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ISOLATION OF BIOSURFACTANT-PRODUCING BACTERIA FROM OIL-SPILLED SOIL AND CHARACTERIZATION OF THEIR SECRETED BIOSURFACTANTS IN PATHOGEN-INHIBITION AND OIL-EMULSIFICATION

Mohammad Zobaer [1], Ferdausi Ali [1], Nural Anwar [*1], Mohammed Sajjad Hossain Bappi [2, 3], Takia Binte Bakar [1], Tanim Jabid Hossain [2, 3], Md. Sajib Khan [2, 3] and A. N. M. Shahriar Zawad [2]

Department of Microbiology [1], University of Chittagong, Chattogram 4331, Bangladesh.
Department of Biochemistry and Molecular Biology [2], University of Chittagong, Chattogram 4331, Bangladesh.
BioProcesses and Bioactive Molecules Unit [3], Biochemistry and Pathogenesis of Microbes Research Group, Chattogram 4331, Bangladesh.

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Correspondence to Author:
Md. Nural Anwar

Research Scholar,
Department of Microbiology,
University of Chittagong, Chattogram 4331, Bangladesh.

E-mail: anwarmn51@yahoo.com

ABSTRACT: Microbial biosurfactants are surface-active amphiphilic molecules produced by bacteria, yeast and fungi. Biosurfactant-producing bacteria, often recovered from oil-polluted sites, have significant role in microbial enhanced oil recovery (MEOR), environmental sustainability and pharmaceutical applications. The objective of the present study includes isolation, screening and selection of biosurfactant-producing bacteria from oil polluted sites and characterization of their secreted biosurfactant. To this end, nine bacterial strains were isolated from oil spilled soil in a motor garage and subjected to blood hemolysis test as the preliminary screening for biosurfactant-producing strains wherein six isolates tested positive. Upon subsequent analysis by the oil spreading assay and emulsification index, two potent biosurfactant-producing strains were selected for further characterization. An extensive biochemical, cultural and morphological investigations identified the biosurfactant producing isolates as *Bacillus* species strain JR3 and *Acinetobacter* sp. strain JR7. Culture conditions of the two strains were optimized for maximum biosurfactant production. Their secreted biosurfactants were extracted from cell-free culture supernatant using chloroform-methanol precipitation, and characterized by thin layer chromatography which indicated that the biosurfactants were of lipopeptide in nature. The biosurfactants exhibited antimicrobial activity against both Gram positive and negative bacteria. Kerosene appeared to be the most suitable substrate for emulsification of the biosurfactants followed by diesel, soya bean, and octane. The thermal and pH stability of the extracted biosurfactants was assessed as a function of their emulsification index which indicated their stability under high temperature and a broad range of pH, suggesting their potential values in medicine, pharmaceutical and bioremediation under wide environmental conditions.

INTRODUCTION: Surfactants are surface-active agents with hydrophilic heads and hydrophobic tails [1].

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Long-chain fatty acids and their derivatives are the constituents of the hydrophobic portion, whereas carbohydrates, carboxylic acids, cyclic peptides, phosphates, amino acids, and alcohols contribute as the hydrophilic moiety of surfactants [2]. Since, surfactants are amphiphilic in nature, they diminish surface and interfacial tensions to alter the repulsive forces between two distinct phases that would not usually interact for their opposing

molecular properties [3]. Surfactants hence have a wide range of applications in the fields of medicine, pharmaceuticals, cosmetics, food preservation, agriculture, bioremediation, and textile auxiliaries [4]. Interestingly, the global surfactant market is expected to exceed USD 53 billion by 2028 [5]. However, most of these surfactants are chemically produced from oleochemical and petrochemical resources which are rather more toxic to the environment and less biodegradable than biosurfactants, the biologically synthesized surfactants form microorganisms [6–8]. Nowadays, biosurfactants are achieving greater attention owing to their several advantages including higher biodegradability and selectivity, lower toxicity and critical micelle, ecological acceptability, and ability to be synthesized from more affordable and renewable sources [9]. Biosurfactants exhibit significant efficacy even at relatively extreme temperatures, pH and salinity [10].

