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CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY BRUCELLA INTERMEDIA AND STUDY OF ITS BIOREMEDIATION AND ANTIMICROBIAL POTENTIAL

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Abstract

Crude oil contamination poses significant environmental challenges due to its complex mixture of hydrocarbons and organic compounds. Oil spills in soil disrupt plant growth, harm microorganisms, kill soil fauna, and contaminate water, leading to habitat loss and environmental degradation. Many bacteria are known for their potential to synthesize biosurfactant sand sustainably remediate hydrocarbon pollution. This study aimed to isolate a potential biosurfactant producing strainfrom environmental samples, optimize the nutrient and physicochemical conditions for its yield, and characterize the biosurfactant type. In addition, the antimicrobial and Microbial Enhanced Oil Recovery (MEOR) potential was also studied using well diffusion assay and sand pack column, respectively. Among the 31 isolates, *Brucella intermedia* was identified as the most potent strain with emulsification value of 45%. It reduced surface tension of garage oil by 37.5%. Optimum yield was obtained in media with 6%inoculum, pH 7.2,containing 4%garage oil,0.5% tryptone and 0.3% potassium nitrate, and incubation at 30°C under shaker conditions for 48 h. Optimum biosurfactant recovery was obtained with chloroform:methanol (2:1). The crude biosurfactant contained 2.345 mg/mL carbohydrate, 1.81 mg/mL proteins and 35.77% lipids. FTIR analysis revealed key functional groups that highlight the amphiphilic nature of the biosurfactant. Studies on the antimicrobial potential of the biosurfactant indicated broad spectrum activity, with more prominent effect against Gram positive bacteria. It also recovered 65%used garage oil and 50% mixed vegetable oil, indicating its potential in MEOR. Overall, the biosurfactant producing *B. intermedia* strain is a potential candidate for industrial applications in the biological and environmental fields.

Keywords: *Brucella intermedia, hydrocarbon pollution, MEOR, oil spill, optimization, sustainable.*

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INTRODUCTION

Rapid industrialization is accompanied with increased petroleum exploration and accidental oil spills [1]. Hydrocarbons are also used in agriculture (in the form of fuel, pesticides and insecticides). The surface runoff from these sites also contaminates nearby soil and water ecosystems [2]. Petroleum hydrocarbons are among the most persistent and hazardous pollutants due to their hydrophobic nature, toxicity, and resistance to natural degradation3. Conventional remediation strategies rely mostly on chemical surfactants, solvents and dispersants to mobilize these pollutants. However, the extensive use of synthetic surfactants has raised serious environmental concerns due to the associated toxicity and low biodegradability [4]. Consequently, there is a need for environmentally sustainable and effective alternatives for pollution control and remediation. Biosurfactants have emerged as promising eco-friendly substitutes for chemical surfactants. These surface-active compounds are produced by a wide range of microorganisms, including bacteria, fungi, and yeasts, and are characterized by their amphiphilic structure consisting of hydrophilic and hydrophobic moieties [5,6]. The unique structural arrangement of biosurfactants significantly reduces surface and interfacial tension between immiscible phases such as oil and water6. Unlike synthetic surfactants, biosurfactants are biodegradable, less toxic, and effective under extreme environmental conditions, making them highly suitable for environmental applications [4]. In bioremediation, biosurfactants play a crucial role in enhancing the bioavailability of hydrophobic pollutants. They emulsify

petroleum hydrocarbons, increasing the surface area accessible to microbial attack and thereby accelerate the biodegradation process [3,6]. Biosurfactants also modify microbial cell surface properties, promoting adhesion to hydrophobic substrates and stimulating the growth and metabolic activity of hydrocarbon-degrading microorganisms. This synergistic interaction between biosurfactants and microbes significantly improves the efficiency of bioremediation processes [7]. A diverse group of microorganisms, particularly those isolated from oil-contaminated environments, has evolved metabolic pathways that allow them to utilize hydrocarbons as carbon and energy sources. Genera such as Pseudomonas, Bacillus, and Rhodococcus are well-known producers of structurally diverse biosurfactants, including glycolipids, lipopeptides and phospholipids [7,8]. Among these, glycolipids such as rhamnolipids, sophorolipids, and trehalolipids are the most extensively studied due to their high surface activity and wide applicability [9,10]. The structural diversity of biosurfactants contributes to their adaptability across various environmental conditions and enhances their biotechnological potential [11].

Beyond environmental remediation, biosurfactants have gained increasing attention in industries such as pharmaceuticals, agriculture, food processing, cosmetics, and petroleum recovery12. Their emulsifying, antimicrobial, and surface-modifying properties enable applications ranging from drug delivery systems and antimicrobial formulations to Microbial Enhanced Oil Recovery (MEOR). In MEOR, biosurfactants facilitate the release of residual oil trapped within porous rock formations, improving recovery efficiency while minimizing environmental impact [13,14].

Despite the efficacy of chemical surfactants used across industries, their environmental footprint remains a significant concern. Certain synthetic surfactants and their degradation products, such as alkylphenols and perfluorinated compounds, are known endocrine disruptors and persistent organic pollutants15. In this context, the present study focuses on the isolation of biosurfactant producing bacterium from oil contaminated environments, optimization of its yield and characterization of biosurfactant type. In addition, the biosurfactant produced was evaluated for itsantimicrobial activity and potential application in MEOR.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All reagents, media, and chemicals used in this study were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India; Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India; and Loba Chemicals Laboratories Pvt. Ltd., Mumbai, India. All reagents were of analytical grade.

2.2 Sample collection

Soil samples were collected from six oil contaminated locations, including a garage in Parel, an oil mill in Sewri, a petroleum pump, and other sites both within and surrounding Mumbai. Samples were aseptically collected in sterile containers or polyethylene bags and transported to the laboratory for further analysis.

2.3 Enrichment of Biosurfactant Producing Organisms

One gram of each soil sample was suspended in distilled water to prepare soil suspensions. The supernatant was inoculated into 100 mL of Minimal Salt Medium (MSM) supplemented with 2% oil as the sole carbon source. Flasks were incubated at room temperature under shaking conditions (130 rpm) for 7–10 days until visible microbial growth was observed.

