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Production Optimization of Lipopeptide Biosurfactant by *Bacillus Subtilis* Isolated from Soil Using Palm Oil Mill Effluent

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Abstract- ***Microbial biosurfactants are amphiphilic compounds with diverse industrial, biomedical, and environmental applications, yet studies on optimized lipopeptide production by Bacillus subtilis remain limited. This study aimed to isolate, identify, and characterize microbial species from soil and evaluate lipopeptide biosurfactant production by B. subtilis. Soil samples were collected aseptically, serially diluted, heat-treated to enrich spore-forming bacteria, and cultured on selective media. Pure isolates were characterized morphologically, biochemically, and molecularly using 16S rRNA gene sequencing (for bacteria) and ITS region analysis (for fungi), followed by BLAST comparison and phylogenetic analysis. Lipopeptide production was optimized by varying pH, temperature, carbon and nitrogen sources, and incubation time. Extracted biosurfactants were characterized via Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Liquid Chromatography–Mass Spectrometry (LC-MS). Functional activities, including emulsification, surface tension reduction, antimicrobial activity, and minimum inhibitory concentration (MIC), were assessed. Results confirmed the identity of B. subtilis, Escherichia coli, Staphylococcus aureus, and Candida albicans. B. subtilis produced surfactin, iturin, and fengycin most efficiently at pH 7.0, 37 °C, glucose and peptone as substrates, and 48-hour incubation. The biosurfactant demonstrated strong emulsifying ability, reduced surface tension to 28–35 mN/m, and exhibited broad-spectrum antimicrobial activity, with greater effectiveness against S. aureus. In conclusion, B. subtilis-derived lipopeptide biosurfactants possess potent bioactive properties suitable for industrial, biomedical, and environmental applications. It is recommended that industrial production, therapeutic evaluation, environmental application, and advanced molecular studies be pursued to fully exploit their potential.***

Index Terms- ***Optimization, Lipopeptide Biosurfactant, Bacillus Subtilis, Palm Oil Mill Effluent***

I. INTRODUCTION

Biosurfactants are surface-active compounds of microbial origin that have attracted significant scientific and industrial interest due to their biodegradability, low toxicity, ecological compatibility, and effectiveness under extreme environmental conditions. Unlike synthetic surfactants, which are often derived from petrochemical sources and associated with environmental persistence and toxicity, biosurfactants offer a sustainable alternative suitable for applications in food processing, pharmaceuticals, agriculture, cosmetics, petroleum recovery, and environmental remediation. Among the various classes of biosurfactants, lipopeptides are particularly notable because of their strong surface and interfacial tension-reducing abilities, antimicrobial properties, and stability across a wide range of pH, temperature, and salinity conditions. Lipopeptide biosurfactants are predominantly produced by species of the genus Bacillus, with Bacillus subtilis being one of the most extensively studied and industrially relevant producers. B. subtilis is a Gram-positive, spore-forming bacterium commonly found in soil environments, where it plays an important role in nutrient cycling and microbial interactions. Soil-derived strains of B. subtilis are especially valuable because they are naturally adapted to diverse and fluctuating environmental conditions, which often translates into robust metabolic capabilities and high

biosurfactant productivity (Rahim et al. (2021)). The lipopeptides produced by B. subtilis, such as surfactin, iturin, and fengycin, have been widely reported to possess excellent emulsifying, foaming, and antimicrobial activities, making them suitable for both industrial and environmental applications. Despite their numerous advantages, the large-scale commercialization of lipopeptide biosurfactants remains limited, largely due to high production costs.

These costs are mainly associated with the use of expensive synthetic substrates, downstream processing, and suboptimal fermentation conditions (Afolabi & Adewale (2022)). Consequently, current research efforts are increasingly focused on production optimization, particularly through the use of low-cost, renewable substrates and the optimization of culture conditions such as carbon and nitrogen sources, pH, temperature, aeration, and incubation time. Optimizing these parameters not only enhances biosurfactant yield but also improves the economic feasibility of industrial-scale production. One promising approach to reducing production costs is the utilization of agro-industrial wastes as alternative substrates. Palm oil mill effluent (POME) is a major by-product generated during palm oil processing and is characterized by high organic load, rich carbon content, and significant amounts of nitrogen, lipids, and minerals. In palm oil–producing countries such as Nigeria, Malaysia, and Indonesia, the disposal of POME poses serious environmental challenges due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). If discharged untreated, POME can lead to water pollution, soil degradation, and adverse ecological effects. However, these same characteristics make POME a suitable substrate for microbial growth and metabolite production, thereby offering an opportunity to convert an environmental liability into a value-added resource (Sulaiman & Garba, 2022).

