Assessment of Rheological Behaviour of Water-in-Oil Emulsions Mediated by Glycolipid Biosurfactant Produced by Bacillus megaterium SPSW1001

Title: Assessment of Rheological behaviour of water-in-oil emulsions mediated by glycolipid biosurfactant produced by *Bacillus megaterium* SPSW1001

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Abstract

A screening programme using mineral salt medium supplemented with n-hexadecane resulted in isolating a *Bacillus megaterium* SPSW1001 which was capable of producing surface active molecules lowering culture medium surface tension to 27.43 ± 0.029 mN/m and interfacial tension to 0.38 ± 0.03 mN/m at 72 h and an emulsification index (E_{24}) (85.63%). The biosurfactant product was further used to assess its effects on the rheological characteristics of oil-in-water emulsion prepared with engine oil. Structural characterization of the biosurfactant product by FTIR revealed a C-O-C stretch in sugar moiety, ester carbonyl linkage group between sugar and fatty acids, respectively, while mass spectral analysis revealed its glycolipid nature, with an *m/z* value of 662.44. The fluid behaviour of oil-in-water emulsion showed a non-Newtonian viscoelastic dilatant flow after yielding exemplified appropriately by Herschel-Bulkley model with 100% confidence of fit. The present study is significant in formulation and handling, processing, and transport of emulsion, and in understanding flocculation characteristics.

Keywords: *Bacillus*; Biosurfactant; Emulsification; Mass spectrometry; Rheology

1. Introduction

Emulsions are defined as two-phase systems which consist of a colloidal dispersion of immiscible fluid (hydrocarbon/oil and water) into each other. Two forms of emulsions are generally formed depending on the disperse phase, water in oil or oil in water emulsions. Emulsions are encountered in various industries such as agriculture, cosmetics, food, petroleum, pharmaceutical, printing, paints, polymer, and textile industries **[1, 2]**. Bioemulsifiers are biosurfactant biopolymers produced by microorganisms, capable of lowering the viscosity of crude oil through the process of emulsification, which is a method frequently utilized in microbial enhanced oil recovery **[3]**.

Some biosurfactants are characterized as bioemulsifiers for their emulsification characteristics which are described in terms of emulsification activity and emulsification stability **[4]**. The various characteristics of bioemulsifiers such as their low toxicity, biodegradability, and efficacy at extreme pH, salinity, and temperature established them as commercially attractive for several industrial applications **[5, 6]**. Biosurfactant productions are often associated with the uptake of hydrocarbons by microbial communities and are mainly synthesized by hydrocarbon-degrading microorganisms. At low concentration, biosurfactants are soluble in the aqueous phase and with an increase in concentration in solutions, it forms micelles. The concentration of biosurfactant where micelles formation begins is known as the critical micelle concentration (CMC). In two-phase system biosurfactants can solubilize/emulsify hydrocarbons/oil at concentrations above the CMC. The most significant advantages of biosurfactants over chemical surfactants are their biodegradability, ecological application, and biosynthesis by various naturally occurring microorganisms **[7,8,9]**. Fatty acids, glycolipids, neutral lipids, lipopeptides, phospholipids, and polymeric compounds are among the several types of biosurfactants that have been studied **[10]**. *Pseudomonas oleovorans* when grown on a hydrophobic substrate, such as n-decane produce extracellular biosurfactants with emulsifying and stabilizing properties of oil in water emulsions **[11].**

Numerous biopolymers, such as polysaccharides that are produced by microorganisms, form viscous solutions at concentrations range 0.05–1.0 % which has a significant role in industrial applications, because of their rheological properties and extreme thermal, ionic, and pH stability **[12]**. Several biopolymers are considered as significant bioemulsifiers because of their ability to stabilize emulsions; extra-cellular polysaccharides produced by *Pseudomonas tralucida* **[13]**, *Enterobacter cloaceae* **[14]**, and *Pseudoalteromonas sp*. **[15]** are examples of such bioemulsifiers. There are several studies reporting on the rheological studies of emulsion where the aqueous phase contains a slight quantity of surfactants **[16]**. Fewer studies have been reported to date on the rheological behaviour of biosurfactant-mediated oil-in-water emulsions. The alternatives to chemically synthesized surfactants are biosurfactants which are more sought-after alternatives at present due to their positive environmental credentials **[17,18,19,20]**.

Understanding the rheological behaviour of biosurfactant-mediated emulsions is one of the challenging tasks in material and fluid sciences. The emulsion's deformability and aggregation cause major problems in predicting rheology. We aimed to visualized emulsion droplet deformation to compare it to the rheological activity of emulsions at various biosurfactant concentrations.

In the present study, an environmental bacterium was isolated from crude oil contaminated soil, characterised and screened for the production of biosurfactant. Surface behaviour and emulsification abilities of the extracted biosurfactant at extreme ionic strength, pH, and elevated temperatures were investigated. The viscosity-biosurfactant concentration behaviour of the water-in-oil type emulsions was also studied to determine the rheological behaviour of fluid and microstructure of flocculated emulsions.

2. Materials and Method

2.1 Sampling and Isolation of Bacterial Strain

Soil samples were obtained from Indian Oil Corporation Limited, Haldia Refinery (22.04 °N, 88.10 °E), India. The carbon, hydrogen, nitrogen, and sulphur value of soil samples obtained from depths between 0 and 12 cm was measured 72.37, 13.04, 0.011, and 2.283 percent (%) respectively by CHNS analyzer (Elementary, Germany, VarioELIII). Analytical grade chemical, glassware, and media components from Sigma and Merck were used. Biosurfactants producing environmental microorganisms were isolated from contaminated (petroleum) soil using the enrichment method. Briefly, 1 g crude oil contaminated soil was added to 250 mL Erlenmeyer flask containing 100 ml of sterile MSM medium supplemented with hydrophobic substrate n-hexadecane 2% (v/v) and incubated in an orbital shaker incubator (CIS-24 Plus, REMI) at 180 rpm for 7 days at 37°C. Samples were collected and serially dilution and a 100 µl sample of dilution 10^{-2} to 10^{-6} were spread onto a mineral salt agar plates supplemented with hydrocarbon n-hexadecane. Plates were incubated at 37 °C for 24–72 h and colonies of bacteria were identified by their distinct morphology and were streaked over the nutrient agar plates to obtain a pure culture **[21]**.

