *Rhamnolipids*

Predominantly synthesized by *Pseudomonas aeruginosa*, rhamnolipids are composed of one or two rhamnose molecules glycosidically linked to one or two β-hydroxy fatty acid chains. They are potent surface-active agents, capable of reducing the surface tension of water from 72 mN/m to approximately 25–30 mN/m. Their excellent emulsifying and foaming properties make them particularly valuable for Microbial Enhanced Oil Recovery (MEOR) and the bioremediation of sites contaminated with hydrophobic pollutants.

- *Trehalolipids*

Produced by *Rhodococcus*, *Mycobacterium*, and related actinomycetes, trehalolipids feature the disaccharide trehalose linked to mycolic acids complex, long-chain α-alkyl, β-hydroxy fatty acids. Their structural complexity confers high efficiency in breaking water-in-oil emulsions, positioning them as potent agents for managing petroleum spills and oil sludge.

- *Sophorolipids*

These are glycolipids synthesized by yeasts such as *Starmerella bombicola*. Their structure consists of the disaccharide sophorose linked to a hydroxy fatty acid, often 17-hydroxyoctadecanoic or 17-hydroxy-Δ9-octadecenoic acid. They commonly exist in two forms: the lactonic form, noted for superior surface tension reduction and antimicrobial activity, and the acidic form, recognized for its higher water solubility and foaming capacity. Their mild, biodegradable nature favors their use in cosmetics, detergents, and personal care products.

- *Mannosylerythritol Lipids (MELs) and Cellobiose Lipids*

MELs, produced by *Pseudozyma* spp., consist of mannosylerythritol as the hydrophilic moiety. They exhibit

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notable self-assembling properties, forming distinctive lyotropic liquid crystals, which has sparked interest in their application for drug and gene delivery systems. Cellobiose lipids, produced by fungi like *Ustilago maydis*, share a similar glycolipid structure but feature cellobiose as the sugar component.

➢ *Lipopeptides and Lipoproteins*

Lipopeptides are cyclic or linear peptides linked to a fatty acid chain, predominantly produced by *Bacillus* species. They are among the most effective biosurfactants known.

- Surfactin, a cyclic lipoheptapeptide from *Bacillus subtilis*, can reduce surface tension to 27 mN/m and is a powerful antiviral, antibacterial, and anti-mycoplasma agent.
- Iturin and fengycin are other well-characterized lipopeptide families from *Bacillus*, primarily recognized for their potent antifungal activities, making them significant in agricultural biocontrol.

➢ *Phospholipids and Fatty Acids*

Certain hydrocarbon-degrading bacteria, such as *Acinetobacter* species, produce phospholipid-rich membrane vesicles or excrete fatty acids with surfactant properties. These molecules can effectively emulsify hydrocarbons, facilitating their uptake and degradation by microbial cells.

➢ *Polymeric Biosurfactants*

These are high-molecular-weight amphiphilic biopolymers with exceptional emulsifying power but generally lower surface tension reduction capability compared to low-molecular-weight biosurfactants.

- Emulsan: A lipopolysaccharide-protein complex from *Acinetobacter calcoaceticus* RAG-1, forming extremely stable oil-in-water emulsions.
- Alasan: A complex of anionic polysaccharide and protein from *Acinetobacter radioresistens*, known for its stability and ability to emulsify a broad range of hydrocarbons.
- Liposan: An extracellular water-soluble emulsifier produced by *Yarrowia lipolytica*, composed of carbohydrate and protein.

➢ *Particulate Biosurfactants*

This category refers to whole microbial cells, membrane vesicles, or other amphiphilic cell surface structures that act collectively to reduce interfacial tension. For instance, the hydrophobic cell surface of *Acinetobacter venetianus* facilitates direct contact with and emulsification of oil droplets.

➢ *Biosurfactant Production via Fermentation*

Biosurfactant synthesis occurs through submerged, solid-state, or biofilm-based fermentations. The choice of carbon source is pivotal; hydrophobic substrates like vegetable oils (e.g., soybean, olive, groundnut oil) or hydrocarbons often induce higher yields. This is because the microorganism must produce surfactants to emulsify these water-immiscible substrates, thereby enhancing their bioavailability for uptake and metabolism.

➢ *Factors Influencing Biosurfactant Production*

- Carbon Source: The nature and concentration of the carbon source are the most critical parameters. Hydrophobic carbon sources generally lead to higher biosurfactant yields. Groundnut oil, rich in oleic (C18:1) and linoleic (C18:2) acids, serves as an excellent precursor for fatty acid chains in biosurfactant molecules.
- Nitrogen Source: The type and concentration of nitrogen (e.g., ammonium salts, nitrate, urea, organic nitrogen like yeast extract) influence cellular metabolism. Often, biosurfactant production is maximized under conditions of nitrogen limitation with an excess carbon source, triggering secondary metabolite synthesis.
- Environmental Parameters: Optimal temperature (typically 25–37°C for mesophiles), pH (often near neutral), and adequate aeration and agitation rates are essential for maximizing microbial growth and product formation. However, excessive shear stress from agitation can be detrimental.

➢ *Physiological Roles of Biosurfactants*

For the producing microorganisms, biosurfactants fulfill critical ecological functions: they facilitate the uptake of hydrophobic nutrients by increasing surface area and bioavailability, aid in microbial motility (e.g., swarming on surfaces), participate in biofilm formation and dispersal, and can provide a competitive advantage through antimicrobial activity.