2.4 Screening of Biosurfactant Producing Bacteria

2.4.1 Drop Collapse Assay

The drop collapse assay was performed as described by Jain et al16. Two microliters of used garage oil were placed on a cavity slide and allowed to equilibrate for 24 h. Subsequently, 5 µL of cell free culturebroth of isolates obtained from different environmental samples was added to the oilcoated area, and the drop diameter was observed after 1 min. A positive result was recorded if the drop diameter exceeded that of deionized water (negative control) by at least 1 mm.

2.4.2 Oil Displacement Assay

Fifty microliters of distilled water was added to a 150 mm Petri dish, followed by 50 µL of crude garage oil to form a surface film. Twenty microliters of culture supernatant was applied to the center of the oil layer, and the diameter of the clear zone was measured after 30 secs.The formation of an oil-free clearing zone indicated biosurfactant production, with larger zonescorrespondingto higher activity [17].

2.4.3 CTAB/Methylene Blue Agar Assay

Extracellular glycolipid production was evaluated using the CTAB agar plate method. Minimal Salt Agar (MSA) was supplemented with 2% carbon source, 0.0005% cetyltrimethylammonium bromide (CTAB), and 0.0002% methylene blue. Bacterial cultures were spotted onto the agar and incubated at 37°C for 72h. Formation of a bluish halo around colonies indicated biosurfactant production [17].

2.4.4 Emulsification Index (E24)

Culture broth was mixed with kerosene in a 1:1 (v/v) ratio, vortexed for 2 mins, and allowed to stand for 24 h. The height of the emulsion layer was measured, and the emulsification index was calculated as indicated in equation 117:

$$\text{E24 (\%)} = \frac{\text{Height of emulsion layer}}{\text{Total height}}\ x\ 100\ldots \text{ Eq. 1}$$

2.4.5 Hemolytic Activity

Hemolytic activity was assessed on nutrient agar supplemented with 5% fresh blood. Bacterial isolates were spotted onto blood agar and incubated at 37°C for 48 h. Clear halos surrounding colonies indicated positive biosurfactant activity.

2.4.6 Surface Tension Measurement

Surface tension reduction of cell-free culture broth was measured using a tensiometer at room temperature. A platinum ring was immersed in 50 mL of culture supernatant, and the force required to pull

the ring through the air-liquid interface was recorded. Sterile MSM served as the negative control [17].

2.5 Identification of potential isolate

Identification was done based on morphological, biochemical and molecular methods. The potential biosurfactantproducing isolate was subjected to molecular identification at Hi-Gx360® Solutions, HiMedia Laboratories Pvt. Ltd. DNA extracted from the isolate was used for cycle sequencing with BDT v3.1 chemistry and analyzed using the NA10 3500XL Genetic Analyzer to obtain the 16S rRNA sequence.

2.6 Extraction of Biosurfactant

Biosurfactant was extracted using the acid precipitation method. A 24 h old culture was centrifuged at 10,000 rpm for 20 mins, and the bacterial pellet was discarded. The supernatant was filtered through a 0.2 µm filter, and its pH was adjusted to 2 using 1 M HCl. The sample was incubated at 4°C overnight for acid precipitation. For further extraction, 50 mL of chilled chloroform:methanol (2:1, v/v) was added to 50 mL of the supernatant in a separating funnel, mixed vigorously, and allowed to separate into two layers. The crude biosurfactant layer was collected and concentrated using a rotary evaporator at 45°C to remove the solvent [18,19].

2.7 Optimization of Biosurfactant Production

2.7.1 Optimization of Physiochemical Parameters

Biosurfactant production was optimized by varying physical and nutrient parameters, including incubation time (24–120 h), optical density (0.2 to 0.9 at 540 nm), inoculum size (1% to 9%), pH (4.5, 5.5, 6.5, 7.2, 7.5, and 8.5), temperature (30°C, 37°C, and 40°C), agitation (shaker vs. static), carbon sources (2% glucose, sucrose, sunflower oil, used sunflower oil, mustard oil, peanut oil, used garage oil, brake oil, and machine oil) and their concentrations (1% to 9%), as well as organic (0.5% yeast extract, meat extract, beef extract, peptone, and tryptone) and inorganic (0.3% KNO_3, $NaNO_3$, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl, and $(NH_4)_2NO_3$) nitrogen sources. Optimization was performed using the One Factor at a Time (OFAT) approach. An 18 h old culture was suspended in sterile phosphate-buffered saline (PBS, pH 7.2) and adjusted to an Optical Density (O.D) of 0.5 at 540 nm. This suspension was used to inoculate 100 mL MSM supplemented with 2% (v/v) used garage oil, with an initial inoculum size of 1% (v/v) [18].

2.7.2 Optimization of Biosurfactant Extraction Method

As downstream processing contributes approximately 60% of total production costs, extraction efficiency was optimized. The most promising isolate was grown in MSM with 2% (v/v) used garage oil for 24 hunder optimum conditions. Various solvent systems were tested for biosurfactant recovery, including chloroform:methanol (2:1), ethyl acetate:methanol (2:1), chilled ethyl acetate, cold dichloromethane, and cold acetone [18,19].

2.8 Partial Purification of Biosurfactants by Thin Layer Chromatography (TLC)

Pre-coated silica gel aluminum TLC plates were used for separation and detection of biosurfactant components. Partially purified biosurfactant (0.1 g) was dissolved in 1 mL methanol, and 20 µL was spotted twice at a distance of 2 cm from the plate bottom, allowing drying between applications. Separate plates were prepared for detection of lipids, carbohydrates, and amino acids. For carbohydrate detection, an additional plate was prepared using 1% rhamnose in distilled water as a standard.The mobile phase comprised chloroform:methanol:acetic acid (65:15:2 v/v). Plates were developed until the solvent front reached the top, air-dried, and visualized using standard reagents [lipids by iodine vapour; amino acids by 1% ninhydrin in acetone, carbohydrates by Molisch's reagent (10% α-naphthol in 96% methanol with concentrated H_2SO_4), and sugars by anthrone reagent (1 g anthrone in 5 mL H_2SO_4, diluted with 95 mL ethanol)]. Development ofyellow-brown, purpleand violet to yellow colour indicated presence oflipids, amino acids and carbohydrates/sugars respectively. Rf values for sugars were calculated using standard methods [19].