2.2 Screening for and Production of Biosurfactant

The starter culture was prepared by inoculating pure bacterial colonies in a nutrient broth medium followed by incubation for 24 h at 37 °C and 180 rpm. For the biosurfactant production, a flask containing 250 mL sterilized MSM media (pH 7.0), were inoculated with 2% n-hexadecane and 1% inoculum and kept for incubation in an orbital shaker at 37 °C at 180 rpm (GeNei-SLM INOS-02-D). Culture broth samples (50 mL) were collected for different time intervals (24 –96 h) every 24 h and centrifuged (10,000 g at 4 °C for 10 mins) to remove microbial cells and obtain cell-free supernatant to screen for biosurfactant production.

2.3 Surface Tension Measurement

The cell-free supernatant was used to measure surface tension (ST) by DCAT 11 tensiometer (Data Physics, Germany) with platinum Wilhelmy plate [22]. Positive and negative controls Triton X-100 solution (0.1 percent v/v), and distilled water were used, respectively.

2.4 Emulsification Index (E_{24})

The emulsification activity of cell-free supernatant containing biosurfactant was evaluated against different hydrocarbons, petroleum oil, and vegetable oil. Several emulsions were prepared systemically at room temperature by vortexing 2/5 part of oil/hydrocarbon and 3/5 part of cell-free supernatant containing biosurfactant for 3-5 mins. The emulsification index (E_{24}) was calculated as previously described by Panjiar et al. [23]. An emulsion is considered stable if E_{24} is equal to or greater than 50% [24].

2.5 Stability of Biosurfactant at extreme pH, ionic strength, and high temperature

Stabilities studies were carried out on cell-free supernatant containing biosurfactant by taking culture broth obtained after 120 h of cultivation, followed by centrifugation at 15,000 g at 4°C for 15 mins. Cell-free supernatant containing biosurfactant was subjected to extreme pH (2-10) with 1N NaOH or 1N HCl using a pH meter (Sartorius PB-14), ionic strength (2-20 %) and high temperature range from 50-70 °C for 24 h as well as in an autoclave for 30 minutes at 121°C (15 psi). The emulsification index and ST values were used to assess the biosurfactant's stability.

2.6 Extraction and FT-IR spectroscopy of Biosurfactant

Cell-free supernatant containing biosurfactant was obtained from fermentation broth by centrifugation (15,000 g for 30 mins at 5 °C), which was then changed to pH 2.0 with 1N HCl and permitted to precipitate for 24 - 48 h. The precipitate obtained was centrifuged at 15,000 g at 4 °C for 30 mins to separate the biosurfactant. The biosurfactant was re-dissolved in deionised water, washed, and lyophilized (BST-LY101, Bionics Scientific Technologies Private Ltd., India) [25]. The lyophilized biosurfactant was encapsulated in 150 mg of potassium bromide (KBr) to prepare disks of the homogenized sample. The infrared spectra were obtained using Perkin Elmer Spectrum RX I, USA with a scanning speed of 2 mm/s in the wavelength ranges from 4000 – 500 cm^{-1}.

2.7 Biosurfactant mass spectrometric analysis

An LC system (Agilent), equipped with binary pumps, and a thermostat autosampler coupled with MS Q-TOF (model: G6550A), was used in the dual AJS ESI mode. The biosurfactant molecular mass was confirmed using MS data from redundant *m/z* peak. A linear gradient method of (A) 0.1 percent formic acid in 100 percent water and (B) 0.1 percent formic acid in 10

% water plus 90 percent acetonitrile was used in the mobile process. The mobile phase's gradient conditions were as follows: at 1200.00 bar using a Hypersil GOLD column c18 (100mm 2.1 mm-3micron), the flow rate was kept steady at 0.300 mL/min for 0-1 min, 95 percent A; 1-20 min, 0 percent A; 20-25 min, 0 percent A; 25-26 min, 95 percent A; 26-30 min, 95 percent A.

2.8 Rheology properties of emulsions (o/w)

The o/w emulsions were prepared using an aqueous solution containing biosurfactant and oil/hydrocarbon. To prepare emulsion of known concentration, 2/5 parts of oil/hydrocarbon and 3/5 parts of different concentrations of an aqueous solution containing biosurfactant (continuous phases) were taken. The biosurfactant was extracted at a regular time interval from cell-free supernatant and different concentrations of biosurfactant rheology of emulsions was investigated. The oil-in-water emulsions were prepared in 400 mL batches at room temperature (28 °C) using a variable speed homogenizer. At the constant speed of homogenizer, shearing was continued for 3-4 mins. The rheological study for an emulsion at different concentrations of biosurfactant was measured by Brookfield viscometer (Brookfield Engineering Laboratories: Model DV3T) to cover a broad range of torques (between 10 to 100%) at room temperature 28°C. For valid measurement, the viscometer was adjusted by the selection of suitable spindle and rotational speeds. The tip of the spindle was at least 10 mm above the vessel base to avoid trapping air bubbles. Point by point shear rate was measured at consecutive 60s steps to varying shear rates. For each point, viscosity was measured to get the flow curves.

2.9 Characterization of Biosurfactant Producing Microorganisms

The 16S rRNA gene sequencing technique was also used for the identification of the bacterial strain to overcome any differences in the results of VITEK 2. Genomic DNA extraction was performed using the phenol-chloroform method **[26]**, and thereafter amplification of gene by PCR. By precipitation of PEG-NaCl, the amplified product was further purified and sequenced by automated DNA (ABI 3730XL, Applied Biosystems Inc.). Using additional internal primers, from both ends sequencing was carried out to read each location twice. The phylogenetic tree of the isolated strain SPSW1001 (Accession No. MW042172) was developed by MEGA 6 software using the neighbour-joining (NJ) approach **[27]**. The 16S sequence obtained was compared to the GenBank sequences using the Simple Local Alignment Search Tool (BLAST). 16S bacterium identification was performed at the sequencing facility of the National Center for Microbial Resources (NCMR), the National Center for Cell Research, Pune.