➢ *Industrial and Environmental Applications*

- Environmental Remediation: Used for bioaugmentation and biostimulation in the cleanup of soils and water contaminated with crude oil, pesticides, and heavy metals.
- Enhanced Oil Recovery (EOR): Injected into reservoirs to mobilize trapped crude oil, improving recovery rates.
- Food Industry: Function as emulsifiers, stabilizers, and anti-adhesive agents in products like ice cream, chocolate, and bakery items.
- Pharmaceuticals and Cosmetics: Incorporated into topical formulations, wound-healing products, and antimicrobial agents due to their bioactive properties and biocompatibility.
- Agriculture: Serve as adjuvants for pesticide formulations, antifungal agents for plant protection, and soil-wetting agents.

➢ *Economic Considerations in Biosurfactant Production*

The high commercial cost of biosurfactants, a major barrier to widespread adoption, is attributed to:

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- Substrate Cost: Can constitute up to 50% of total production expenses.
- Low Volumetric Productivity: Yields are often lower than those of chemical processes.
- Expensive Downstream Processing: Recovery and purification from fermentation broths can be complex and costly. Strategies to improve economic viability center on using low-cost or waste substrates (like groundnut oil), developing hyper-producing strains via metabolic engineering, and optimizing fermentation and recovery processes.

➢ *Advantages and Limitations*

- *Advantages:*

✓ Sustainable production from renewable resources.
✓ High biodegradability and low toxicity.
✓ Functional efficiency under extreme conditions.
✓ Structural diversity enabling specialized applications (e.g., antimicrobial, anti-adhesive).

- *Limitations:*

✓ High production costs relative to synthetic surfactants.
✓ Potential pathogenicity of some producer strains (e.g., *P. aeruginosa*).
✓ Challenges in consistent large-scale production and purification.
✓ Stringent regulatory hurdles for application in food and healthcare.

This review underscores the immense potential of biosurfactants as versatile, green biochemicals. It also highlights the strategic importance of research focused on optimizing production processes, particularly through the use of inexpensive agro-industrial substrates like groundnut oil, to bridge the gap between laboratory promise and commercial-scale reality.

## III. MATERIALS AND METHODS

➢ *Study Area*

This study was conducted within the Microbiology Laboratory of the Department of Microbiology, University of Maiduguri. The laboratory provided the requisite infrastructure for microbial cultivation, biochemical analyses, and product characterization, including autoclaves, incubators, centrifuges, and sterile workstations. Ambient conditions were regulated to minimize exogenous contamination. All chemicals, reagents, and media components were of analytical grade, sourced from certified suppliers.

The broader geographical context is Maiduguri, the capital of Borno State in northeastern Nigeria, situated at approximately 11.15°N latitude and 30.05°E longitude. The region experiences a semi-arid climate, typified by low annual rainfall (300–500 mm) and high diurnal temperatures ranging from 22°C to over 40°C during the peak of the dry season (March–June). The vegetation is primarily Sudan savannah, and the predominant soil type is sandy loam.