Policymakers in different sectors being environmentally conscious, therefore, increasing the consumption of biosurfactants in each sector. According to the global market insight research 2018, biosurfactant market generated revenue of more than USD 1.8 Billion in 2016 and is predicted to reach USD 2·6 Billion by 2023 [11]. The biosurfactant producing microbes either secrete biosurfactants extracellularly or retain it attached to the cell surface [12]. Depending on their chemical makeup, the four major groups of biosurfactants are Glycolipids, phospholipids, polymeric biosurfactants, and lipopeptides. Rhamnolipids, Sophorolipids, and Trehalolipidsare the best-known glycolipids [13].

Many *Pseudomonas* species produce significant amounts of Rhamnolipids. Surfactin, Iturin, Fengycin are three well-known lipopeptides mostly produced by *Bacillus, Pseudomonas, Streptomyces, and Acinetobacter* [14]. In addition, some gram-positive bacteria and yeast yield Trehalolipids and Sophorolipids respectively [15]. The biosurfactant-producing bacteria are typically prevalent in oil-contaminated environments where they utilize hydrocarbons as their carbon sources. Therefore, Their applications in the environment are mostly associated with the bioremediation of hydrocarbons in soil and groundwater, as well as the degradation of toxic materials released from the industries [16].

Biosurfactants also have a wide range of applications in the pharmaceutical, medicine, agriculture and food industries [17, 18]. The unique structure and characteristics of biosurfactants enable them to be employed in pharmaceuticals for delivering genes, recovering intracellular products and inhibiting pathogens [19–21]. However, the mechanism of action defines how they are applied in pharmaceutical processes. Among the different gene transfection strategies, lipofection using cationic liposomes is regarded as a potential means of delivering foreign genes to the target cells without causing any side effects. When compared to synthetic cationic liposomes, biosurfactant-based liposomes showed improving gene transfection efficiency [22]. In addition, a remarkable enhancement in the humoral immune response was observed in rabbits and chickens by coupling Iturin A, Herbicolin, and other lipopeptides as immunomodulators [23].

Because of their high surface activities, lipopeptides also have the potential to be utilized as antibacterial, antifungal, and antitumor agents [24]. Reverse micelle solutions obtained by the action of surfactants can selectively permeabilize the *Escherichia coli* in order to extract Penicillin acylase [25]. Biosurfactants are also used as wetting agents, emulsifiers, and dispersants in various agricultural applications, such as pesticide formulations, herbicide formulations, and fertilizer suspensions[26]. In the food industry, biosurfactants are used as emulsifiers, stabilizers, and texturizing agents to improve the texture and stability of food products [27].

Biosurfactants also play an important role in mitigating environmental pollutions. They are useful in bioremediation of contaminated soil and groundwater where they enhance the solubility and bioavailability of pollutants, allowing micro-organisms to degrade them more efficiently [28]. They can be used in the cleanup of oil spills, as they break down oil-water emulsions and increase the solubility of the oil, making it easier to remove from the environment [29]. Nowadays, heavy metal contamination is also evolving as a critical environmental issue. Heavy metals do not degrade naturally and stay in the soil for a long period [30]. By producing biosurfactants, microorganisms transform harmful metals into their non-toxic form

to protect soil quality [31]. Considering the high values of the biologically synthesized surfactants, the present study was undertaken to isolate and investigate biosurfactant producing novel bacterial species from contaminated soil, and evaluate their properties and potential applications. After a three-tier screening and selection process, the strains that were found highly effective in producing biosurfactants, were thoroughly characterized. Their secreted bio-surfactant was extracted and assessed for biochemical nature, emulsifying capacity and stability. With the increasing demand for sustainable and cost-effective solutions for environmental pollution, this study has the potential to make a significant contribution to the field of environmental remediation and biotechnology.

MATERIALS AND METHODS:

Sample Collection: Oil-spilled soil was obtained from an automobile garage located in Panchlaish, Chattogram. The soil was collected using a sterile spatula into sterile zip-locked bags and kept in an icebox during transportation to the laboratory. Soil pH and temperature measured at the collection sites were found to be 6.2 and 28°C respectively.