2.9 Quantitative Estimation of Biosurfactant Components

2.9.1 Carbohydrate Content

Total carbohydrate content was quantified using the anthrone assay20. The anthrone reagent forms a blue-green complex upon reaction with carbohydrates under acidic conditions. Precisely, carbohydrate dehydration by sulfuric acid produces furfural derivatives (pentoses) or hydroxymethylfurfural (hexoses), which condense with anthrone to yield a color proportional to carbohydrate concentration. Absorbance was measured at 620 nm using a spectrophotometer. The concentration of carbohydrate was calculated with the help of standard rhamnose curve.

2.9.2 Protein Content

Protein content was determined using the Folin-Lowry method with BSA as a standard21. The method involves cupric ion binding to proteins in alkaline medium (Biuret reaction) and subsequent reduction of Folin-Ciocalteu (FC) reagent by aromatic residues, forming a blue complex which can be measured at 660 nm.The concentration of proteins was calculated with the help of a standard BSA curve.

2.9.3 Lipid Content

Lipids were extracted from 1 mL of dried biosurfactant using chloroform:methanol (2:1 v/v), and total content (%) was estimated gravimetrically. The mixture was shaken, allowed to separate, and the lower organic

layer containing lipids was collected in pre-weighed beakers. Beakers were dried at 130°C for 30 mins and then at 55°C for 3 h. Total lipid content was calculated using equation 2.

$$\%\ Lipids = \frac{W1 - W3}{W1}\ X\ 100\ ...\ Eq.2$$

Where W1 = initial sample weight and W3 = final lipid weight. Extraction was repeated until a constant weight was achieved.

2.10 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed to identify functional groups in the biosurfactant. Spectra were recorded using a PerkinElmer Spectrum IR instrument in the range 450–4000 cm^{-1}, with 4 cm^{-1} resolution. Four scans were averaged using a $LiTaO_3$ detector.

2.11 Antimicrobial Activity of Partially Purified Biosurfactant

Antimicrobial activity was evaluated using the agar well diffusion method. Mueller-Hinton agar plates were inoculated with 0.1 mL of 18–24hold bacterial cultures. Wells (6 mm) were filled with 50 µL of culture supernatant or partially purified biosurfactant.Sterile distilled water served as a negative control. Plates were incubated at 37°C for 24 h, and inhibition zones were measured to assess antimicrobial activity.

2.12 Microbial Enhanced Oil Recovery

The efficacy of biosurfactant in oil recovery was evaluated using the sand pack column method. Two glass columns were packed with 50 g pre-treated sand (acid-washed, water-rinsed, dried at 100°C) and plugged with glass wool. One column was saturated with 20 mL of a mixed oil sample (used garage oil, brake oil, machine oil, sunflower oil, used sunflower oil, peanut oil, mustard oil); the other column served as a water control.Columns were stabilized for 24 h. Biosurfactant solution (100 mL, 2%) was added to the columns; distilled water served as a negative control, and 1 mg/mL SDS as a positive control. After overnight percolation, effluent was collected, and residual oil in the sand was extracted with n-hexane. Solvent was evaporated at 50°C, and recovered oil was quantified using Eq. 320:

$$\text{Oil removed}\ (\%) = \frac{\text{Initial oil}\ (g) - \text{Remaining oil}\ (g)}{\text{Initial oil}\ (g)}\ X\ 100\ ...\ Eq.3$$

3. RESULTS AND DISCUSSIONS

3.1 Screening of Potential Biosurfactant Producer

A total of 31 bacterial cultures were obtained in this study (Table 1).Among these, 28 isolates produced clear zones on the oil surface (oil displacement test), demonstrating biosurfactant activity (Fig. 1b). Precisely, biosurfactants modify the contact angle at the oil-water interface, generating surface tension differences that lead to oil droplet displacement [21].The diameter of the clear zones varied among isolates, reflecting differences in surface activity.The drop collapse test is another rapid qualitative screening method for biosurfactant activity (Fig. 1a). Sixteenisolates showed complete drop collapse within one minute, indicating strong surface-active properties. Eight isolates showed a positive CTAB test. The CTAB agar test differentiated anionic biosurfactant producers from those that may have synthesized neutral or cationic compounds. This diversity highlights the structural variability of biosurfactants produced by environmental isolates [19]. Emulsification activity (Fig 1c) is a key functional property of biosurfactants, particularly relevant to hydrocarbon bioremediation22.Among the screened isolates, NA1, NA2, and NA10 exhibited significant emulsification, with E24 values greater than 40%. Isolate NA10 showed the highest emulsification index (45%), followed by NA2 (40.58%). Surface tension measurements revealed that isolate NA10 exhibited the maximum reduction, lowering surface tension by 37.5% (to approximately 25 dynes/cm) when grown in MSM containing 2% used garage oil, supporting its strong surfaceactive potential. This isolate, however, did not exhibit hemolysis on blood agar. Several studies have shown that not all biosurfactants exhibit hemolytic properties, as hemolysis depends on biosurfactant type and interaction with erythrocyte membranes23. Therefore, reliance on hemolysis alone may lead to false-negative conclusions. Overall, the combined results demonstrate that isolate NA10 is a promising biosurfactant producer, exhibiting strong emulsification ability and significant surface tension reduction. Oil-contaminated environments naturally enrich biosurfactant producing microorganisms, as these compounds enhance hydrocarbon availability and uptake by reducing surface and interfacial tension24.Previous studies have reported a varied range of emulsification efficiency among environmental isolates. For example, *Bacillus subtilis* A1 demonstrated strong emulsifying capability with an E24 value of 76%25. A recent review highlighted high emulsification indices among Bacillus species such as *Bacillus velezensis*, reaching ~97%in some cases under optimized conditions26. Among Gram-negative bacteria, Pseudoxanthomonas sp. G3 showed E24 values around 72.9%, along with potential applications in enhanced oil recovery27. Hydrocarbonoclastic (hydrocarbon degrading) bacteria identified as Achromobacter, Pseudomonas, and Serratia isolates from petroleum sludge exhibited E24 values in the range of ~64–72%, indicating effective emulsification relevant to petroleum biodegradation [28].