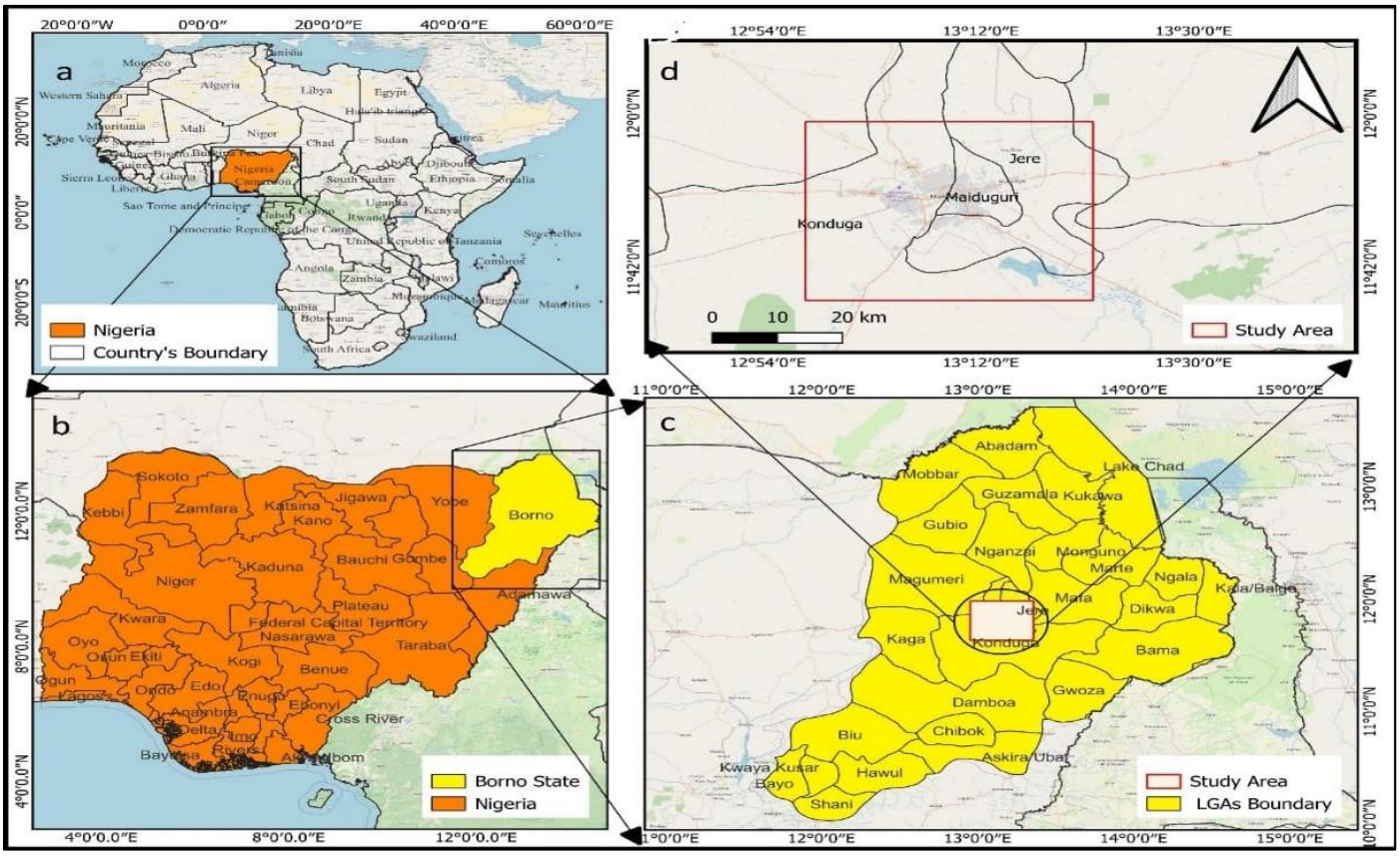


Fig 1 Map Detailed Map of Maiduguri, Borno State Clearly Illustrating the Study Area

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➢ *Sampling Site*

Soil samples were obtained from a site with a documented history of hydrocarbon contamination to increase the likelihood of isolating proficient biosurfactant-producing microorganisms. The selected site was adjacent to an active groundnut oil processing unit, where recurrent spills of lipid-rich material were evident. Such an environment exerts selective pressure for microbiota capable of metabolizing hydrophobic compounds.

➢ *Sample Collection*

Composite soil samples were collected to ensure representative sampling. From a point within the designated site, approximately 40 grams of soil were aseptically retrieved from a depth of 5–10 cm after removing the surface litter. Each subsample was placed into a sterile, labelled polythene bag using a pre-sterilized spatula. The subsample were subsequently homogenized in the laboratory to form a single composite sample. A portion was processed immediately for microbiological analysis, while the remainder was stored at 4°C for subsequent use.

➢ *Media Preparation*

All culture media were prepared with deionized water, and pH was adjusted prior to sterilization by autoclaving at 121°C for 15 minutes at 15 psi. Strict aseptic techniques were observed throughout.

- *Nutrient Agar (NA)*

Nutrient Agar was employed for the initial isolation and enumeration of heterotrophic bacteria. Its composition per liter was: Peptone (5.0 g), Beef Extract (3.0 g), Sodium Chloride (5.0 g), and Agar (15.0 g). The medium was sterilized and dispensed into Petri dishes.

- *Mineral Salt (MS) Medium*

A defined Mineral Salt medium served as the basal medium for screening and cultivating biosurfactant-producing isolates, with groundnut oil as the sole carbon source. Its composition per liter was:

✓ $K_2HPO_4$: 2.0 g
✓ $KH_2PO_4$: 2.0 g
✓ $MgSO_4 \cdot 7H_2O$: 0.2 g
✓ NaCl: 0.1 g
✓ $CaCl_2 \cdot 2H_2O$: 0.02 g
✓ Trace Element Solution: 1.0 mL

The trace element solution contained (per liter): $FeSO_4 \cdot 7H_2O$ (0.01 g), $MnSO_4 \cdot H_2O$ (0.001 g), $ZnSO_4 \cdot 7H_2O$ (0.001 g), and $CuSO_4 \cdot 5H_2O$ (0.0001 g). Filter-sterilized groundnut oil was added aseptically to the sterilized basal medium at a concentration of 2% (v/v) unless otherwise stated.

➢ *Enumeration and Isolation of Bacteria*

Total viable bacterial counts were determined using the standard spread plate technique. Ten grams of the composite soil sample were added to 90 mL of sterile 0.85% (w/v) physiological saline and shaken vigorously for 10 minutes to prepare a $10^{-1}$ dilution. Serial decimal dilutions up to $10^{-7}$ were prepared.

From appropriate dilutions ($10^{-5}$, $10^{-6}$, $10^{-7}$), 0.1 mL aliquots were spread onto the surface of pre-dried Nutrient Agar plates in triplicate. Plates were incubated at 30°C for 24–48 hours. Distinct colonies were counted, and the result was expressed as Colony Forming Units per gram of soil (CFU/g). Morphologically unique colonies were selected, purified by repeated streaking, and maintained on NA slants at 4°C for further study.

➢ *Screening for Biosurfactant Production*

- *Cultivation and Biosurfactant Harvest*

Selected bacterial isolates were screened for biosurfactant production in liquid MS medium. Erlenmeyer flasks (250 mL) containing 100 mL of MS broth supplemented with 2% (v/v) groundnut oil were inoculated with a standardized cell suspension (1% v/v, $OD_{600} \approx 0.8$). The cultures were incubated at 35°C with shaking at 150 rpm for 10 days. Culture aliquots were withdrawn at 48-hour intervals. Cells were removed by centrifugation at 10,000 × g for 20 minutes at 4°C. The resultant cell-free supernatant was used for subsequent biosurfactant activity assays.