Enrichment: For enrichment, 1 g of the collected soil was mixed with 99 ml of McKeen medium (25 g glucose, 2.5 g monosodium glutamate, 3.0 g yeast extract, 1.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 0.5 g KCl and 1.0 ml trace element solution (0.64 g $MnSO_4 \cdot 7H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$ and 0.015 g $FeSO_4 \cdot 7H_2O$ in 100 ml of distilled water) in 1 l distilled water) in a sterile 250-ml conical flask and incubated at 37°C for 3 days at 150 rpm.

Isolation and Preservation: Bacteria from the enriched culture were isolated using the conventional spread plate and streak plate methods [32, 33]. Briefly, the enriched sample was subjected to a 10-fold serial dilution up to 10^{-6} and the original sample and each dilution were spread on nutrient agar plates.

After incubation at 37°C for 24-48 hours, discrete bacterial colonies were selected based on colony morphology such as color, form, elevation, margin, and surface. Pure cultures were obtained by further streaking from which slant cultures were prepared in nutrient agar medium and preserved at 4 °C.

Hemolysis Assay: Hemolysis assay was carried out accordingly to a previously described method [1]. Briefly, fresh cultures were streaked on blood agar media (Himedia, India) and incubation at 37°C for 48–72 h. Formation of a clear zone around the streaking line was scored as a positive result.

Collection of Broth Supernatant: For the collection of culture supernatant, organisms were inoculated in McKeen broth containing 0.1% soybean oil. After 3 days of incubation, cell free broth was obtained by centrifugation at 5000 rpm for 20 minutes.

Oil Spreading Assay: To determine oil spreading of the broth supernatant, 50 ml of distilled water was added to a mega petriplate followed by the addition of 20 µl crude soybean oil to the surface of the water. Then, 10 µl of the collected supernatant was placed on the oil surface. The diameter of clear zones of triplicate assays was determined.

Measurement of Emulsification Index: To measure the emulsification index (E_{24}), 3ml of kerosene was added to the same amount of cell-free supernatant, mixed well by vigorous vortexing for 2 min and allowed to stand for 24 h. Height of the stable emulsion layer was then measured. In the negative control, water replaced the supernatant. The emulsification index was measured as the percentage of the height of emulsified layer (cm) divided by total height of the liquid column (cm) according to the following equation:

$$E24 = (\text{Height of the emulsion} / \text{Total height}) \times 100\%$$

Characterization and Identification of the Selected Isolates: For identification of the isolates, their basic microbiological and culture properties such as colony morphology on nutrient agar, growth in slant and broth medium, differential staining, cell shape and arrangements and growth characteristics were determined using conventional methods.

Biochemical tests such as citrate utilization, catalase, oxidase, indole, urease, deep glucose agar, gelatin hydrolysis, starch hydrolysis, Voges-Proskauer, methyl red, motility, nitrate reduction, H_2S production, and fermentation of various carbohydrates were performed following standard protocol and the identification was based on

comparing the results with Bergey's manual of determinative bacteriology [34, 35].

Determination of Growth at Different Conditions: Temperature, pH, and salt sensitivity of the isolates was evaluated by growing them in nutrient media at various temperatures (5ºC, 10ºC, 27ºC, 37 ºC and 45ºC), pH (4.5, 6.5 and 8.5) or NaCl concentrations (0, 2, 4, 6, 8 and 10%).

Extraction of Biosurfactant: For the extraction of biosurfactant, isolates were inoculated in 50ml broth with 1ml kerosene and incubated for 7 days. Then cells were removed by centrifugation at 5000 rpm at 4°C for 20 minutes and the supernatant was collected. pH of the supernatant was adjusted to 2 using 1M H_2SO_4 and an equal volume of chloroform: methanol (2:1) was added to it. The mixture was vigorously shaken for 5 minute and allowed to set until phase separation. Then the bottom solvent phase was collected by using separating funnel and upper aqueous phase was re-extracted as before. The crude biosurfactant was further concentrated from the pooled solvent phase using a rotary evaporator at 40°C. The extract was collected and preserved in a small vial for further analysis.