3. Results and Discussion

3.1 Isolation and Screening of Bacterial Strain

The higher frequency of biosurfactant producers in the water-insoluble substrates is possibly due to the enrichment of microorganisms capable of utilizing these substrates as substrates, which are usually those able to gain access to these hydrophobic substrates through producing biosurfactants. After including several steps of the enrichment process, 8 bacterial strains were isolated, and the most potent strain SPSW1001 was selected for further studies as it produces biosurfactant after 24 h of growth with maximum emulsification activity and reduction in surface tension. Strain SPSW1001 was found to be aerobic, rod-shaped, and gram-positive. Colony features of isolated SPSW1001 on nutrient agar plates after 24 h incubation at 37 ºC showed circular configuration, 2-3 mm size, entire margin, convex elevation, and pale yellow with smooth and glistening texture. The physiological properties of strain SPSW1001 indicate optimum growth at acidic pH 6. Growth of bacterial strain SPSW1001 was noted at temperatures 37 °C and 30 °C, whereas sodium chloride (NaCl) tolerance was reported at a maximum concentration of 8%. Bacterial strain SPSW1001 generates acid from various carbon sources, and the results of the biochemical tests using conventional method are listed in Table 1. The bacterial strain SPSW1001 cell-free supernatant showed tensioactive characteristics by broth medium reduction of ST to 27.4±0.08 mNm^{-1} and interfacial tension to 0.32±0.08 mNm^{-1} when hydrophobic substrate n-hexadecane was used as substrate. ST control measurements showed 70.69±0.01 mNm^{-1} in the case of distilled water, whereas reduction of ST in Triton X-100 solution was noted as 22.59±0.01 mNm^{-1}. Biosurfactants of *Bacillus aryabhattai* SPS1001 were reported to give ST reduction to 24.3 ±0.01 mNm^{-1} **[21]**. In comparison another biosurfactant producing *Lactobacillus helveticus* MRTL 91 showed a surface tension lowering to 39.5 mNm^{-1} **[28]**.

3.2 16S ribosomal RNA (rRNA) Sequence Identification

Physiological features such as morphology, growth, and biochemical analyses of the SPSW1001 colonies distinguish it from the closest phylogenetic neighbour *Bacillus megaterium* NBRC 15308. The alignment result of the 16S rRNA gene sequence reveals the similarity of alignment of the strain SPSW1001 with the *Bacillus* genus (Fig. 1). Strain SPSW1001 shows a similarity of 99.52 percent with the nearest type strain *Bacillus megaterium* NBRC 15308(T) ATCC (Accession No. JJMH01000057) in the NCBI database which was isolated from an unknown source **[29]** (Fig. 1). *Bacillus megaterium* SPSW1001 partial 16S rRNA gene sequences was submitted under the accession number MW042172 to the GenBank database as mentioned on the phylogenetic tree (Fig. 1). The phylogenetic tree with the sum of the length of the branch (0.37864428) is shown. Bootstrap values greater than 50% (expressed as a percentage of 1000 replicates) were observed next to the branches **[30]**. Evolutionary distances have been determined by the maximum composite likelihood method **[31]**. Comparison of automated biochemical identification VITEK 2 system with that of molecular method 16S rRNA gene sequencing for identification of *Bacillus* species reported 99.52% similarity and 93% probability with *Bacillus megaterium*. If the homologous rate was noted to be above 99% then the results

would be considered valid. The extent of homology shown by the 16S rRNA gene sequence of ~1200 bp reveals 99.52 percent similarity with *Bacillus megaterium* NBRC 15308 (T) (Accession no. JJMH01000057) with its closest neighbor in the database. Hence, the bacterial strain was identified as *Bacillus megaterium* SPSW1001.

3.3 Emulsion formation and stabilizing capacity

The produced biosurfactant showed emulsifying activity against substrates that are hydrophobic, such as hydrocarbons, petroleum oil, and vegetable oil. The results of the experiment reveal that the produced biosurfactant has the capacity of forming stable emulsion for specific hydrophobic substrates. The cell-free supernatant containing biosurfactant showed an emulsification index (E_{24}) against various hydrocarbons and oil, which includes n-heptane, toluene, engine oil, diesel oil, and coconut oil. Engine oil (trade name Castrol) showed a relatively higher emulsification index (E_{24}) percentage (85.63%) followed by coconut oil (vegetable oil) (81.54%) produced from cell-free supernatant containing biosurfactant after the 5th day of bacterial growth. *Pseudomonas oleovorans* was reported to have an emulsification index (E_{24}) higher than 50% in the case of hydrocarbons, namely, n-hexane, n-hexadecane, xylene, benzene, chloroform, and toluene **[32]**. For aliphatic hydrocarbon n-hexadecane and aromatic hydrocarbon xylene, the concentration of biosurfactant required was 0.6% w/v for the emulsion stability **[33]**. The emulsifying activity for EPS concentration (0.1 wt. %) was measured using n-hexadecane as substrate **[34]**. Exopolysaccharides *produced by Pseudomonas oleovorans* produced emulsion with olive oil; with a 65% emulsification index value **[32]**. The positive control sodium do-decyl sulphate (chemical surfactant) has emulsifying index of 82% with engine oil. Tween 20, Tween 80, and Triton X-100 (chemical surfactants) had emulsification index values of 43%, 40%, and 38%, respectively, with aromatic hydrocarbon xylene **[35]**, while the exo-polysaccharides *produced by Pseudomonas oleovorans* had an E_{24} of 60% **[32]**. Emulsification activity against motor lubricant oil was also reported by the biosurfactant produced by *Bacillus megaterium* **[36]**.