Table 1: Biosurfactant producing potential of bacterial isolates

Isolate No.	Collection Site	Drop collapse test	Oil displacement test	CTAB agar method	Emulsification index %	% decrease in surface tension
NA 1	Garage (Lalbagh)	+	+	+	40	10.9
NA 2		+	+	+	40.58	1.83
NA 3		+	-	-	-	3.66
NA 4		+	+	-	15	8.45
NA 5	Garage (Parel)	+	+	+	-	0.5
NA 6	Garage (Lalbagh)	+	+	+	12.25	-
NA 7	Oil mill(Konkan)	+	+	+	6.66	-
NA 8		+	+	-	-	1.08
NA 9	Garage (Lalbagh)	+	+	-	16.12	-
NA 10	Oil mill(sewri)	+	++	+	45	37.5
NA 11		+	+	+	12	-
NA12		+	+	-	18.75	-
NA 13		+	+	-	16.12	-
NA 14	Garage (Konkan)	-	+	-	9.09	-
NA 15		-	+	-	19.03	-
NA 16	Petroleum pump(Parel)	+	+	-	-	-
NA 17		+	-	+	-	-
NA 18		+	+	-	20	5.60
NA 19		-	+	-	16.12	-
NA 20	Garage (Parel)	-	+	-	-	5.03
NA 21		-	+	-	9.67	5.25
NA 22		-	+	-	-	-
NA 23		+	+	-	28.12	-
NA 24		-	+	-	15.62	-
NA 25	Petroleum pump(Parel)	-	+	-	12.12	-
NA 26		-	+	-	15.62	-
NA 27		-	-	-	12.60	-
NA 28		-	+	-	9.67	-
NA 29	Garage (Lalbagh)	-	+	-	22.58	-
NA 30		-	+	-	12	-
NA 31		-	+	-	5.09	-

+: positive; ++ strongly positive; -: negative

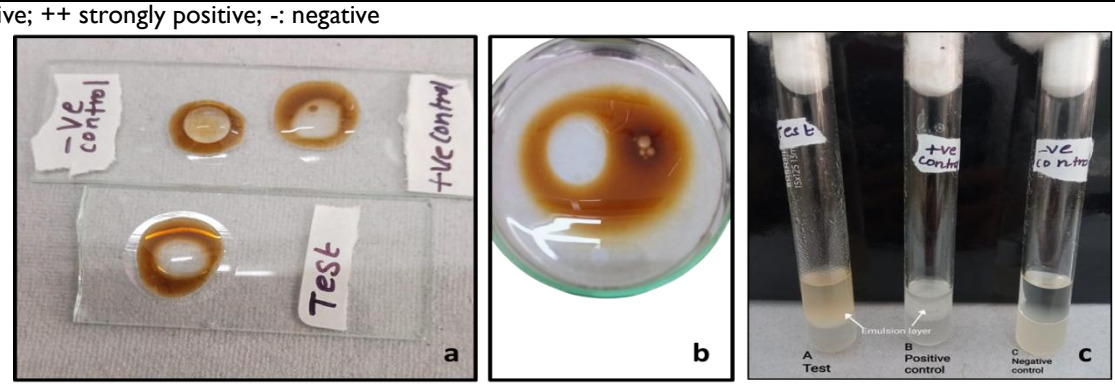

Fig.1: Isolate NA10 showing positive (a) drop collapse test (b) oil displacement test and (c) emulsification layer

3.2 Identification of potential isolate

Gram staining of isolate NA10 revealed Gram-negative cells. Biochemical characterization showed that isolate NA10 was catalase-positive, oxidase-positive, and urease-positive, while tests for nitrate reduction and lysine decarboxylase were negative. The isolate did not ferment 1% galactose, sucrose, xylose, or arabinose. The observed biochemical profile was consistent across repeated experiments, indicating stable metabolic characteristics.BLASTN analysis against the NCBI database revealed highest sequence identity (99.711%) with *Brucella intermedia* LMG 3301 (Fig. 2). Phylogenetic analysis (Fig. 3) based on 16S rRNA sequences demonstrated that isolate 24P110_007_NA10 clustered closely with recognized Brucella species. A strong evolutionary relationship was observed with *B. intermedia* LMG 3301 and *B. inopinata* BO1, supported by a bootstrap value of 93. Additional clustering with *B. ceti*, *B. abortus*, *B. melitensis*, *B. canis*, and *B. suis* further confirmed its placement within the Brucella genus.

BLAST ANALYSIS RESULT

Program Name: BLASTN Program Version: 2.12.0 Database used: NCBI Type strain

qseqid	sseqid	bitscore	score	qcovs	pident
24P110_007_NA10	NR_026039.1_Brucella_intermedia_LMG_3301_16S_ribosomal_RNA,_partial_sequence	2534	1372	100	99.711
24P110_007_NA10	NR_116161.1_Brucella_inopinata_BO1_16S_ribosomal_RNA,_partial_sequence	2470	1337	99	99.126
24P110_007_NA10	NR_074286.1_Brucella_canis_strain_ATCC_23365_16S_ribosomal_RNA,_complete_sequence	2462	1333	100	98.772
24P110_007_NA10	NR_074111.1_Brucella_melitensis_strain_16M_16S_ribosomal_RNA,_partial_sequence	2462	1333	100	98.772
24P110_007_NA10	NR_074146.1_Brucella_ovis_ATCC_25840_16S_ribosomal_RNA,_partial_sequence	2462	1333	100	98.772
24P110_007_NA10	NR_103935.2_Brucella_suis_1330_16S_ribosomal_RNA,_complete_sequence	2462	1333	100	98.772
24P110_007_NA10	NR_074336.2_Brucella_microti_strain_CCM_4915_16S_ribosomal_RNA,_complete_sequence	2462	1333	100	98.772
24P110_007_NA10	NR_042460.1_Brucella_abortus_strain_544_16S_ribosomal_RNA,_partial_sequence	2447	1325	99	98.765
24P110_007_NA10	NR_042462.1_Brucella_pinnipedialis_strain_NCTC_12890_16S_ribosomal_RNA,_partial_sequence	2447	1325	99	98.765
24P110_007_NA10	NR_042463.1_Brucella_ceti_strain_NCTC_12891_16S_ribosomal_RNA,_partial_sequence	2447	1325	99	98.765