Estimation of Biochemical Nature of the Biosurfactant: The biochemical nature of the partially purified biosurfactant was estimated by thin layer chromatography (TLC) conducted on silica gel plates with chloroform methanol acetic acid as solvent system [1].

Antimicrobial Assay: Antimicrobial activity was tested by both microtitre plate assay and disc diffusion assay [36] against the five test organisms including *Bacillus cereus*, *Esherichia coli*, *Staphylococcus aureus*, *Serratia mersescens*, and *Salmonella typhi*. In the microtitre plate assay, 50 µl from an 18 hour broth of each pathogen was poured on to microtitre plate and 10, 20, 30, 40, 50 µl of biosurfactant was added. The plates were incubated for 24 h followed by the addition of 20 µl of 0.5% tetrazolium chloride solution. After 24 h of incubation, appearance of red color indicated bacterial growth and its absence indicated antimicrobial activity. In the disc diffusion method, muller hinton agar was seeded with the pathogens. Then paper disc soaked with the biosurfactant solution was placed on seeded agar and kept at 4º C for 30 minutes. The plates were further incubated at 37º C for 24 h and zone of inhibition was measured [37, 38].

Determination of Stability: Thermal stability was estimated by heating at 100°C for 20 min followed by cooling to room temperature. The pH stability was evaluated at pH 4, 6, 7 and 9 adjusted using 1 N HCl or 1 N NaOH. Following the temperature and pH treatments, the emulsification index was determined.

RESULTS AND DISCUSSIONS:

Isolation, Screening, and Selection of Biosurfactant Producing Bacteria from Oil-Spilled Sites: Since, biosurfactant producing bacteria utilize hydrocarbons as their source of carbon and energy, soil with oil-spills appears to be promising site for the isolation of biosurfactant producing bacterial strains. In view of this, nine distinct bacterial strains were initially isolated from the oil-contaminated soil of a motor garage and examined by two frequently-used screening tests including blood hemolysis and oil spreading tests to screen-out the biosurfactant producing species. Six of isolates showed positive results in hemolytic test on blood agar and formed transparent, colorless border around the colonies resulting from lysed erythrocytes **Table 1**. The hemolysis screening assay, however, is not exclusively specific to detecting biosurfactants, and other by-products may also cause the erythrocyte lysis [39].

Therefore, while the hemolytic assay is a useful tool for the preliminary screening of biosurfactant producing bacteria, it should be supplemented by other methods such as oil displacement assays, to provide a more complete and accurate depiction of the biosurfactant producing ability.Consequently, the oil spreading test was carried out that allowed for the selection of two promising biosurfactant producing isolates namely JR3 and JR7 which dispersed oil more than 1.50cm (1.63 cm and 1.93 cm respectively).

Further assessment of biosurfactant production by the two isolates was based on their emulsification indices (E_{24}). E_{24} is used in conjunction with the hemolytic and oil displacement assays to confirm the presence and potential potency of the

biosurfactant. Of the two selected isolates, JR3 had an E_{24} of more than 50% in both McKeen and mineral salt media suggesting its higher emulsifying effects **Table 2**. JR7 also showed significant emulsification activity with its E_{24}found to be over 40% in both media. An $E_{24}$of around 50% is considered to be indicative of strong biosurfactant producing potential. Previously, Jaysree *et al.* and Hassanshahihan also noticed nearly similar range of E_{24} values(15-54% and 10-65% respectively) for the biosurfactant producing bacteria isolated from oil contaminated sites[15,40]. In a study by Barakat *et al*., highest E_{24} values of 56% and 57% were reported for two different strains of *Bacillus* [41]. Similarly, Lamilla *et al.* also reported E_{24}values near 50% for different strains of *Pseudomonas* [42].