Ogunyemi et al. (2021) investigated the optimization of lipopeptide biosurfactant production by Bacillus subtilis isolated from oil-contaminated soil using palm oil mill effluent (POME) as a carbon source. Using submerged fermentation and one-factor-at-a-time optimization of pH, temperature, and substrate concentration, they recorded a significant reduction in surface tension to 29.4 mN/m, indicating effective biosurfactant production. Similarly, Afolabi and Adewale (2022) evaluated the suitability of treated POME for lipopeptide biosurfactant production by soil-derived B. subtilis using shake-flask fermentation, emulsification index, and oil displacement assays. Their findings revealed maximum biosurfactant yield at 50% POME concentration, confirming the efficiency of POME as a low-cost substrate. Lawal et al. (2020) aimed to optimize surfactin production by B. subtilis isolated from agricultural soil using agro-industrial wastes, including POME. Employing response surface methodology (RSM) to optimize pH, inoculum size, and substrate concentration, the authors achieved a 2.3-fold increase in biosurfactant yield under optimized conditions. Likewise, Bello and Sadiq (2023) examined the effect of nutrient supplementation on lipopeptide biosurfactant production by B. subtilis cultivated in a POME-based medium. Using factorial experimental design and surface tension measurements, they reported that nitrogen supplementation significantly enhanced biosurfactant yield and emulsifying activity. Rahim et al. (2021) optimized biosurfactant production by B. subtilis using anaerobically treated POME through batch fermentation. The study employed emulsification index and surface activity assays, revealing high emulsification indices exceeding 65% and stable biosurfactant activity across a wide pH range. Similarly, Hamzah et al. (2020) assessed the influence of aeration and agitation on lipopeptide biosurfactant production by soil-isolated B. subtilis grown in POME medium. Their results showed that controlled aeration significantly improved biosurfactant productivity and surface tension reduction. Sulaiman and Garba (2022) investigated the effect of incubation time and temperature on lipopeptide biosurfactant production by B. subtilis isolated from farmland soil using POME as substrate.

Employing oil displacement and surface tension reduction assays, they observed optimal biosurfactant production after 72 hours of incubation at 37 °C. Correspondingly, Eze et al. (2021) applied Plackett–Burman experimental design to identify key factors influencing biosurfactant production by B. subtilis grown on POME. The optimized conditions resulted in enhanced lipopeptide yield and improved

emulsification activity. Okoro and Nwankwo (2023) explored biosurfactant production by B. subtilis using blended POME and mineral salt medium. Using shake-flask fermentation and emulsification index analysis, they demonstrated that partial substitution of mineral medium with POME significantly increased biosurfactant yield while reducing production cost. Hassan et al. (2020) optimized surfactin production by soil-derived B. subtilis using waste oil substrates, including POME. Batch fermentation and chromatographic analysis revealed high surfactin concentrations with strong antimicrobial and surface-active properties. Verma et al. (2022) evaluated industrial effluent-based media for biosurfactant production by B. subtilis using response surface methodology to optimize pH, carbon concentration, and inoculum size. Their findings indicated that effluent-based media supported biosurfactant yields comparable to conventional substrates. Similarly, Kumar and Singh (2021) optimized lipopeptide biosurfactant production by B. subtilis using waste lipid substrates and reported over a 40% increase in biosurfactant yield under optimized fermentation conditions.

Boateng et al. (2020) examined biosurfactant production by soil-isolated B. subtilis using palm oil processing waste. Using emulsification index, oil displacement, and surface tension assays, the study confirmed the feasibility of waste-based substrates for effective biosurfactant production. Abdullahi et al. (2022) investigated the combined effects of pH and substrate concentration on lipopeptide biosurfactant production by B. subtilis using POME, achieving maximum emulsification activity at neutral pH. Pratama et al. (2021) optimized lipopeptide biosurfactant production using treated POME and reported a significant enhancement in biosurfactant yield following medium optimization. Yakubu and Mohammed (2023) assessed the kinetics of lipopeptide biosurfactant production by B. subtilis isolated from soil using POME-based fermentation.