Fig. 2 (A) shows the emulsification index of cell-free supernatant containing biosurfactant of strain SPSW1001 at regular time intervals against various oils and hydrocarbons. The stability of biosurfactant was investigated by measuring emulsification index against engine oil and surface tension reduction in cell-free supernatant containing biosurfactant at various pH (2-10), NaCl (2-12 %), and temperature ranges. At room temperature, the effect of pH on the stability of cell-free supernatant containing biosurfactant obtained after 72 hours was investigated. With an increase in pH from 7 an increase in emulsification index to 89.29% occurs and surface tension remains unchanged at pH 12, whereas by lowering pH to 2 a reduction in emulsification index to 82.98% occurs, and surface tension to 23.56 ± 0.02 mNm^{-1}. *Yarrowia lipolytica* NCIM 3589 grown in *n*-hexadecane shows stability over pH range 3–9 with emulsification index recorded between 72–80 % and a pH above 9 activities was reduced **[37]**. Maintaining more than 50% emulsification index (E_{24}) after 24 h of its formation determines the stabilizing ability of an emulsifier **[33]**.

In the case of ionic strength, the emulsification index and surface tension remained almost constant at the concentration (2-12%), no significant change was measured in both emulsification index and surface tension measurements. Stability of emulsions at 4.0 M concentrations of NaCl was reported in *Pseudomonas fluorescens* produced biosurfactant **[38]**. The thermal stability of cell-free supernatant containing biosurfactant was also studied which shows an increase in emulsification index with the increase in temperature (50-121 °C) and no loss of surface activity as there was no significant change in surface tension readings. Anionic surfactants possess greater stability at high temperatures when compared to cationic surfactants due to hydrogen-bond at the head group **[39]**. The combined effect of two factors pH (2-12) and temperature (50 and 70 °C) was studied simultaneously to monitor the stability of cell-free supernatant containing biosurfactant on emulsion stability (in days with engine oil). Experimental results revealed that with increasing pH (>7), the emulsion stability was more than 70 days at temperatures 50 °C and 70 °C. Emulsion stability was less at pH 6 when compared to pH 4 (Fig. 2 (B).

3.4 FT-IR of Biosurfactant

Biosurfactant produced by the isolated bacterium SPSW1001 was recovered from the cell-free supernatant by centrifugation of the fermentation broth and further precipitated by HCl, lyophilized, and characterised by FT-IR (Fig. 3). The biosurfactant FT-IR spectra show a large peak at 3498 cm^{-1} which is characteristic of the O-H stretch vibrations and, due to H-bonding, suggests the presence of polysaccharides. The peaks at 2320 cm^{-1} and 2779 cm^{-1} display the stretch of the C–H band (CH_2–CH_3) in the aliphatic chain. The FTIR peaks of *Bacillus megaterium* at 2929 cm^{-1} represent the CH aliphatic stretching **[40]**. The absorption peak at 1528 cm^{-1} reflects an ester carbonyl group (C=O in COOH). The existence of the functional group –COOH in the molecule was confirmed by peaks in the region of 1446–1369 cm^{-1} for the bending of the hydroxyl (-OH) group. The peak of absorption at 1068 cm^{-1} was assigned to C–O–C which reveals the sugar molecules in the biosurfactant. Absorption peaks of around 1076 cm^{-1} were also been identified as C–O–C stretch in sugar moiety **[41]**. The observed bands are similar to the structure previously described for glycolipidic moieties **[42].**

3.5 Mass spectrometric analysis of biosurfactant

The molecular weight of the glycolipid biosurfactant produced by *Bacillus megaterium* SPSW 1001 was determined using LC-MS QTOF analysis. A protonated molecule [M + H] at *m*/*z* 663.4 (Fig. 4) was detected in mass spectra, corresponding to the molecular weight (*m*/*z* 662.44) of a parental compound. The glycolipid's molecular mass was determined to be 662.44 Da (Table 2), and its molecular weight was found to be the same as that of previously recorded glycolipid biosurfactants produced by *Bacillus megaterium* **[43]**. This result corroborated the findings of Abdel-Mawgoud et al. **[44]** who stated that most observed glycolipid biosurfactants have molecular masses ranging from 302–803 Da.

3.6 Biosurfactant production and emulsion flow behavior

Biosurfactant production by isolated bacterium SPSW1001 culture initiates at log phase. The concentration of biosurfactant at exponential phase was recorded 0.31 wt.% whereas in the stationary phase concentration of biosurfactant was measured maximum (0.43 wt.%). The stationary phase is often associated with scarcity of nutrients and the production of biosurfactants may be associated with nutrient-deficient conditions. The flow behaviour of oil-in-water emulsions prepared with engine oil and various concentrations of aqueous solutions containing biosurfactant was investigated using the RV-2 spindle at 28 °C.

The non-Newtonian (viscoelastic) dilatant behaviour of oil-in-water emulsions was recorded at biosurfactant concentrations (0.13, 0.17, 0.24, 0.31, and 0.43 wt.%) by shear stress versus shear rate plot (Fig. 5 (B)). The graph shows the relationship between viscosity and shear rate as a function of biosurfactant concentration in the range of 0.13-0.24 wt.% was not high initially but at high biosurfactant concentration 0.31 and 0.43 wt.%, a sharp rise in viscosity is observed (Fig. 5 (A)) which indicates that the biosurfactant is forming (reverse) micelles. In the case of micelle forming biosurfactants, the consistency in the viscosity is not predicted as observed in Fig. 5 (A) such types of emulsions systems are likely to show high viscosity at intermediate biosurfactants concentrations as a result of micelles interference. Another reason for discontinuation in the viscosity-biosurfactant concentration plot may be possible because of the inversion phenomenon i.e., inversion from aqueous solutions containing biosurfactant-continuous system to oil-continuous system and vice-versa. As the aqueous solutions containing biosurfactant display these attributes**,** hence the biosurfactant when dissolved in oil to prepare emulsions forms reverse micelles. For example, in the case of micelle-forming aqueous solutions of non-ionic chemical surfactant, Triton X-100 the viscosity-concentration behavior at the intermediate concentrations of surfactant shows a sharp rise in viscosity (Fig. 6) because of the occurrence of micelle interference. Hence, the viscosity drops abruptly leading to inversion from an aqueous-continuous system to a surfactant continuous system. Hence the aqueous solutions containing biosurfactant are dissolved in oil forming (reverse) micelles, as shown in the current study (see Fig. 5(A).