- *Preliminary Activity Assays*

✓ *Drop Collapse Test*

This qualitative assay, as described by Bodour and Miller-Maier (1998), was used. Briefly, 2 µL of groundnut oil was placed on a Parafilm-coated surface. Subsequently, 5 µL of cell-free supernatant was gently placed onto the center of the oil droplet. The behavior of the droplet was observed for one minute. Immediate spreading or collapse of the oil droplet was recorded as a positive result, indicating the presence of surface-active compounds. The time to complete collapse was noted.

✓ *Oil Spreading Assay*

The method followed was adapted from Morikawa et al. (2000). A Petri dish was filled with 50 mL of distilled water, onto which 100 µL of groundnut oil was carefully added to form a thin film. A drop of the cell-free supernatant (10 µL) was then gently placed onto the center of the oil film. The diameter of the clear zone formed due to the displacement of oil was measured after 30 seconds. A larger displacement zone correlates with higher surface activity.

✓ *Emulsification Index ($E_{24}$)*

The emulsification capacity of the biosurfactant-containing supernatant was quantified using the method of Cooper and Goldenberg (1987). Equal volumes (5 mL each) of the cell-free supernatant and a hydrocarbon substrate (n-hexadecane, kerosene, diesel, or crude oil) were combined in a graduated test tube. The mixture was vortexed at high speed for 2 minutes and then allowed to stand undisturbed for 24 hours at room temperature. The $E_{24}$ index was calculated using the formula:

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

A higher $E_{24}$ value indicates superior emulsifying activity and stability. The isolate demonstrating the most consistent and potent activity across all preliminary assays was selected for further characterization.

## IV. RESULTS

- *Screening for Biosurfactant Production Using the Oil-Spreading Assay*

The oil-spreading assay served as an initial qualitative screen to evaluate the secretion of surface-active metabolites by the isolated bacterial strains. This test measures the displacement of an oil film on a water surface, which correlates directly with the sample's ability to reduce surface tension.

The results, summarized in Table 1, revealed a pronounced disparity in biosurfactant production between the two candidate isolates. *Micrococcus* sp. LB11 demonstrated superior surface activity, generating a substantial clear zone with a diameter of 15 mm within 22 seconds. In stark contrast, *Bacillus* sp. LC21 produced a minimal displacement zone of only 0.9 mm, which required a longer period of 42 seconds to manifest. The significant difference in both the magnitude of oil displacement and the reaction kinetics indicates that *Micrococcus* sp. LB11 synthesizes and secretes surface-active compounds of considerably higher potency or concentration under the tested conditions.

Table 1 Screening of Bacterial Isolates for Biosurfactant Production Using the Oil-Spreading Assay.

| Isolate | Oil Displacement Zone (mm) | Tittime to formation(s) |
|---|---|---|
| *Bacillus* sp. LC21 | 0.9 | 4242 |
| *Micrococcus* sp. LB11 | 15.0 | 2222 |

mm = millimetre; s = Seconds. Values Represent the Mean of Triplicate Measurements.