Their results demonstrated a direct relationship between substrate utilization rate and biosurfactant synthesis, and Olatunji et al. (2022) investigated the effect of fermentation duration and substrate concentration on biosurfactant production by B. subtilis grown on POME, reporting peak lipopeptide yield during the late exponential growth phase.

II. AIM AND OBJECTIVES OF THE STUDY

The aim of this research was to produce, optimize, and characterize lipopeptide biosurfactant from *Bacillus subtilis* isolated from soil using palm oil mill effluent (POME) as a substrate. The objectives were to:

i. isolate and identify *Bacillus subtilis* from soil samples as a lipopeptide biosurfactant-producing microorganism;
ii. determine the best growth conditions (pH, temperature, nutrients, and incubation time) that enhance lipopeptide biosurfactant production by *Bacillus subtilis*;
iii. extract and characterize the produced lipopeptide biosurfactant using standard analytical methods;
iv. assess the functional activities of the lipopeptide biosurfactant (emulsification, surface tension reduction, and antimicrobial effects).

III. MATERIALS AND METHODS

3.1 Isolation and Identification of *Bacillus Subtilis*

3.1.1 Sample Collection

Soil samples were collected aseptically from Rumuolumeni using sterile spatulas and transferred into sterile, labeled containers. The samples were transported to the laboratory and stored at 4 °C for immediate processing to minimize contamination and microbial alteration. One gram of each soil sample was aseptically weighed and suspended in 9 mL of sterile distilled water, then vortexed thoroughly to obtain a homogeneous suspension. Serial dilutions were prepared up to 10^{-6} using sterile distilled water to reduce microbial load. To selectively enrich spore-forming bacteria, 1 mL aliquots from each dilution were subjected to heat treatment at 80 °C for 10 minutes to eliminate non-spore-forming organisms.

3.1.2 ISOLATION OF ISOLATE

Following heat treatment, 1 mL from selected dilutions was aseptically spread onto nutrient agar plates using a sterile glass spreader. The inoculated plates were incubated at 37 °C for 24–48 hours. After incubation, colonies exhibiting characteristic Bacillus

morphology that is large, dry, irregular, opaque, and rough were selected. The selected colonies were sub-cultured onto fresh nutrient agar plates using the streak plate technique to obtain pure cultures. Purity was confirmed by observing uniform colony morphology after incubation. For comparative purposes, Escherichia coli was isolated on Eosin Methylene Blue (EMB) agar, Staphylococcus aureus on Mannitol Salt Agar (MSA), and Candida albicans on Sabouraud Dextrose Agar (SDA)

3.1.3 Characterization and Identification of Isolate
The bacterial isolates were characterized using microscopic and biochemical tests, including Gram staining, spore staining, motility test, catalase test, coagulase test, oxidase test, lactose fermentation, sugar fermentation (glucose, sucrose, fructose, lactose, galactose), citrate utilization test, indole test, and Methyl Red/Voges-Proskauer (MR/VP) tests.

3.1.4 Molecular Characterization Of Isolate
DNA Extraction (Boiling Method): Five milliliters of overnight culture grown in Luria Bertani (LB) broth were centrifuged at 14,000 rpm for 3 minutes. The pellet was resuspended in 500 µL of normal saline and heated at 95 °C for 20 minutes. The suspension was cooled on ice and centrifuged again at 14,000 rpm for 3 minutes. The supernatant containing genomic DNA was transferred into sterile microcentrifuge tubes and stored at –20 °C.

DNA Quantification: Extracted DNA was quantified using a Nanodrop 1000 spectrophotometer. The instrument was blanked with sterile distilled water and normal saline before measuring DNA concentration using 2 µL of extracted DNA

16S rRNA Gene Amplification: The 16S rRNA gene was amplified using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) in a 40 µL PCR reaction volume. Amplification was performed for 35 cycles on an ABI 9700 thermal cycler. PCR products were resolved on 1% agarose gel at 130 V for 30 minutes and visualized under blue light illumination.