Oil-in-water emulsions prepared from cell-free supernatant containing biosurfactant solution and engine oil showed a non-Newtonian behavior at different concentrations of biosurfactant (Table 2). The flow behaviour of emulsions from shear stress versus shear rate plot (Fig. 5 (B)) reveals non-Newtonian shear-thickening (dilatant) behaviour after yielding; which is best explained by the Herschel-Bulkely model with 100% confidence of fit (Table 3) at higher concentrations of biosurfactant (0.43 wt.%). The confidence of fit for the Hershel-Bulkley model was measured between 98.7 to 100% with an increase in biosurfactant concentration in water-in-oil emulsions. Yield stress in an emulsion also increases with the increase in biosurfactant concentration (Table

2). Water-in-oil emulsions prepared from cell-free supernatant containing different concentration of biosurfactant (0.13, 0.17, 0.24, 0.31 and 0.43 wt.%) are considered to be extremely flocculated. In these o/w emulsions, the degree of flocculation increases with an increase in concentrations of biosurfactant. The shear-thickening behaviour was noted with an increase in the shear rate in the emulsions as a result of the progressive formation of flocculated structure. As a result, the apparent viscosity of o/w emulsions increased in tandem with the biosurfactant concentration. Freitas et al. **[32]** reported an increase of viscosity of extracellular polysaccharides (produced by *Pseudomonas oleovorans*) solutions with increasing concentration.

3.7 Microstructure of Emulsion

The rheological properties of an emulsion can also be illustrated by its microstructure. Microscopic view of the emulsions sample at different concentrations of biosurfactant indicated that droplets of emulsion were flocculated and contained small clusters of droplets called flocs when they aggregated together to form these flocs clusters. The viscoelastic behavior of emulsions with shear-thickening flow after yielding could be because of the formation of flocs-aggregate structure. The presence of biosurfactant causes flocculation of the emulsion's droplets, thus affecting the rheological behavior of emulsion. From Fig. 7 (C) it is evident that emulsions were highly flocculated at the highest concentrations of biosurfactant (0.43 wt.%), thus a droplets aggregates to form a network of interconnected structures, while simultaneously with an increase in biosurfactant in continuous phase, the size of a droplet decreases. The increase in yield stress occurs as a result of the formation of a network of interconnected emulsion droplets due to the rise in the degree of flocculation (due to depletion force) with the increase in biosurfactant concentrations. Hence, the yield stress and strength of interconnected structures; depends upon the degree of flocculation of the droplets and on the dispersed droplets. The shear thickening behavior is likely because of the progressive formation of the structure of flocculated emulsions with the rise in the shear rate. The plot of viscosity versus shear rate for emulsions with various biosurfactant concentrations (Fig. 5) showed that viscosity increased as the shear rate increased, suggesting that the microstructures developed. The formation of a network structure between the emulsion droplets is caused by yield stress. Binks et al. **[45]** reported in their stability studies on water-in-oil emulsions that flocculation occurs due to microemulsion droplets (diameter < 26 nm) in the continuous oil phase. An optical microscope image of emulsion samples of different biosurfactant concentrations is presented in Fig. 7.

Conclusion

It is concluded that biosurfactant concentration strongly influences the rheological properties of emulsions. The viscosity-biosurfactant concentration behaviour of the emulsions reveals that biosurfactants forms reverse micelles. However, due to depletion forces, a high biosurfactant concentration (in continuous phase) induces flocculation of emulsion droplets. The study of

rheological properties of biosurfactant-mediated emulsions is important because of their industrial applications, especially in emulsion formulation and handling, processing, transport, and storage; it also aids in understanding emulsion flocculation characteristics.

Declarations

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Not applicable

Consent for publication

Not applicable

Code availability

Not Applicable

Authors Contributions

Varsha Singh: designed the study, performed the experiments, analyzed the results, wrote and revised the manuscript.
Zairah Waris: performed the experiments and analyzed the results, wrote and revised the manuscript.
Ibrahim M. Banat: analyzed the results and revised the manuscript.
Padmini Padmanabhan: designed the study, analysed the results, and revised the manuscript.
Sriparna Saha: revised the manuscript and participated in the study. Manuscript was read and approved by all authors

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Competing interests

The authors Varsha Singh, Zairah Waris, Ibrahim M. Banat, Sriparna Saha, and Padmini Padmanabhan declare that they have no conflicts of interest regarding the publication of this article.

Availability of data and materials

Not applicable.