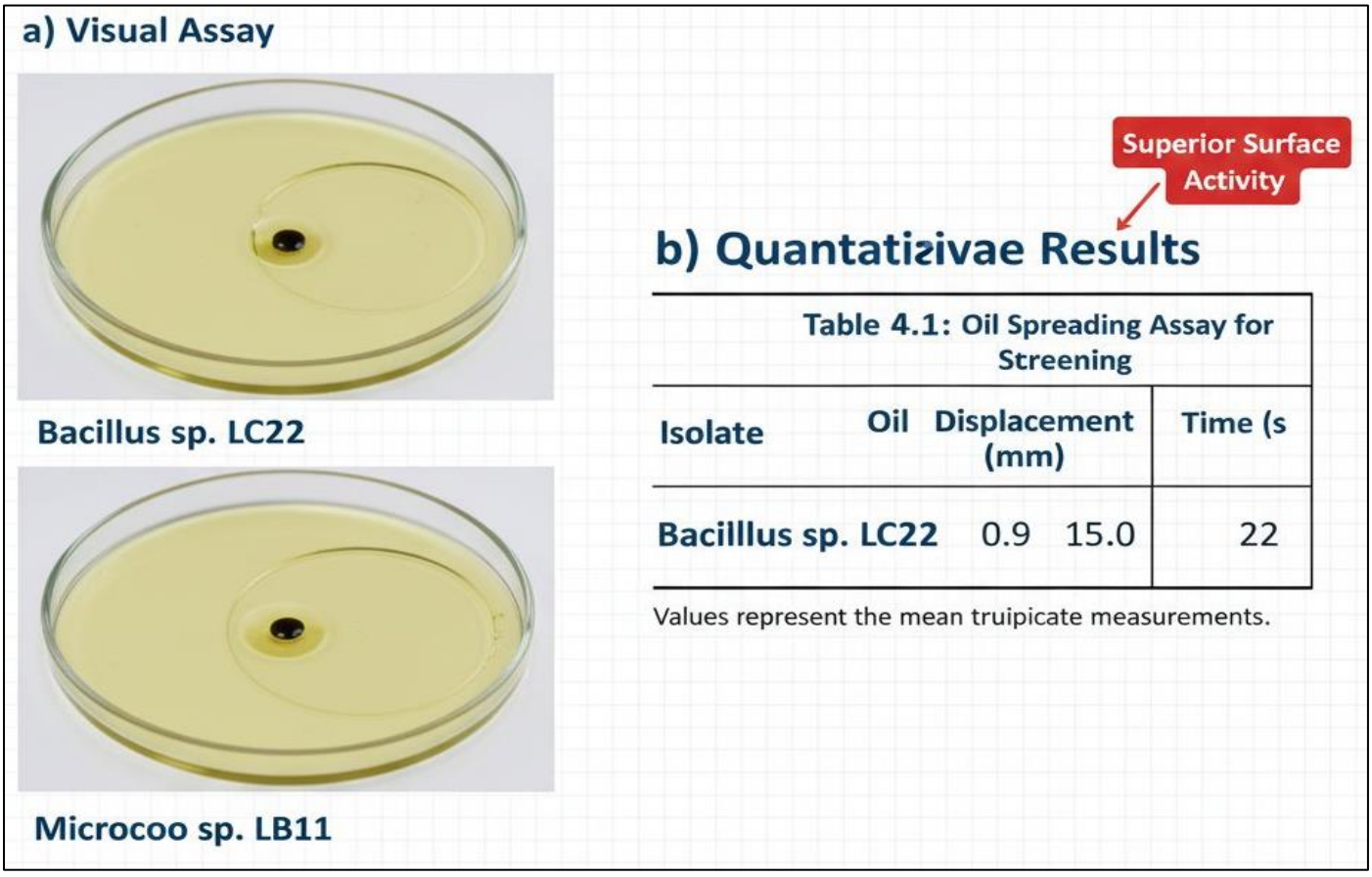


Fig 2 Screening of Bacterial Isolates for Biosurfactant Production Using the Oil-Spreading Assay

- *Screening for Biosurfactant Production Using the Drop-Collapse Assay*

The drop-collapse assay provided a supplementary functional assessment of biosurfactant presence by evaluating the destabilization of a hydrophobic oil droplet. A positive result, indicated by the spreading and collapse of the droplet, confirms the reduction of interfacial tension between the aqueous supernatant and the oil.

As presented in Table 2, both isolates yielded positive results, confirming their capacity to produce biosurfactants. *Bacillus* sp. LC21 caused the oil droplet to collapse to a diameter of 40 mm within 45

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seconds. *Micrococcus* sp. LB11 produced a marginally larger final collapse diameter of 43 mm but required a slightly longer duration of 53 seconds. While the larger collapse diameter for *Micrococcus* sp. LB11 suggests effective interfacial activity, the faster reaction time observed for *Bacillus* sp. LC21 points to a potentially more rapid interaction of its biosurfactant with the oil-water interface.

Table 2 Screening of Bacterial Isolates for Biosurfactant Production Using the Drop-Collapse Assay.

| Isolate | Collapsed Drop Diameter (mm) | Time to Collapse (s) |
|---|---|---|
| *Bacillus* sp. LC21 | 40 | 45 |
| *Micrococcus* sp. LB11 | 43 | 53 |