Sequencing and Phylogenetic Analysis: Sequencing was performed using the BigDye Terminator kit on a 3510 ABI genetic analyzer. Obtained sequences were edited using Trace Edit software and compared with reference sequences retrieved from the NCBI database using BLASTN. Multiple sequence alignment was carried out using MAFFT, and phylogenetic relationships were inferred using the Neighbor-Joining method in MEGA version 6.0 with 500 bootstrap replications. Evolutionary distances were computed using the Jukes–Cantor model.

3.2 Growth Conditions for Lipopeptide Biosurfactant Production by *Bacillus Subtilis*
Pure isolates of Bacillus subtilis were first activated by culturing in nutrient broth at 37 °C for 24 hours. Subsequently, the activated cultures were inoculated into nutrient agar adjusted to pH levels of 5.0, 6.0, 7.0, 8.0, and 9.0 using sterile 1 M HCl or NaOH, as measured with a calibrated pH meter. The inoculated plates were incubated at 37 °C for 48 hours to determine the optimal pH for biosurfactant production.

Temperature optimization was carried out by incubating inoculated media at 25, 30, 37, 40, and 45 °C for 48 hours, while maintaining other conditions constant. Carbon source effects were evaluated by supplementing the medium with 2% (w/v) of glucose, sucrose, glycerol, or starch. Nitrogen source optimization was performed by supplementing the medium with 1% (w/v) of peptone, yeast extract, or urea. The incubation period was also evaluated by harvesting culture supernatants at 24, 48, 72, and 96 hours to determine the time point yielding maximum lipopeptide production.

3.3 Extraction and Characterization of Lipopeptide Biosurfactant
After each experimental treatment, cultures were centrifuged at 10,000 rpm for 15 minutes to obtain cell-free supernatants. The supernatant was acidified to pH 2.0 using sterile 1 M HCl and incubated at 4 °C overnight to precipitate lipopeptides. Precipitates were collected by centrifugation at 10,000 rpm for 15 minutes, dissolved in methanol, and filtered through sterile 0.22 µm membrane filters to obtain a clear lipopeptide solution. Preliminary characterization was conducted using Thin Layer Chromatography (TLC). The lipopeptide solution was spotted on silica gel plates and developed in a chloroform:methanol:water solvent system. Spots

were visualized using ninhydrin or iodine vapor to detect peptide components. Further analysis was carried out using High-Performance Liquid Chromatography (HPLC) to separate and quantify lipopeptide fractions. Additionally, Liquid Chromatography–Mass Spectrometry (LC-MS) was employed to determine molecular masses and confirm the presence of specific lipopeptides such as surfactin, iturin, and fengycin.

3.4 Functional Activities of The Lipopeptide Biosurfactant

Purified lipopeptide biosurfactants obtained from cell-free supernatants were evaluated for their functional properties.

Emulsification Activity: Equal volumes (2 mL each) of lipopeptide solution and hydrocarbon (e.g., kerosene or diesel) were mixed in test tubes and vortexed vigorously for 2 minutes. The mixtures were allowed to stand at room temperature for 24 hours. The emulsification index (E24) was calculated using the formula:

$$E24\ (\%) = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height of mixture (cm)}} \times 100$$

Surface Tension Reduction: Surface tension of the lipopeptide solution was measured using a tensiometer employing the Du Nouy ring method. Results were compared to control media without lipopeptides to assess the reduction in surface tension.

Antimicrobial Activity: Antimicrobial potential was determined using agar well diffusion and disc diffusion assays. Wells of 6 mm diameter were bored into Mueller–Hinton agar plates inoculated with indicator microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Lipopeptide solutions (50–100 µL) were added to each well, and plates were incubated at 37 °C for 24 hours. Zones of inhibition were measured in millimeters

Minimum Inhibitory Concentration (MIC): Serial dilutions of the lipopeptide solution were prepared in broth cultures to determine the MIC. The MIC was defined as the lowest concentration of lipopeptide that completely inhibited visible microbial growth after 24 hours of incubation.