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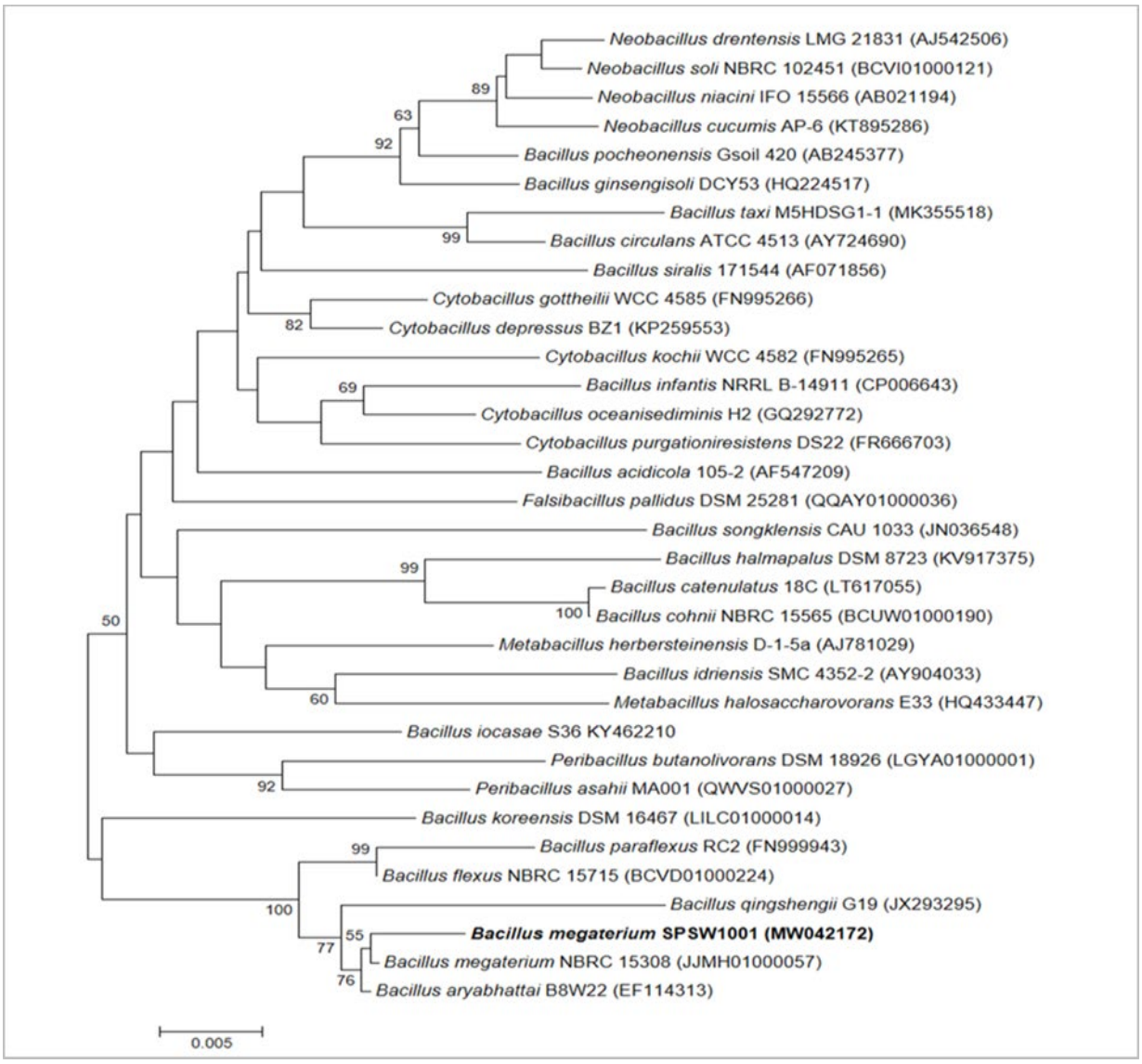

Fig. 1 Phylogenetic tree of the isolate *Bacillus megaterium* SPSW1001

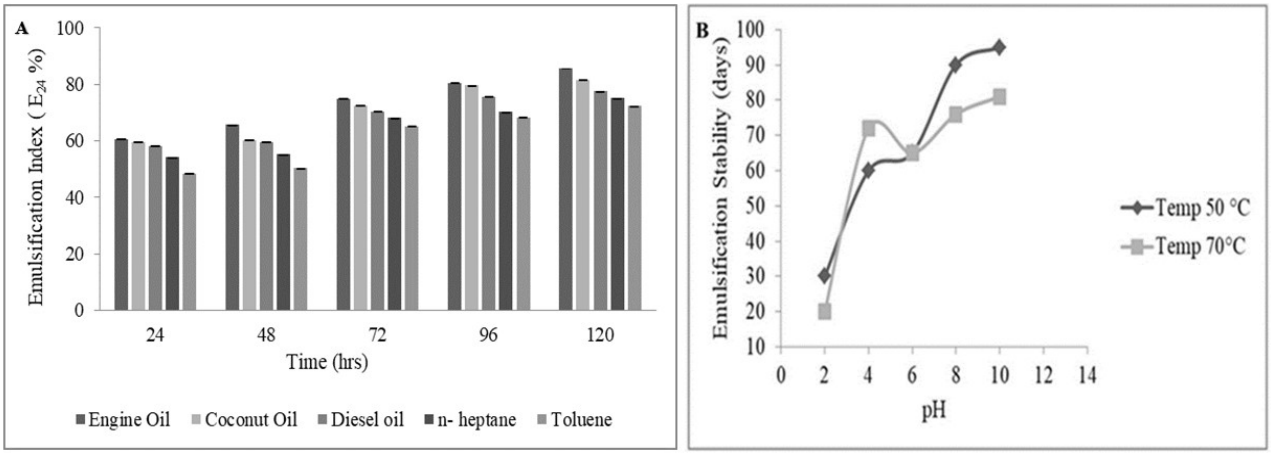

Fig. 2 [A] Emulsification index (E_{24}) of the cell-free supernatant containing biosurfactant with different hydrocarbons and oils at regular time intervals. The error bars are not visible because the standard deviation was within the area occupied by the symbol; **[B]** Combined effect of different pH and temperature on the emulsification stability of engine oil.