*mm = Millimetre; s = Seconds.*

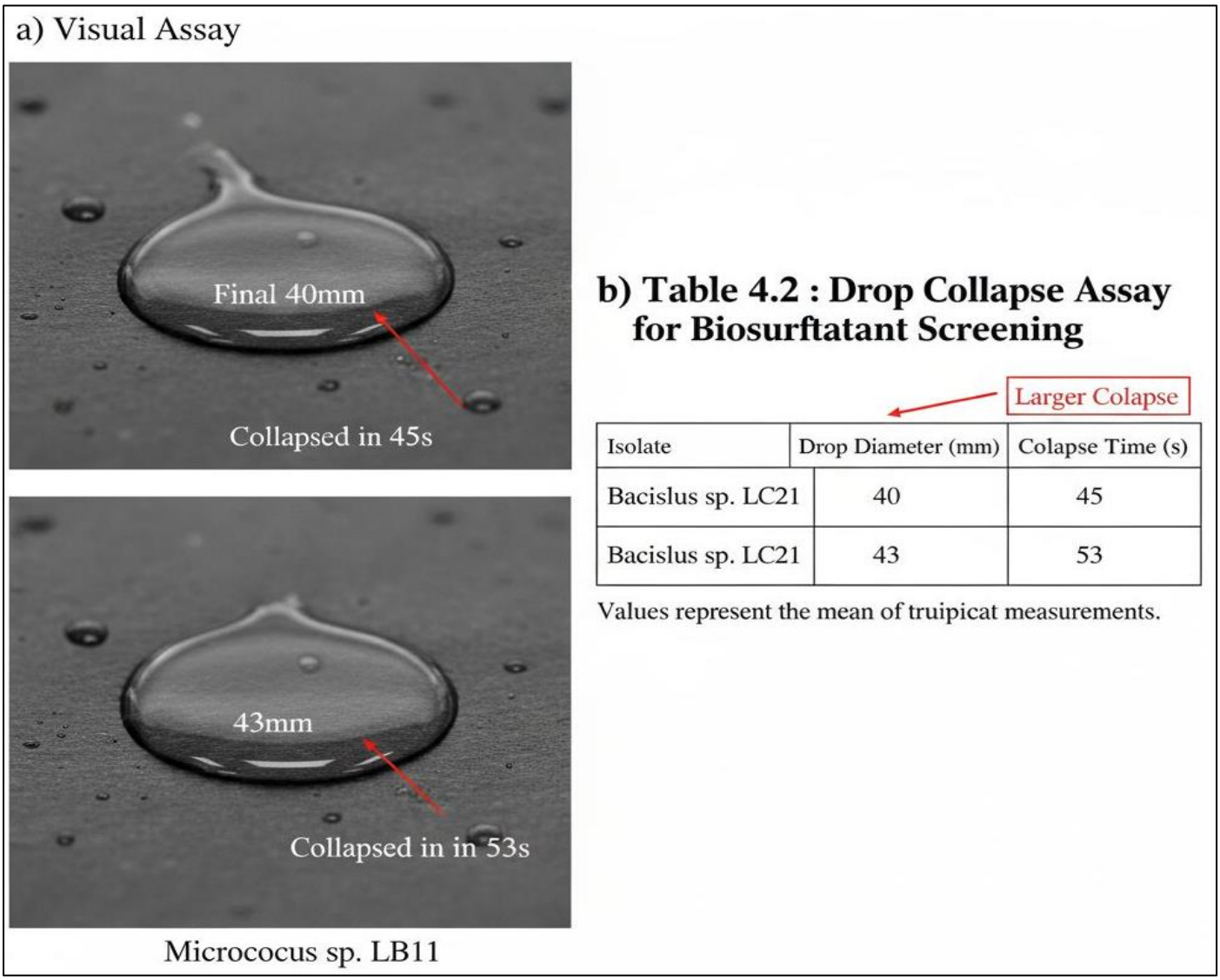


Fig 3 Screening of Bacterial Isolates for Biosurfactant Production Using the Drop-Collapse Assay.

- *Evaluation of Emulsification Potential Using the Emulsification Index ($E_{24}$)*

The emulsification index ($E_{24}$) assay was employed to quantify the ability of the biosurfactants to form and stabilize hydrocarbon-in-water emulsions, a key functional property for many applications. The stability of the emulsion over 24 hours was also qualitatively assessed.

The quantitative results, detailed in Table 3, show that both isolates produced biosurfactants capable of emulsifying groundnut oil. *Bacillus* sp. LC21 generated an emulsion layer of 10.4 mm, while *Micrococcus* sp. LB11 produced a comparable height of 10.32 mm. A critical distinguishing factor, however, was the observed stability of these emulsions. The emulsion formed by *Micrococcus* sp. LB11 retained its integrity with minimal phase separation over the 24-hour period (rated 'High' stability), whereas the emulsion from *Bacillus* sp. LC21 showed noticeable breakdown ('Moderate' stability). This indicates that the biosurfactant synthesized by *Micrococcus* sp. LB11 possesses superior emulsifying strength or chemical properties that confer greater long-term emulsion stability.

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Table 3 Emulsification Activity of Bacterial Isolates Against Groundnut Oil.

| Isolate | Hydrocarbon Substrate | Emulsion Height (mm) | Emulsion Stability (24 h) |
|---|---|---|---|
| *Bacillus* sp. LC21 | Groundnut Oil | 10.4 | Moderate |
| *Micrococcus* sp. LB11 | Groundnut Oil | 10.32 | High |