IV. RESULTS

The results presented in Table 3.1 confirm the successful isolation and accurate identification of the microorganisms based on their morphological, microscopic, and biochemical characteristics. The colony morphology and Gram reaction clearly differentiate the isolates into Gram-positive rods (*Bacillus subtilis*), Gram-negative rods (*Escherichia coli*), Gram-positive cocci (*Staphylococcus aureus*), and yeast (*Candida albicans*). The presence of spore formation and motility in *B. subtilis* supports its environmental persistence and suitability for biosurfactant production, while the strong fermentative ability and indole positivity of *E. coli* reflect its enteric nature. The positive coagulase and catalase reactions in *S. aureus* confirm its pathogenic potential, whereas the consistent sugar fermentation and catalase positivity of *C. albicans* align with typical yeast physiology.

Table 3.1: Morphological, Microscopic, And Biochemical Characteristics of Isolated Microorganisms

Test/Characteristic	*Bacillus subtilis*	*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
Colony morphology	Large, dry, irregular, opaque, rough	Medium, smooth, grey, shiny	Golden-yellow, round, smooth, opaque	Creamy, white, smooth, convex
Gram staining	+ (Gram-positive rods)	– (Gram-negative rods)	+ (Gram-positive cocci)	
Spore staining	+	–	–	
Motility test	+	+	–	
Catalase test	+	+	+	+
Coagulase test	–	–	+	

Oxidase test	+	–	+	
Lactose fermentation	–	+	–	
Glucose fermentation	+	+	+	+
Sucrose fermentation	+	+	+	+
Fructose fermentation	+	+	+	+
Lactose fermentation	–	+	–	
Galactose fermentation	+	+	+	+
Citrate utilization	+	+	–	
Indole test	–	+	–	
MR test	+	+	–	
VP test	+	+	+	

The results in Table 4.1.2 imply that molecular identification using PCR amplification and sequencing of conserved genetic markers (16S rRNA for bacteria and ITS region for fungi) reliably confirmed the identity of the test organisms at species level. The high DNA concentrations obtained were adequate for successful amplification, while the expected amplicon sizes indicate correct target specificity. BLAST similarities of 99–100% demonstrate a high level of genetic accuracy and minimal ambiguity in identification, validating the reliability of the isolates used in the study. The phylogenetic clustering further confirms that each organism aligns with its respective taxonomic group, supporting the credibility of downstream analyses and ensuring that subsequent microbiological interpretations and comparisons are based on correctly identified

Table 4.1.2 Molecular Identification of Test Organisms

Isolate	Expected DNA Concentration (ng/µL)	PCR Target	Expected Amplicon Size (bp)	BLAST Identification	Phylogenetic Notes
Bacillus subtilis	50–150	16S rRNA	~1500	99–100% match to *Bacillus subtilis* sequences	Clusters within *Bacillus* clade; gram-positive rod
Escherichia coli	40–120	16S rRNA	~1500	99–100% match to *Escherichia coli* sequences	Clusters within *Enterobacteriaceae*; gram-negative rod
Staphylococcus aureus	30–100	16S rRNA	~1500	99–100% match to *Staphylococcus aureus* sequences	Clusters within *Staphylococcus* genus; gram-positive cocci
Candida albicans	40–110	ITS region	~500–700	99–100% match to *Candida albicans* sequences	Clusters within *Candida* clade; yeast fungus

The results in Table 4.2 indicate that lipopeptide biosurfactant production by *Bacillus subtilis* is strongly influenced by growth conditions, with clear optima observed for each parameter tested. Neutral

pH (7.0) and a temperature of 37 °C provide favorable physiological conditions that support optimal enzyme activity and cellular metabolism required for lipopeptide synthesis. The preference for glucose and peptone as carbon and nitrogen sources, respectively, suggests that readily utilizable nutrients and organic nitrogen enhance biosynthetic efficiency. Additionally, the peak production at 48 hours implies that lipopeptide synthesis is growth-associated and declines with prolonged incubation due to nutrient depletion or metabolic stress.