Fig. 2: BLAST analysis reportof potential biosurfactant producer

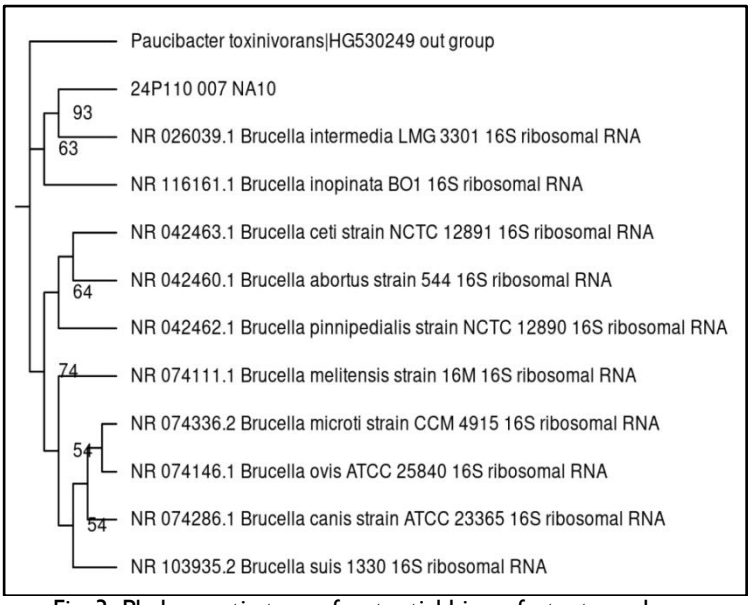

Fig. 3: Phylogenetic tree of potential biosurfactant producer

3.3 Optimization of environmental and nutritional parameters

The optimum parameters for biosurfactant production by *B. intermedia* LMG 3301 is represented in Fig. 4. The maximum yield was obtained in MSM medium (pH 7.2) supplemented with 4% used garage oil, 0.5% tryptone and 0.3% potassium nitrate. The optimum inoculum size was noted as6%culture suspension of 0.4 OD540nm. The optimum incubation conditions were 30°C, shaker conditions and 48h.

The observed results demonstrated that biosurfactant production was dependent on growth phase, nutrient availability, as well as environmental conditions. For example, maximum yield at 48 h suggests that biosurfactant synthesis likely peaks during the late exponential phase, after which the observed reduction in yield may be due to exhaustion of nutrient and substrates [29]. The optimum temperature of 30°C suggests mesophilic behavior of the isolated strain, and is consistent with many previously reported biosurfactant producing environmental isolates [30-33]. Enhanced yield under shaker conditions is logical, as oxygen plays a key role in fatty acid metabolism [34]. Interestingly, used garage oil was the most effective carbon source for inducing biosurfactant production. This may be likely due to prior environmental adaptation of the isolate to hydrocarbon rich (oil polluted) environments [35]. Additionally, the optimized concentration of garage oil in this study may have resulted in availability of balanced amount of substrate while preventing inhibitory effects. Tryptone is a complex organic nitrogen source that likely supported cell growth, and hence improved biosurfactant yield indirectly [36]. Although biosurfactant yield was optimum in presence of potassium nitrate, other inorganic nitrogen sourcesalsosupported its production significantly. It is well known that inorganic nitrogen sources are more easily assimilated by bacteria compared to complex organic nitrogen sources, hence is more likely that these compounds were also utilized by the bacteria for cell growth, and indirectly supported biosurfactant production [37].