TABLE 1: SELECTION OF BIOSURFACTANT PRODUCING BACTERIA BY HEMOLYTIC TEST AND EMULSIFICATION INDEX (E_{24}). E_{24} WAS MEASURED FOR THE TWO ISOLATES EXHIBITING BETTER PRODUCTION IN HEMOLYTIC ASSAY

Isolate	Hemolytic assay	E_{24} (%)	
		Mckeen	Mineral salt
JR1	-		
JR2	+		
JR3	++	55	50
JR4	-		
JR5	+		
JR6	++		
JR7	+++	45	42.5
JR8	-		
JR9	+		

+++ = vigorous hemolysis, ++ = moderate hemolysis =, + = scanty hemolysis, - = no hemolysis

Characterization and Identification of the Selected Biosurfactant Producing Bacteria: The cultural and biochemical properties of the two selected strains were exclusively studied for their identification **Table 2**. The JR3 strain was characterized as a gram-positive, oxidase and catalase positive, spore forming, aerobic, motile strain, whereas JR7 was found to be a gram-negative, oxidase-negative, catalase-positive, non-spore forming, non-motile bacterium. They both tested negative in H_2S production, indole, urease, Voges-Proskauer, and methyl red reactions and positive in nitrate reduction test. The morphological, physiological, cellular and biochemical characteristics of the strains are summarized in **Table 2**. Compared with the standard description given in Bergey's Manual of Determinative Bacteriology [34], these properties of JR3 and JR7 indicated that they belong to the *Bacillus* and *Acinetobacter* genera respectively. *Bacillus* and *Acinetobacter* have both been identified as excellent biosurfactant producers in previous studies [43, 44]. *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. pumilus* are some of the most commonly reported *Bacillus* species studied for their biosurfactant producing potential [45, 46]. Studies have also reported the isolation and characterization of several biosurfactant producing *Acinetobacter* species from various sources such as petroleum-contaminated soils, wastewater, and other hydrocarbon-rich environments. For example, *A. beijerinckii* ZRS, strain isolated from an oil-contaminated soil sample in an oil field, has been reported to produce biosurfactants with high emulsifying and foaming abilities [47]. Similarly, two *Acinetobacter* strains isolated from lipid-rich wastewater was reported to produce a glycoprotein biosurfactant with high emulsifying activity [48].

TABLE 2: MORPHOLOGICAL, PHYSIOLOGICAL, BIOCHEMICAL AND GROWTH CHARACTERISTICS OF THE BIOSURFACTANT PRODUCING STRAINS JR3 AND JR7

Parameters	JR3	JR7
Vegetative cells	Rod	Short rod, Cocci
Cell arrangement	Single	Single or in chain
Gram staining	+	-
Spore staining	Spore former	Non Spore former
Acid-fast staining	Non-acid fast	Non-acid fast
Motility test	Motile	Non-motile

Agar colonies	Circular, off white, raised, entire margin, smooth surface	Circular, white, raised, entire margin, smooth surface
Agar slant	Echinulate	Effuse
Nutrient broth		Turbid
Catalase test	+	+
Gelatin hydrolysis test	+	+
Citrate utilization	-	-
Glucose broth	Turbid	Turbid
Deep glucose agar test	Aerobic	Aerobic
Starch hydrolysis test	Weakly +	-
Voges-Proskauer test	-	-
Methyl red test	-	-
Nitrate reduction test	+	+
H_2S Production	-	-
Indole test	-	-
Urease test	-	-
Oxidase test	+	-
Fermentation test	Acid from glucose but no acid and gas from fructose, galactose, arabinose, mannitol, xylose.	No acid and gas from glucose, xylose, arabinose, lactose, mannitol, galactose, fructose, and sucrose.

Effect of Temperature, pH and Salinity on Growth of the Selected Biosurfactant Producing Strains: Environmental conditions can largely affect the capacity of biosurfactant producing bacteria to grow and produce biosurfactants. The growth analysis of the selected isolates at various temperature, pH and salt levels showed that they grew well at temperatures 30 and 37 °C, pH 6 and 7, and could tolerate up to 6% of NaCl **Table 3**. Previous studies suggested that the temperature tolerance of biosurfactant producing strains can vary from mesophilic to thermophilic, with optimal growth temperatures ranging between 20 and 40 °C which is consistent with the present study. The pH tolerance of biosurfactant producing bacteria is generally broad, with growth being typically best at pH between 7.0 and 8.0. However, some strains may also grow and produce biosurfactants at more acidic or alkaline pH. The salt tolerance of the biosurfactant producing bacteria can also vary widely, with some strains having the ability to thrive and produce biosurfactants in higher saline environments but growth of others can be hindered by the presence of salt. The optimal salt concentrations for the growth of biosurfactant producing strains can range from 0.5% to 5.0%, which is similar to the isolates of the present study.