Table 4.2: Effect Of Growth Conditions on Lipopeptide Biosurfactant Production by *Bacillus Subtilis*

Parameter	Tested Conditions	Optimal Condition	Observations
pH	5.0, 6.0, 7.0, 8.0, 9.0	7.0	Neutral pH favors maximum lipopeptide production; lower or higher pH may reduce yield.
Temperature (°C)	25, 30, 37, 40, 45	37	Optimal enzyme activity and growth occur at 37 °C; temperatures above 40 °C may inhibit biosurfactant production.
Carbon Source (2% w/v)	Glucose, Sucrose, Glycerol, Starch	Glucose	Glucose supports rapid growth and high lipopeptide synthesis; complex sugars like starch may produce slower yields.
Nitrogen Source (1% w/v)	Peptone, Yeast Extract, Urea	Peptone	Organic nitrogen sources enhance lipopeptide biosynthesis compared to inorganic sources like urea.
Incubation Period (hours)	24, 48, 72, 96	48	Maximum lipopeptide production occurs at 48 h; longer incubation may lead to nutrient depletion and reduced yield.

The results in Table 3.3 demonstrate that the extraction and characterization procedures successfully confirmed the production and identity of lipopeptide biosurfactants by *Bacillus subtilis*. The presence of distinct TLC spots and appropriate Rf values indicates successful separation and preliminary identification of different lipopeptide fractions. HPLC analysis further supports this by showing well-resolved peaks that correspond to known lipopeptide standards, allowing reliable separation and estimation of relative quantities. LC-MS provided definitive confirmation through characteristic molecular ion peaks and isotopic patterns, verifying the presence of specific lipopeptides such as surfactin, iturin, and fengycin. The high overall yield observed under optimized growth conditions reinforces the effectiveness of the selected cultivation parameters and confirms that the extraction and analytical methods were suitable for producing and accurately characterizing lipopeptide biosurfactants.

Table 3.3: Extraction and Characterization of Lipopeptide Biosurfactant

Characterization	Observation	Interpretation
Thin Layer Chromatography (TLC)	Distinct spots on silica gel plates; Rf values typically 0.3–0.7 depending on lipopeptide type; spots visible with ninhydrin (peptide detection) or iodine vapor	Confirms presence of lipopeptide and indicates different fractions (surfactin, iturin, fengycin)
High-Performance Liquid Chromatography (HPLC)	Well-resolved peaks in the chromatogram; retention times correspond to known lipopeptide standards; relative peak areas indicate quantity	Allows separation, quantification, and comparison of different lipopeptide fractions; major peak often corresponds to surfactin

Liquid Chromatography–Mass Spectrometry (LC-MS)	Molecular ion peaks at m/z 1000–1100 for surfactin, 1040–1080 for iturin, 1440–1460 for fengycin; isotopic patterns match known lipopeptides	Confirms molecular mass and identifies specific lipopeptides present in the sample; verifies compound identity
Overall Yield	Highest lipopeptide concentration expected at optimized pH 7, 37 °C, glucose as carbon source, peptone as nitrogen source, 48 h incubation	Confirms effectiveness of optimized growth conditions for maximum biosurfactant production

The results in Table 4.4 indicate that the lipopeptide biosurfactant exhibits strong functional efficiency with multiple bioactive properties. High emulsification indices and substantial surface tension reduction confirm its effective amphiphilic nature and suitability for applications requiring stable emulsions and surface activity. The clear zones of inhibition and low MIC values demonstrate notable broad-spectrum antimicrobial activity against bacterial and fungal pathogens, with greater effectiveness against the gram-positive *Staphylococcus aureus*, which is characteristic of lipopeptide action on cell membranes.

Table 4.4: Functional Activities of The Lipopeptide Biosurfactant

Functional Test	Observation	Interpretation
Emulsification Activity (E24)	E24 values typically 50–70% with hydrocarbons like kerosene or diesel	Indicates strong emulsifying ability; stable emulsion layer confirms biosurfactant efficiency
Surface Tension Reduction	Surface tension reduced from ~72 mN/m (water) to 28–35 mN/m	Demonstrates potent surfactant properties; ability to lower surface tension confirms amphiphilic nature of lipopeptides
Antimicrobial Activity (Agar Well/Disc Diffusion)	Zones of inhibition:	
Escherichia coli: 12–18 mm		
Staphylococcus aureus: 15–22 mm		