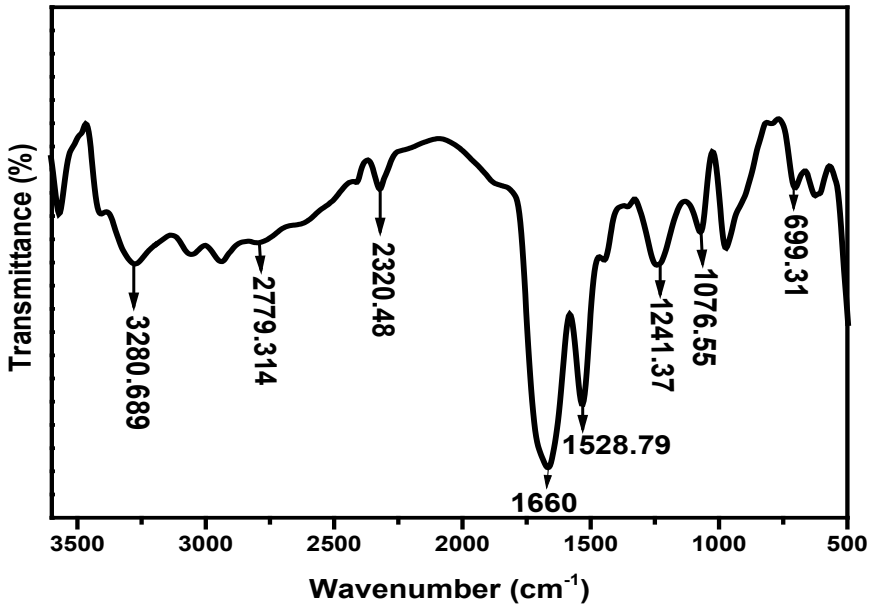

Fig. 3 The FTIR spectra of biosurfactant produced by strain SPSW1001

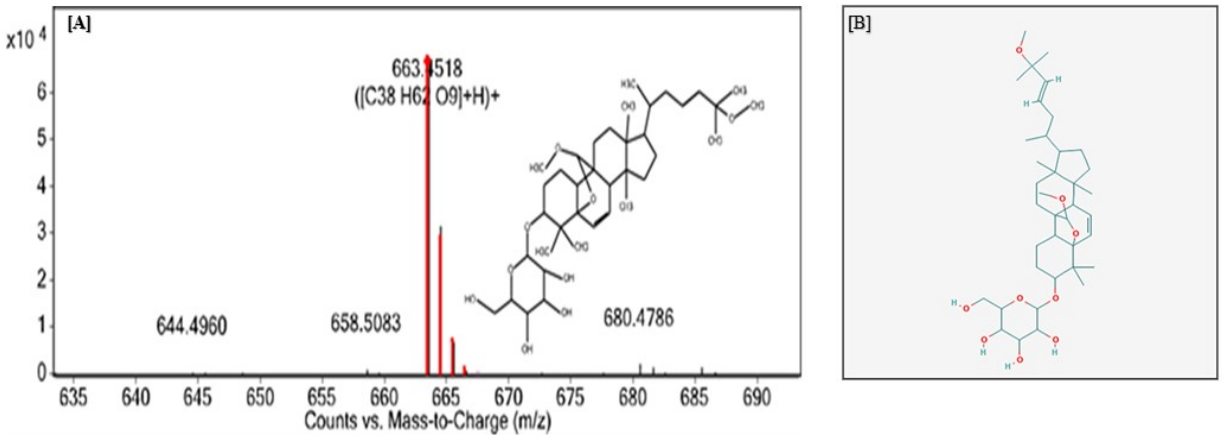

Fig. 4 [A] Mass spectrum of glycolipid biosurfactant produced by *Bacillus megaterium* SPSW1001; [B] Structure of glycolipid biosurfactant Goyaglycoside c

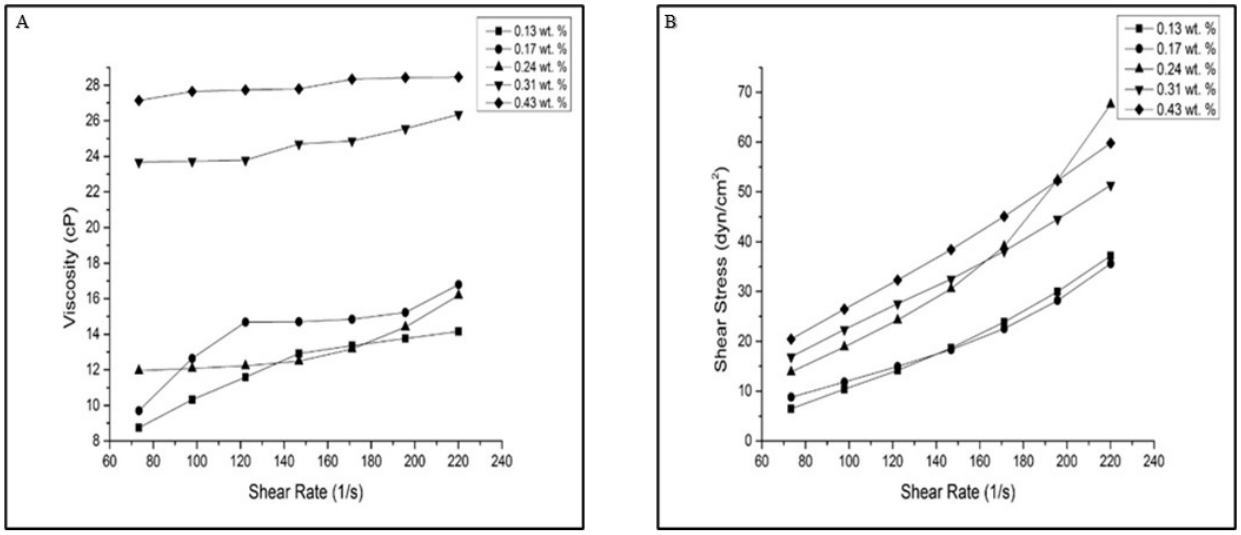

Fig. 5 [A] Changes in viscosity vs. shear rate plot at different concentrations of biosurfactant at 28 °C for water-in-oil emulsions; [B] Shear stress versus shear rates at different concentration of biosurfactant at 28 °C for water-in-oil emulsion