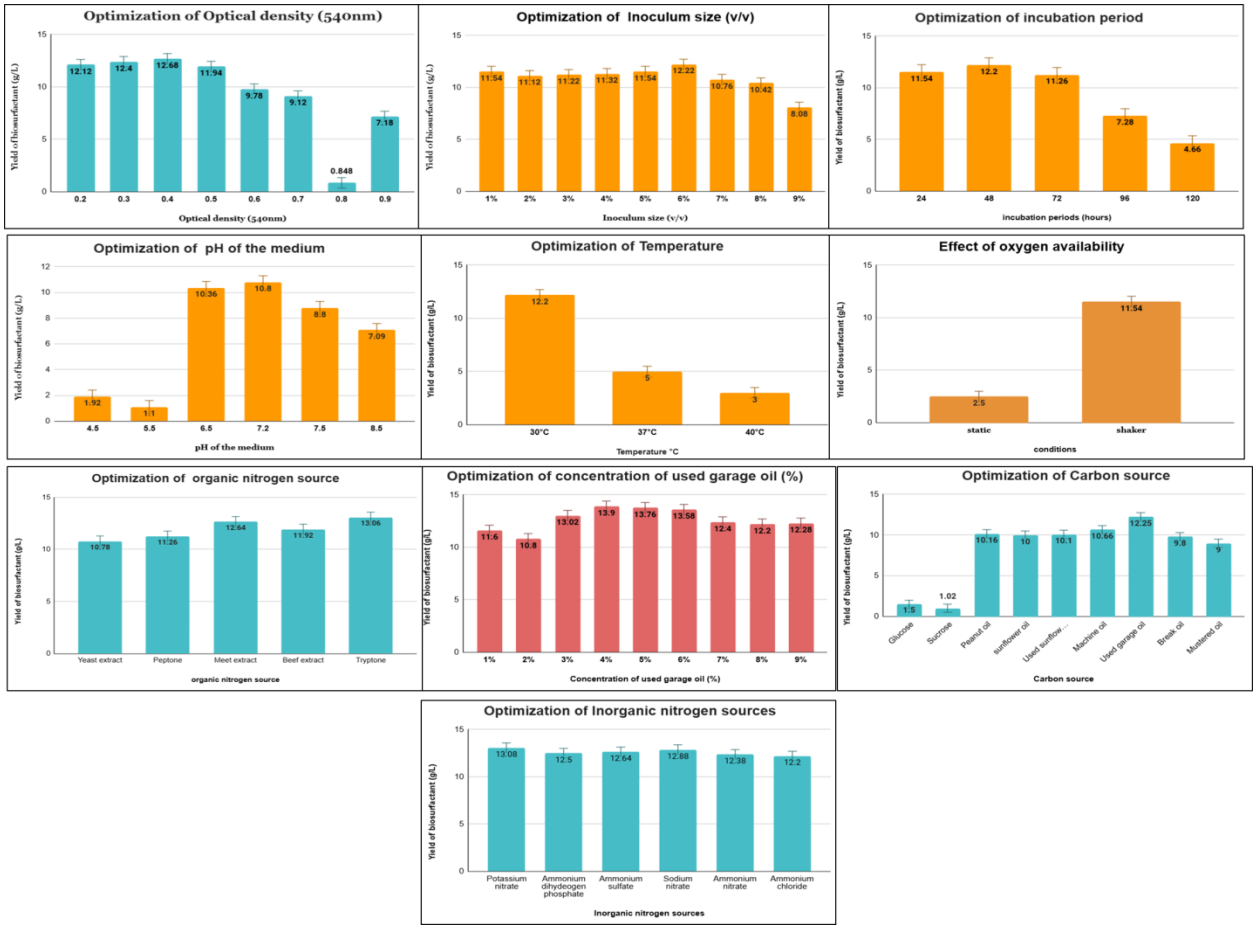

Fig. 4: Optimization of physicochemical and nutrient parameters for biosurfactant production by *B. intermedia* LMG 3301

Similar to our findings, optimum biosurfactant production by *Pseudomonas aeruginosa* PBSC1, isolated from mangrove ecosystem in Pichavaram (Boathouse), TamilNadu, India, was obtained in MSM (pH 7) containing 1% crude oil, glycerol, sodium nitrate and trace elements on incubation at 30°C38. The optimum conditions for biosurfactant production by *B. brevis* were 33°C incubation temperature at pH 8 for 10 days in media with 8.5 g/L glucose39. The optimal medium for biosurfactant production by Vibrio sp. 3B-2 contained 0.5% lactose, 1.1% yeast extract, 2% sodium chloride, and 0.1% disodium hydrogen phosphate. The optimum temperature for this strain was 28°C40. Among fungal strains, *Candida mogii* showed optimum yield in presence oflicuri oil, glucose, NH4NO3 and yeast extract41. Under optimum conditions, *C. mogii*lowered water surface tension from 71.04 mNm−1 to 28.66 mNm−1, with a critical micelle

concentration of 0.8 gL−1. *Candida tropicalis* produced 7.36 gL−1biosurfactant in presence of 2.5% waste frying oil, 2.5%, corn steep liquor, 2.5% molasses, and 2% inoculum in a 50L bioreactor [42].

3.4 Optimization of solvent extraction method

By using suitable extraction method, media development, process optimization, strain improvement, biosurfactant yield can be enhanced. Common techniques for biosurfactant recovery include solvent extraction, ammonium sulphate precipitation and acid precipitation, centrifugation [43]. The solvent extraction method is more frequently used for biosurfactant recovery as it a rapid and efficient technique44,45.Among the extraction methods tested in this study, chloroform:methanol (2:1) yielded the highest biosurfactant recovery (Fig. 5).

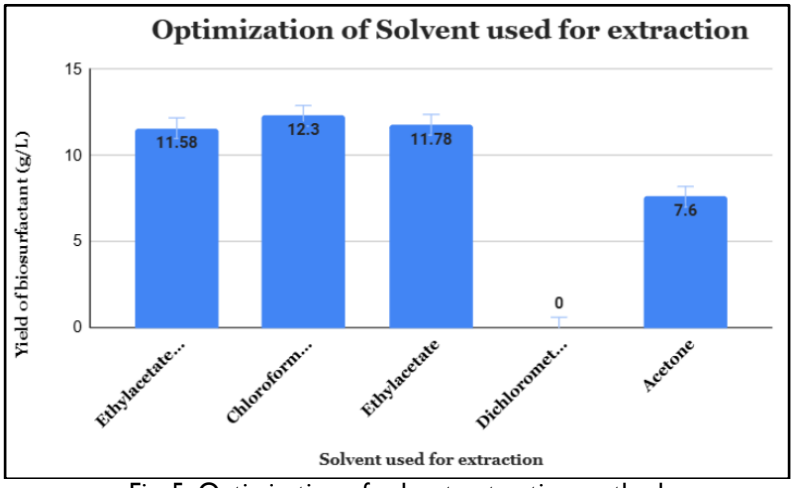

Fig. 5: Optimization of solvent extraction method

3.5 Characterization of Biosurfactant

The Fig. 6 represents the dried biosurfactant obtained from thegrowth medium maintained under optimum conditions and extracted using a chloroform and methanol solvent mixture. TLC indicated absence or negligible amount of rhamnose sugar, and presence of amino acids in the biosurfactant. Quantitative analysis of biosurfactantcomponents revealed presence of 23.45 µg/mL carbohydrates, 120.68 µg/mL proteins and 35.77% lipids. Overall, these findings indicated that the biosurfactant was predominantly composed of lipid and protein components with minimal carbohydrate content.