*mm = Millimetre.*

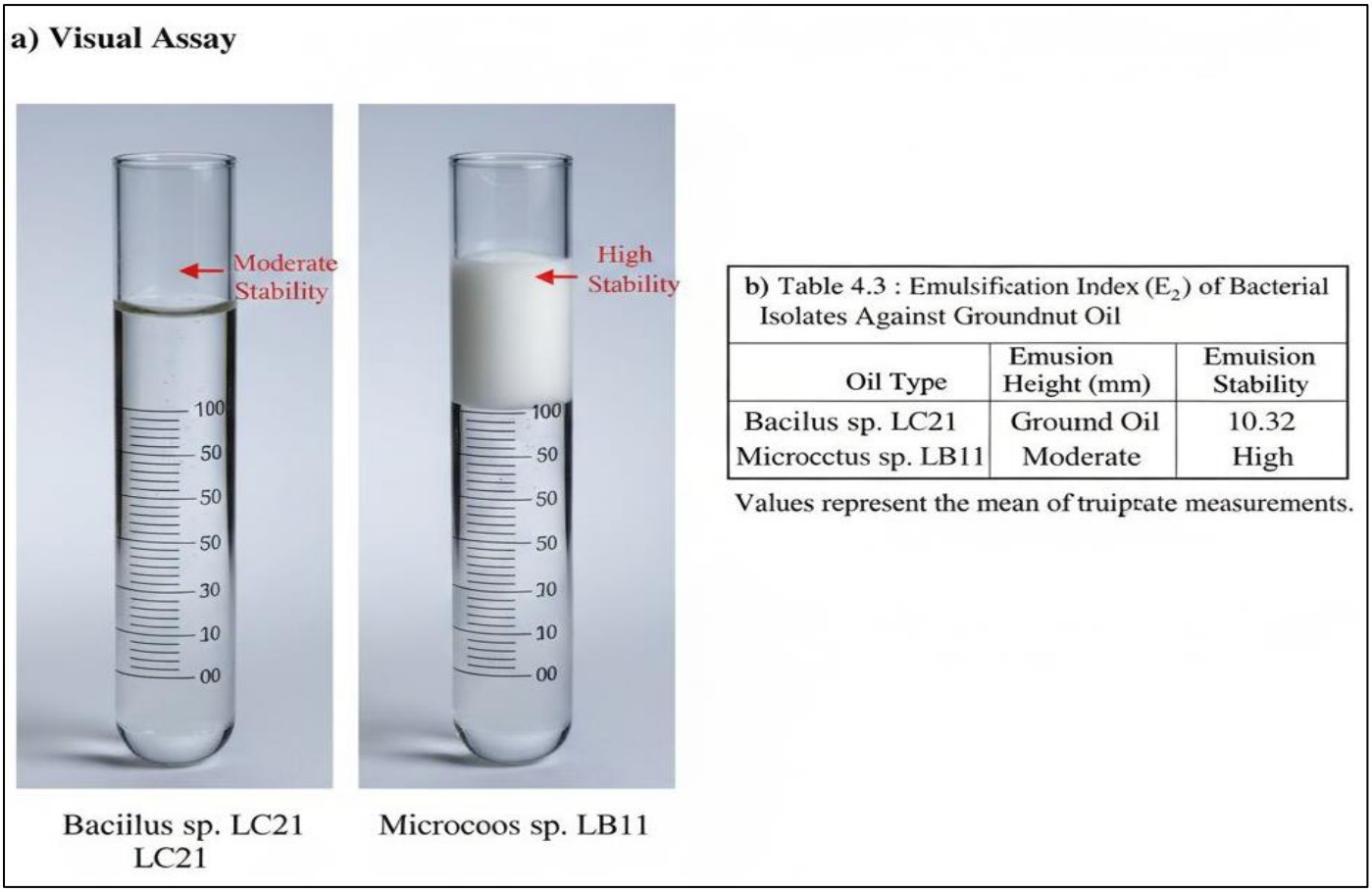


Fig 4 Emulsification Activity of Bacterial Isolates Against Groundnut Oil.

## V. DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

➢ *Discussion*

This study successfully evaluated the biosurfactant-producing potential of bacterial isolates from a hydrocarbon-impacted environment. The application of three complementary assays oil spreading, drop collapse, and emulsification index ($E_{24}$) provided a multifaceted assessment of surface-active properties, revealing distinct functional profiles for *Micrococcus* sp. LB11 and *Bacillus* sp. LC21.

The oil-spreading assay, a sensitive indicator of surface tension reduction, demonstrated a pronounced difference in activity. *Micrococcus* sp. LB11 generated a substantial displacement zone (15 mm) rapidly, indicative of the secretion of potent surface-active compounds. In contrast, the minimal activity of *Bacillus* sp. LC11 in this assay suggests either a lower concentration or a different mechanism of interfacial interaction of its biosurfactant under these specific conditions.

The drop-collapse assay, which measures the ability to disrupt a hydrophobic interface, yielded positive results for both isolates but with differing kinetics. The faster collapse time observed for *Bacillus* sp. LC21 implies its biosurfactant may act more rapidly at the oil-water interface. However, the final collapsed drop diameter was marginally larger for *Micrococcus* sp. LB11, hinting at potentially greater overall interfacial activity once equilibrium is approached. This dichotomy underscores that biosurfactants from different microbial origins can exhibit unique kinetic and equilibrium properties, influenced by their molecular architecture and critical micelle concentration.

The most telling distinction emerged from the emulsification index test. While both isolates formed emulsions of similar initial height, the emulsion stabilized by *Micrococcus* sp. LB11 exhibited superior long-term stability. High emulsion stability is a characteristic often associated with high-molecular-weight biosurfactants, such as glycoproteins or polymeric surfactants, which form stronger interfacial films. The moderate stability of the emulsion from *Bacillus* sp. LC21 aligns with the typical profile of low-molecular-weight lipopeptides, like surfactin,

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which are excellent at reducing surface tension but may produce less rigid interfacial layers. Consequently, *Micrococcus* sp. LB11 appears to synthesize a biosurfactant with structural properties highly conducive to forming durable emulsions, a critical asset for applications in bioremediation and oil recovery where long-term stability is paramount.

Collectively, these findings illustrate a key principle in biosurfactant science: functional performance is assay-dependent. An isolate may excel in one functional test while displaying moderate activity in another, reflecting the specific physicochemical demand of each assay. The robust and stable emulsification activity of *Micrococcus* sp. LB11, coupled with its strong performance in the oil-spreading test, positions it as a highly promising candidate for processes requiring effective and stable dispersion of hydrophobic substrates.