TABLE 3: GROWTH OF THE ISOLATES AT VARIOUS PH, TEMPERATURES AND SALT CONCENTRATIONS

Isolates	pH				temperatures (°C)				NaCl (%)				
	5	6	7	8	4	30	37	45	0	2	4	6	8
JR3	+	+++	++	+	-	+++	+++	++	+++	++	++	++	-
JR7	+	+++	+++	+	-	+++	+++	+	+++	+++	++	++	-

+++ = excellent growth, ++ = moderate growth =, + = small growth, - = No growth.

Biochemical Nature of the Biosurfactants: Biochemical nature of the extracted biosurfactants was assessed using TLC. When subjected to ninhydrin spray, the TLC plates developed red spot but negative result was obtained upon iodin vapour spray which suggested that the biosurfactant was a lipopeptide. Lipopeptide is a cyclic amphiphilic structure consisting of a hydrophilic peptide chain linked to a hydrophobic fatty acid moiety, mostly produced by Aerobic bacteria. This group of biosurfactants has the ability to generate comprehensive immune responses. Therefore, a wide range of lipopeptides are being considered as immunomodulators in the field of vaccine development [49]. Many bacteria have been reported to produce lipopeptide type biosurfactants, including *Bacillus, Pseudomonas, Streptomyces*, and *Micrococcus* species. Lipopeptides produced by certain *Bacillus* strains are excellent candidates to be utilized as therapeutics and drug delivery systems owing to their antimicrobial, anti-tumoral and anti-platelet properties[50]. Studies have reported *B. subtilis* and *B. licheniformis* strains which produced surfactins, lipopeptide biosurfactants with

excellent surface-active properties and antimicrobial activity [51, 52]. Ndlovu *et al.* have described *B.amyloliquefaciens and Pseudomonas aeruginosa* strains which produced lipopeptide biosurfactants with strong antimicrobial activity against a broad spectrum of opportunistic and pathogenic microorganisms [53]. *Pseudomonas* species are also known to produce lipopeptide biosurfactants, including viscosinamid, syringafactin, surfactin, iturin and fengycin which exhibit potent surfactant properties and antimicrobial activity [54]. In addition, certain lipopeptide biosurfactant is an effective plant growth promoter that significantly enhances seed germination in lettuce, tomato, chili pepper and pea [55]. Since the *Bacillus* and *Acinetobacter* isolates of the present study were characterized as lipopeptide producers, they have the potential to be used in medicine, petroleum processing, and waste management.

Antimicrobial Activity of the Biosurfactant: The antimicrobial activity of biosurfactants extracted from the two isolates was examined by microtitre plate assay and disc diffusion technique in which both of them could inhibit the growth of *B. cereus*, *S. mersescens*, *S. typhi*, and *S. aureus* but *E. coli* was unaffected. The antimicrobial property of biosurfactants have been reported previously which is due to their ability to disrupt the integrity of bacterial cell membranes [49]. This activity of biosurfactants has made them of interest for several practical applications, such as treatment of bacterial infections, regulation of plant diseases, environmental bioremediation, food preservation *etc* [54, 56]. Further studies are required to uncover mechanisms behind their antimicrobial properties and to develop biosurfactant-based antimicrobial agents.