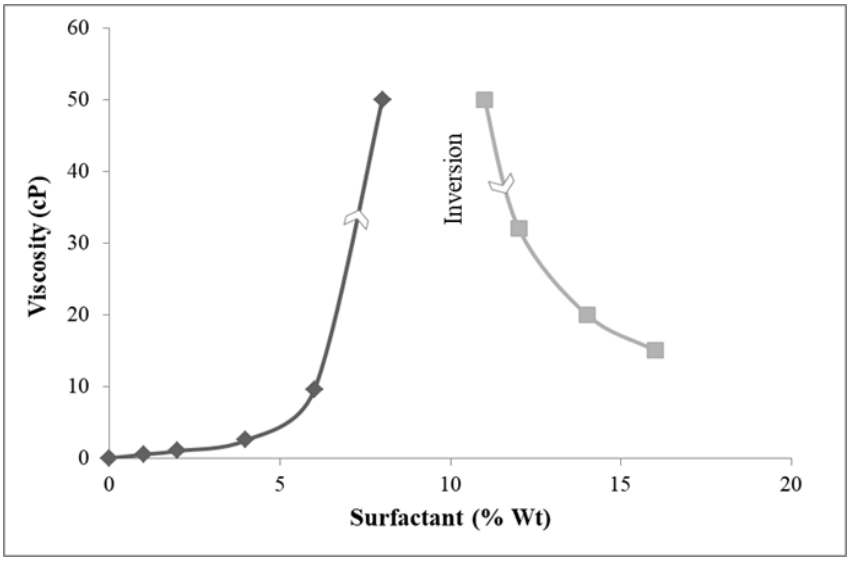

Fig. 6 Viscosity-concentration behaviour of micelle-forming aqueous solutions of the chemical surfactant Triton X-100 at 28 ºC

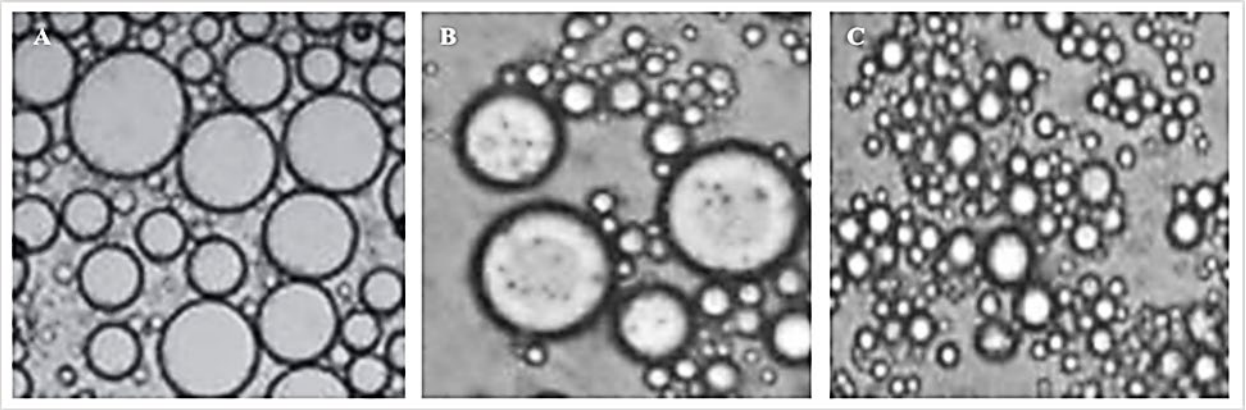

Fig. 7 Optical microscope image of emulsions (at 100X magnification) at biosurfactant concentrations (A) 0.170 wt.%; (B) 0.31 wt.%; (C) 0.43 wt.%

Table 1 Conventional biochemical test results of strain SPSW1011

Characteristics	**Strain SPSW1011**
Aerobic growth	+
Arabinose	+
Citrate utilisation test	-
Casein test	-
Catalase test	+
Esculin hydrolysis	+
Growth at 45 °C	-
Glucose fermentation test[a]	A^-G^+
Gelatin hydrolysis	-
H_2S gas production	-
Indole test	-
Lactose fermentation test[a]	A^-G^+
Mannitol fermentation test [a]	A^-G^+
Motility	+
Methyl red test	+
NaCl tolerance	8%
Nitrate reduction	+
Oxidase test	+
Starch hydrolysis	-
Sorbitol	+
Sucrose	+
Urease test	-
Voges Proskauer test	-

+: positive reaction; —: negative reaction

A^-G^+ indicates acid negative, gas positive; A^+G^- indicates acid positive, gas negative; A^-G^- indicates acid negative, gas negative

Table 2 Characterized biosurfactant glycolipid molecular formula and mass

Compound Name	**Formula**	**m/z**	**Mass**
Goyaglycoside c	$C_{38}H_{62}O_9$	663.4518	662.4446

Table 3. Various parameters of oil-in-water emulsions at regular time intervals

S.No.	Conc. of Biosurfactant (wt.%)	Model Fitted	Plastic viscosity (cP)	Yield Stress (dyn/cm^2)	Consistency Index (cP)	Flow Index	Confidence of Fit (%)	Fluid Behaviour
1.	0.13	Bingham	35.5	16.9	-	-	87.7	Non-Newtonian Viscoplastic - Dilatant flow after yielding
		Power Law	-	-	2.88	1.42	92.9	
		Herschel-Bulkley	-	3.21	0.003	2.69	98.7	
2.	0.17	Bingham	17.6	5.87	-	-	92.2	Non-Newtonian Viscoplastic - Dilatant flow after yielding
		Power Law	-	-	3.97	1.24	92.4	
		Herschel-Bulkley	-	4.21	0.014	2.27	99.8	
3.	0.24	Bingham	21.8	12.3	-	-	94.9	Non-Newtonian Viscoplastic - Dilatant flow after yielding
		Power Law	-	-	0.766	1.57	95.1	
		Herschel-Bulkley	-	6.95	0.053	2.05	99.9	
4.	0.31	Bingham	22.8	14.3	-	-	99.2	Non-Newtonian Viscoplastic - Dilatant flow after yielding
		Power Law	-	-	0.866	1.4	98.1	
		Herschel-Bulkley	-	9.76	0.053	1.894	100	
5.	0.43	Bingham	17.2	6.7	-	-	99.2	Non-Newtonian
		Power	-	-	1.79	1.39	98.0	

		Law						
		Herschel-Bulkley	-	11.9	32.9	2.25	99.9	Viscoplastic - Dilatant flow after yielding