The Fig. 7 represents the FTIR spectrum of biosurfactant produced by *B. intermedia* LMG 3301. It revealed the presence of key functional groups characteristic of amphiphilic biomolecules. A broad absorption band at 3244.24 cm^{-1} corresponded to O–H stretching vibrations, indicating the presence of hydroxyl groups typically associated with carbohydrates, alcohols, or carboxylic acids. The strong peak observed at 2923.43 cm^{-1} was assigned to C–H stretching vibrations of aliphatic chains, confirming the presence of hydrophobic lipid components such as fatty acids. A band at 1633.46 cm^{-1} suggested carbonyl (C=O) stretching, which may be attributed to amide or ester linkages, indicating proteinaceous or lipid-associated structures. The peak at 1438.42 cm^{-1} further supported the presence of aliphatic chains through CH_2 and CH_3 bending vibrations. Prominent absorption at 1060.01 cm^{-1} corresponded to C–O stretching vibrations, while the band at 944.60 cm^{-1} was associated with polysaccharide or glycosidic linkages, collectively indicating the presence of carbohydrate moieties. A minor band at 2360.08 cm^{-1} was likely due to atmospheric CO_2 interference, whereas the low-frequency band at 494.74 cm^{-1}, within the fingerprint region, may represent complex bending vibrations possibly associated with sulfated groups or metal interactions. Overall, the FTIR profile demonstrates the coexistence of hydroxyl, aliphatic, carbonyl, and glycosidic functional groups, supporting the amphiphilic nature of the biosurfactant and suggesting a complex structure comprising lipid, protein, and carbohydrate components.

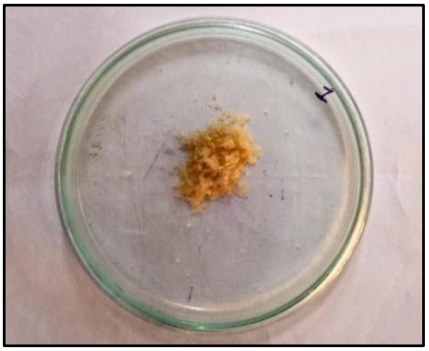

Fig.6: Dried biosurfactant obtained from *B. intermedia* LMG 3301

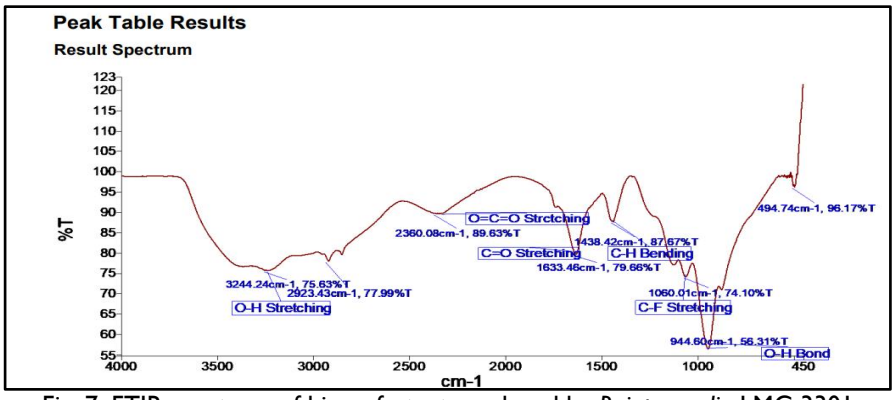

Fig. 7: FTIR spectrum of biosurfactant produced by *B. intermedia* LMG 3301

High lipid content is a defining characteristic of effective biosurfactants, as the hydrophobic fatty acid chains are responsible for factors such as surface activity, emulsification, and interaction with hydrocarbons [46]. Similar lipid proportions (ranging between 28-50%) have been reported for lipopeptide and glycolipoprotein biosurfactants produced by environmental isolates adapted to oil-contaminated habitats [47-49]. Lipopeptide biosurfactants are well known for their role in enhancing crude oil degradation by increasing hydrocarbon bioavailability [50]. Also, a positive correlation between protein biosurfactants and hydrocarbon degradation efficiency has been reported previously51.Several studies have demonstrated that biosurfactants produced by bacteria commonly possess mixed amphiphilic structures comprising lipid, carbohydrate, and sometimes protein moieties, as revealed by TLC and FTIR analyses. For instance, glycolipid biosurfactants from *Pseudomonas otitidis* and *Achromobacterxylosoxidans* were characterized by prominent FTIR bands corresponding to O–H stretching, aliphatic C–H stretching, carbonyl groups, and C–O vibrations, confirming the presence of carbohydrate-linked fatty acid chains and strong surface activity through reduced surface tension and high emulsification indices [52,53]. Similarly, rhamnolipids from *Pseudomonas plecoglossicida* showed distinct carbohydrate and lipid functional groups [54]. In contrast, biosurfactants from *Bacillus amyloliquefaciens* and other Bacillus spp. were predominantly lipopeptides, characterized by peptide-associated carbonyl bands around 1630–1650 cm^{-1} and positive ninhydrin reactions on TLC55. Studies on bacterial isolates obtained from wastewaters further revealed that both glycolipid and lipopeptide biosurfactants often coexist, exhibiting overlapping FTIR signatures of lipids, carbohydrates, and peptides [56], as observed in our study.

3.6 Antimicrobial Activity of the Partially Purified Biosurfactant

The biosurfactant produced by *B. intermedia* exhibited broad-spectrum antimicrobial activity, with zones of inhibition observed between 14mm and 16mm (Fig. 8).

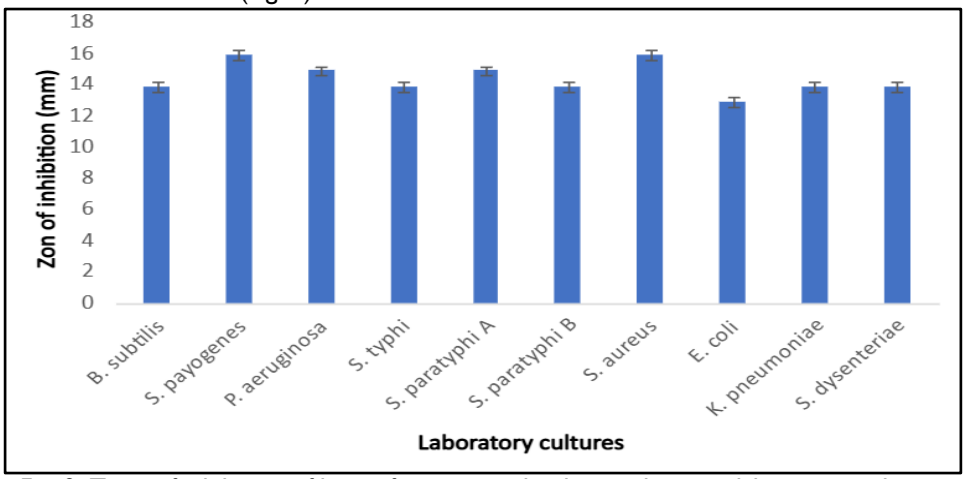

Fig. 8: Zone of inhibition of biosurfactant sample observed against laboratory cultures