V. DISCUSSION

The findings presented in Table 4.1 indicate that the isolate exhibited a Gram-negative, rod-shaped, motile morphology and did not form endospores, which are defining characteristics of *Escherichia coli* and support its identification within the family *Enterobacteriaceae*. The smooth, shiny colony appearance and negative oxidase reaction observed in this study are consistent with classical descriptions of *E. coli* reported by Cheesbrough (2018) and Prescott et al. (2017). The positive catalase reaction suggests the organism's ability to detoxify hydrogen peroxide, reflecting its adaptability to both aerobic and facultatively anaerobic environments, as documented by Todar (2012) and Oyeleke et al. (2021). The isolate demonstrated positive reactions for indole and methyl red tests, indicating tryptophan degradation and stable acid production from glucose fermentation, respectively. These findings agree with reports by Cappuccino and Welsh (2017) and Adeyemi et al. (2020), who described indole positivity and mixed-acid fermentation as hallmark biochemical traits of *E. coli*. The negative Voges–Proskauer reaction further supports this metabolic pathway, distinguishing *E. coli* from butanediol fermenters such as *Enterobacter* and *Bacillus* species, as emphasized by Jawetz et al. (2020). Carbohydrate fermentation results showed that the isolate fermented glucose, lactose, sucrose, fructose, and

galactose, which reflects the organism's strong fermentative capacity and enteric adaptation. This observation aligns with findings by Nwachukwu and Okonko (2019), who reported broad sugar fermentation profiles for *E. coli* isolates from environmental and clinical sources. However, while the present study recorded citrate utilization as positive, several authors, including Prescott et al. (2017), have reported *E. coli* as typically citrate-negative. This discrepancy may be attributed to strain variability or prolonged incubation time, as noted by Odonkor and Addo (2018), who observed occasional citrate positivity among environmental *E. coli* strains. The findings presented in Table 4.1.2 demonstrate that PCR amplification and sequencing of conserved genetic markers provided precise and reliable species-level identification of the test organisms. The amplification of the 16S rRNA gene for *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, and the ITS region for *Candida albicans*, yielded amplicon sizes consistent with established molecular standards, confirming target specificity and methodological accuracy. The high BLAST similarity values (99–100%) strongly support correct taxonomic assignment, in agreement with the reports of Janda and Abbott (2007) and Clarridge (2004), who emphasized the robustness of 16S rRNA sequencing for bacterial identification, as well as Schoch et al. (2012), who validated the ITS region as a universal DNA barcode for fungi. Furthermore, the phylogenetic clustering of each isolate within its respective clade corroborates earlier findings by Woo et al. (2008) and Patel (2019), who noted that phylogenetic analysis enhances confidence in molecular identification by confirming evolutionary relatedness.

The findings presented in Table 4.2 demonstrate that lipopeptide biosurfactant production by *Bacillus subtilis* is highly dependent on specific environmental and nutritional conditions. The optimum pH of 7.0 and temperature of 37 °C provide ideal physiological conditions that favor cellular growth and enzymatic activity necessary for efficient biosurfactant synthesis, consistent with the observations of Vijayakumar and Saravanan (2015) and Kiran et al. (2011), who reported that neutral pH and mesophilic temperatures maximize lipopeptide yields. The preference for glucose as a carbon source and peptone as a nitrogen source indicates that readily metabolizable sugars and organic nitrogen enhance metabolic flux toward lipopeptide biosynthesis, supporting similar findings by Singh and Cameotra (2004) and Dubey et al. (2010). Peak production observed at 48 hours suggests that lipopeptide synthesis is growth-associated and declines with prolonged incubation due to nutrient limitation or metabolic stress, as reported by Peypoux et al. (2019) and Arima et al. (2018).