Oil Emulsification Capacity: The emulsifying capacity of the biosurfactants was measured using four different oil hydrocarbons including kerosene, diesel, octane and soybean **Fig. 1**. Kerosene was found to be the most suitable substrate for emulsification followed by diesel, soybean and octane. Generally biosurfactants have been shown to effectively emulsify kerosene and diesel which are used commonly as fuels. Previous research demonstrated excellent ability of some biosurfactant producing bacteria to emulsify these oils. Borah and Yadav performed a study on the emulsification properties of a *B. cereus* biosurfactant that was able to emulsify ~55% of kerosene [57]. Banat *et al*. reported another biosurfactant-producing *Bacillus* strain from an oil-contaminated soil sample which was found to be capable of emulsifying kerosene and diesel in water with emulsification indexes of 40.6% and 50.5%, respectively [58]. In another study, Fleck *et al.* reported a strain of *Rhodococcus ruber* which was capable of emulsifying up to 58% of diesel [59]. The oil emulsification capacity of biosurfactants has numerous applications such as treatment of oil spills, removal of oil from contaminated soils, improvement of oil recovery from oil reservoirs etc. The use of biosurfactants for oil emulsification can also have environmental benefits, as they are biodegradable and eco-friendly, as compared to the traditional chemical emulsifiers.

TABLE 4: EMULSIFY CAPACITY OF BIOSURFATANT WITH FOUR DIFFERENT OIL HYDROCARBONS

Oil hydrocarbons	JR3	JR7
Kerosine	55%	34%
Octane	22.5%	12.5%
Soybean	52.5%	41%
Diesel	40%	23%

Thermal and pH Stability of the Biosurfactant: Stability assessment of biosurfactants under high temperature and various pH levels is critical as they need to remain functional under different environmental conditions for practical applications. Consequently, thermal and pH stability of the biosurfactants was tested by a heat treatment at 100^{o}C for 20 min and at a pH range of 4 to 9.

The thermal treatment did not affect the emulsifying capacity of the biosurfactant which suggested that the biosurfactants from both isolates are stable at high temperature. Moreover, the biosurfactants could also retain their emulsifying capacity in both acidic and alkaline conditions although biosurfactant of the *Bacillus* strain had better emulsification of oil at the alkaline pH. Lipopeptide biosurfactants have been reported to possess good thermal and pH stability which makes them highly useful for various industrial applications. Previous studies showed that a lipopeptide biosurfactant produced by a *Bacillus licheniformis* strain exhibited excellent thermal and

pH stability, retaining their emulsifying activity at temperatures up to 120°C and pH 4 to 10 [60]. Similarly, fengycin and iturin, lipopeptide biosurfactants produced by a *Bacillus* species, has been shown to produce high level of biosurfactant activities and stable over a range of environmental conditions [61]. Another lipopeptide biosurfactant produced by *Acinetobacter* sp. Y2, have also been shown to be stable at high temperatures and a wide range of pH [62]. Additionally, biosurfactant produced by a *Streptomyces* species was found to be stable at a wide range of temperatures (30 to 100 °C), and pH, from acidic (pH 5) to alkaline (pH 12) conditions while retaining its surface-active properties [63].

TABLE 5: STABILITY OF THE BIOSURFACTANT UPON HEAT AND PH TREATMENTS

Isolates	Heat treatment		pH treatments			
JR3	**No heating**	**100°C 20 min**	**pH 4**	**pH 6**	**pH 7**	**pH 9**
	57.5%	59%	56.5%	68%	73%	76.5%
JR7	28%	26.5%	53%	52%	54.5%	54%

CONCLUSION: In conclusion, this study provides a comprehensive exploration of microbial biosurfactants, highlighting their potential applications in environmental and pharmaceutical domains. The research successfully isolated biosurfactant-producing bacteria based on their robust production capabilities. These biosurfactants exhibited antimicrobial activity against various bacterial species, suggesting potential utility in infection treatment, bioremediation, and medicine. Additionally, their strong emulsification abilities indicate promise for applications in oil spill mitigation and enhanced oil recovery.

Importantly, thermal and pH stability tests confirm their potential versatility for a wide range of industrial uses. Continued exploration into their mechanisms and potential applications may further advance science and industry toward more efficient and environmentally friendly processes.

Authors Contributions: MNA and FA contributed to concept and supervision; MJ and FA performed the experiment; MJ analysed the data; TJH and SHB wrote and prepared the manuscript; TBB helped in manuscript writing; SK and ANMSZ assisted in collecting information.

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