Biosurfactants exert antimicrobial activity mainly through disruption of bacterial cell membranes due to their amphiphilic nature. The hydrophobic moieties insert into lipid bilayers and hydrophilic heads remain exposed, leading to increased membrane permeability, leakage of intracellular contents, and eventual cell death [46]. By lowering surface and interfacial tension, biosurfactants also destabilize membrane-associated structures, interfere with nutrient transport, and impair essential metabolic processes [57]. In addition, biosurfactants inhibit bacterial adhesion and disrupt established biofilms by altering surface hydrophobicity and degrading the extracellular polymeric matrix [58]. Similar to our study, aglycollipoprotein biosurfactant produced by*Lactiplantibacillus plantarum* showed broad spectrum antibacterialactivity at low MIC values [59].

3.7 Microbial Enhanced Oil Recovery

Sand pack column studies demonstrated that the crude biosurfactant produced by *B. intermedia* significantly enhanced

oil recovery compared to water (Fig. 9). Precisely, 65% garage oil and 50%mixed vegetable oil was recovered as against 10–12%recovery with distilled water. The chemical surfactant SDS, however, showed higher recovery efficiencies (80–90%) in both cases.Comparative analysis with previously reported biosurfactants indicates that the recovery efficiency of *B. intermedia* is higher than *Bacillussubtilis*B30 (31%)60, whereas it is lower than that observed for *Pseudomonasaeruginosa*N33 (≈95%)16.

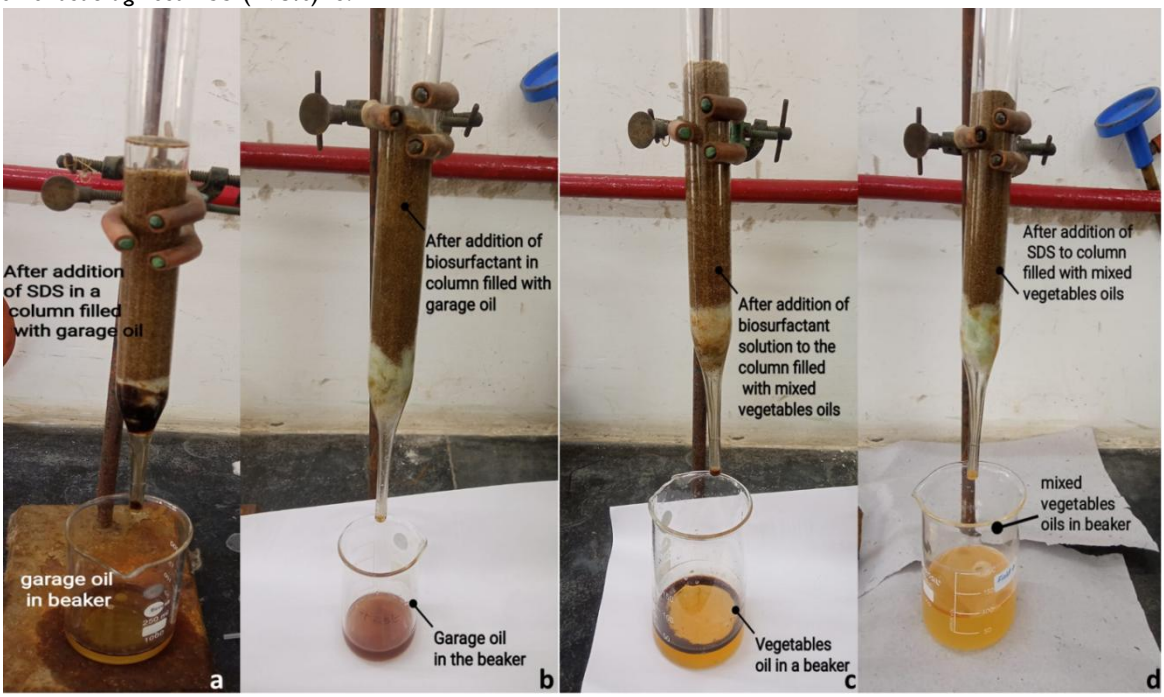

Fig. 8: Recovery of garage oil from sand pack column
The figure shows (a) recovery of garage oil after addition of SDS; (b) recovery of garage oil after addition of biosurfactant (c) recovery of mixed vegetable oil after addition of biosurfactant and (d) recovery of mixed vegetable oil after addition of SDS

4. CONCLUSION

The present study demonstrates promising applications of biosurfactant produced *B. intermedia*. Physicochemical characterization using TLC, biochemical assays, and FTIR analysis confirmed the amphiphilic nature of the biosurfactant, and suggested a complex glycolipo-protein structure. The broad spectrum antibacterial activity, along with non-hemolytic nature of the biosurfactant indicated low cytotoxicity and supported its safety for various environmental and industrial applications. Although its recovery efficiency was lower than that of the chemical surfactant SDS, the biosurfactant offers clear advantages in terms of biodegradability, environmental compatibility, and safety. Overall, these findings highlight the potential of *B. intermedia* as a sustainable and effective biosurfactant producer for applications in MEOR, antimicrobial formulations, and environmental remediation.

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Not Declared

7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

8. INFORMED CONSENT

Not applicable

9. ETHICAL STATEMENT

Not applicable

10. AUTHOR CONTRIBUTION

Dr. Anuradha S Pendse – Concept, Design, Analysis.
Ms. Namita Shamsundar Ghadigaonkar – Data collection, writing.
Ms. Tejashree Shivram Phepade – Analysis.

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