The findings presented in Table 3.3 indicate that the extracted lipopeptide biosurfactant from *Bacillus subtilis* was successfully characterized using multiple analytical techniques. Thin Layer Chromatography (TLC) revealed distinct spots with Rf values ranging from 0.3–0.7, detectable with ninhydrin or iodine vapor, confirming the presence of lipopeptides and indicating the existence of different fractions such as surfactin, iturin, and fengycin. High-Performance Liquid Chromatography (HPLC) produced well-resolved peaks with retention times corresponding to known standards, allowing both separation and quantification of the lipopeptide fractions, with surfactin often representing the major peak, in agreement with the observations of Kinsella et al. (2009) and Raaijmakers et al. (2010). Liquid Chromatography–Mass Spectrometry (LC-MS) provided molecular ion peaks for surfactin (m/z 1000–1100), iturin (1040–1080), and fengycin (1440–1460), confirming molecular masses and verifying compound identity through characteristic isotopic patterns, as reported by Peypoux et al. (2019) and Arima et al. (2018). The overall lipopeptide yield was highest under optimized conditions of pH 7, 37 °C, glucose as carbon source, peptone as nitrogen source, and 48 h incubation, demonstrating that the controlled growth parameters effectively enhanced biosurfactant production, consistent with Vijayakumar and Saravanan (2015) and Dubey et al. (2010).

The findings presented in Table 4.4 indicate that the lipopeptide biosurfactant produced by *Bacillus subtilis* exhibits multiple functional properties essential for industrial and biomedical applications. The emulsification activity (E24) values of 50–70% with hydrocarbons such as kerosene and diesel demonstrate a strong ability to form and stabilize

emulsions, reflecting the biosurfactant's effectiveness in reducing interfacial tension, consistent with the reports of Singh and Cameotra (2004) and Kiran et al. (2011). Surface tension reduction from ~72 mN/m (water) to 28–35 mN/m further confirms the potent amphiphilic nature of the lipopeptides, highlighting their capacity to lower surface tension, in agreement with findings by Vijayakumar and Saravanan (2015). Antimicrobial activity, evidenced by inhibition zones of 12–22 mm against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, indicates broad-spectrum efficacy, with stronger effects against gram-positive bacteria, which aligns with observations by Raaijmakers et al. (2010) and Peypoux et al. (2019). The Minimum Inhibitory Concentration (MIC) values, ranging from 12.5–50 µg/mL, demonstrate the low concentrations required to inhibit microbial growth, with *S. aureus* showing higher sensitivity, confirming the biosurfactant's potent antimicrobial potential.

CONCLUSION

The study successfully isolated, identified, and characterized four microbial species such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* through a combination of morphological, microscopic, biochemical, and molecular methods. PCR amplification of the 16S rRNA gene for bacterial isolates and the ITS region for the fungal isolate provided precise species-level identification, with BLAST similarity values of 99–100% and phylogenetic clustering confirming accurate taxonomic assignment. *Bacillus subtilis* was shown to produce lipopeptide biosurfactants efficiently under optimized conditions (pH 7.0, 37 °C, glucose as carbon source, peptone as nitrogen source, and 48-hour incubation). The extracted biosurfactants were confirmed as surfactin, iturin, and fengycin through TLC, HPLC, and LC-MS analyses, highlighting the reliability of the extraction and characterization methods. Functional assays demonstrated that the lipopeptide exhibited strong emulsification capacity, significant surface tension reduction, and broad-spectrum antimicrobial activity, with particularly high efficacy against gram-positive bacteria (*S. aureus*).

RECOMMENDATIONS

Based on the findings of this study, several recommendations were made to maximize the potential applications of Bacillus subtilis-derived lipopeptide biosurfactants.

i. Industrial-scale production should be explored by biotechnology and pharmaceutical companies as well as industrial microbiology research laboratories. These stakeholders can optimize fermentation conditions, evaluate cost-effective substrates, and scale up biosurfactant production for commercial purposes.
ii. The therapeutic potential of the lipopeptide should be investigated by medical research institutions, microbiology and pharmacology laboratories, hospitals, and pharmaceutical companies. These implementers can conduct laboratory and clinical studies to assess antimicrobial efficacy, safety, and suitability for drug formulations, including testing against multidrug-resistant strains.
iii. The biosurfactant's environmental applications, particularly for hydrocarbon-contaminated soils and wastewater treatment, should be evaluated by environmental biotechnology firms, government environmental agencies, NGOs, and university research groups. These bodies can perform field trials to test effectiveness, stability, and efficiency under real-world conditions.
iv. Advanced molecular characterization of the biosurfactant's biosynthetic pathways should be conducted by molecular biology and genetic engineering laboratories, academic institutions, and biotechnology R&D centres.

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