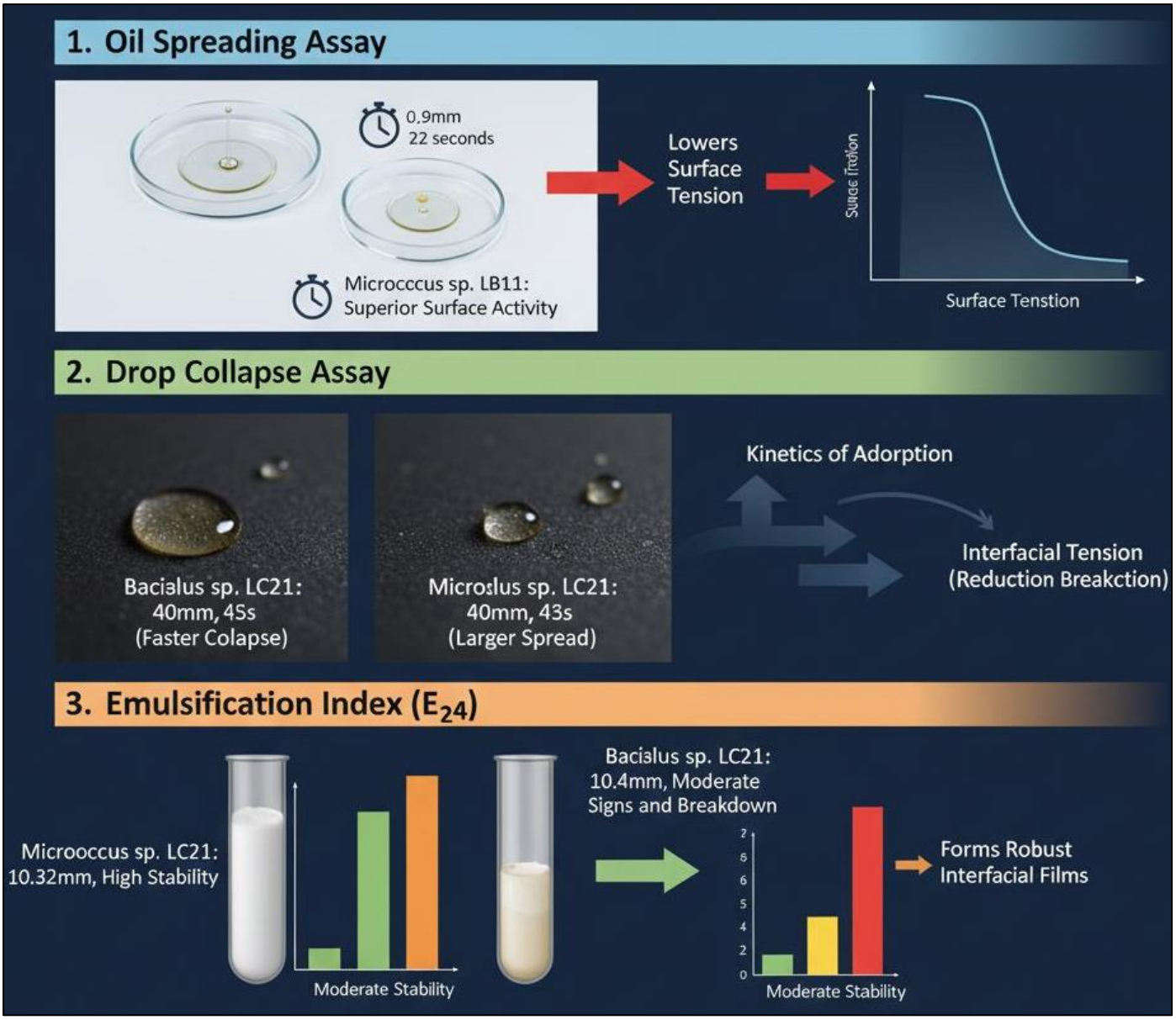

Fig 5 Overall Discussion on Comparative Analysis

➢ *Conclusion*

This investigation confirms the presence of diverse biosurfactant-producing bacteria within an oil-contaminated niche and successfully identifies isolates with distinct functional capabilities. *Micrococcus* sp. LB11 demonstrated consistently high biosurfactant activity, characterized by effective surface tension reduction and, most significantly, the ability to form highly stable emulsions. These properties strongly suggest the production of a high-molecular-weight biosurfactant, potentially of a glycoprotein nature, which is of considerable interest for industrial applications demanding persistent emulsification.

While *Bacillus* sp. LC21 exhibited a rapid action profile in the drop-collapse assay, its overall performance across the screening platform was less consistent than that

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of *Micrococcus* sp. LB11. Therefore, based on the composite analytical evidence particularly the critical parameter of emulsion stability *Micrococcus* sp. LB11 emerges as the most promising isolate identified in this study. Its proficiency in utilizing groundnut oil as a sole carbon source further enhances its economic appeal for scalable bioprocess development. This work underscores the potential of exploiting specialized environmental microbiota and low-cost agro-industrial substrates for the sustainable production of high-value biosurfactants.

➢ *Recommendations*

To advance the findings of this study towards application, the following strategic recommendations are proposed:

- Focused Strain Development and Process Optimization: *Micrococcus* sp. LB11 should be prioritized for subsequent research. Studies should aim to optimize its cultivation parameters (e.g., C:N ratio, pH, aeration, incubation time) using groundnut oil to maximize biosurfactant yield and productivity. Scale-up studies in bioreactors are essential to assess performance under controlled conditions.
- Comprehensive Biosurfactant Characterization: The biosurfactant produced by *Micrococcus* sp. LB11 must be extracted and purified using standard solvent extraction and chromatographic techniques. Its chemical identity should be elucidated through Fourier-Transform Infrared Spectroscopy (FTIR) for functional groups, Gas Chromatography-Mass Spectrometry (GC-MS) for lipid components, and nuclear magnetic resonance (NMR) spectroscopy for detailed structural analysis. This will confirm its hypothesized glycoprotein nature and define its exact structure.
- Rigorous Application Testing: The purified biosurfactant should be evaluated for efficacy in target applications. This includes:

✓ Bioremediation: Testing its ability to enhance the solubilization and biodegradation of crude oil and other hydrophobic pollutants in soil and water microcosms.
✓ Enhanced Oil Recovery (EOR): Assessing its efficiency in mobilizing residual oil from sandstone cores in laboratory-scale flooding experiments.
✓ Industrial Formulations: Investigating its suitability as an emulsifier or stabilizer in model cosmetic, pharmaceutical, or food-grade emulsion systems, subject to necessary biocompatibility and regulatory screening.

- Economic and Substrate Flexibility Analysis: A techno-economic assessment should be conducted to evaluate the feasibility of large-scale production using groundnut oil. Concurrently, research should explore the strain's ability to valorize other low-cost, renewable lipid wastes (e.g., used cooking oil, palm oil mill effluent) to ensure substrate flexibility and cost resilience.
- Molecular and Genetic Exploration: To understand and potentially enhance biosurfactant synthesis, genomic analysis of *Micrococcus* sp. LB11 should be undertaken to identify gene clusters responsible for production. Proteomic and transcriptomic studies under inducing conditions could reveal key regulatory pathways, paving the way for future genetic or metabolic engineering strategies to improve yield and tailor functionality.

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