REVIEW

Characterization and performance of a new lipopeptide biosurfactant producing strain *Bacillus Subtilis* R1-2

Gui-Na Qi[1,2], **Guo-Jun Li**[1,2], **Lei Zhou**[1,3], **Lian-He Wu**[2], **Yi-Fan Liu**[1,3], **Jin-Feng Liu**[2],**Hong-Ze Gang**[1,3], **Shi-Zhong Yang**[1,3], **Bo-Zhong Mu**[1,3*]

[1] State Key Laboratory of Bioreactor Engineering, School of Chemistry and Molecular Engineering, East China University of Science and Technology, Shanghai 200237, China

[2] Daqing Huali Biotechnology Co. Ltd, Daqing 163000, China

[3] MOE Engineering Research Center of Microbial Enhanced Oil Recovery, East China University of Science and Technology, Shanghai 200237, China

Abstract: Biosurfactants produced by native microorganisms have excellent surface activity and ideal environmental compatibility, and the mining of the biosurfactant-producing strains has become a key focus in the field of applied and environmental biotechnology. In this paper, we report a new lipopeptide-producing strain isolated from the production water of Daqing oilfield in China and identified as *Bacillus Subtilis* R1-2 based on 16S rRNA gene sequence analyses. A combination of ESI-MS and FT-IR analyses revealed that the strain R1-2 produced the surfactin family containing four members of the C_{12}-surfactin, C_{13}-surfactin, C_{14}-surfactin and C_{15}-surfactin, which is a representative family of the lipopeptide biosurfactants. The lipopeptide biosurfactant produced by the strain R1-2 exhibits excellent surface activity and good thermal stability over a temperature range between 20°C and 100°C and pH range between 3 and 14, and has a strong salt tolerance to NaCl concentration up to 140 g/L. In addition, the lipopeptide biosurfactant demonstrates significant properties in changing the contact angles of oil reservoir core slices from 86.2° to 39.0° and the wettability from strong oil-wet to strong water-wet, and therefore, resulted in a good oil removing ability with an efficiency of 64.84%, suggesting that the lipopeptide-producing strain R1-2 is promising in applications in environmental bioremediation and enhanced oil recovery.

Keywords: *Bacillus Subtilis*, lipopeptide biosurfactant, wettability alteration, oil washing ability

Correspondence to: Bo-Zhong Mu, State Key Laboratory of Bioreactor Engineering, School of Chemistry and Molecular Engineering, East China University of Science and Technology, Shanghai 200237, China; MOE Engineering Research Center of Microbial Enhanced Oil Recovery, East China University of Science and Technology, Shanghai 200237, China; E-mail: bzmu@ecust.edu.cn

Received: July 20, 2024; **Accepted:** August 20, 2024; **Published Online:** September 4, 2024

Citation: Qi, G.-N., Li, G.-J., Zhou, L., Wu, L.-H., Liu, Y.-F., Liu, J.-F., Gang, H.-Z., Yang, S.-Z., Mu, B.-Z., 2024. Characterization and performance of a new lipopeptide biosurfactant producing strain *Bacillus Subtilis* R1-2Characterization and performance of a new lipopeptide biosurfactant producing strain *Bacillus Subtilis* R1-2. *Applied Environmental Biotechnology*, 9(2): 5-15. http://doi.org/10.26789/AEB.2024.02.002

1 Introduction

Biosurfactants produced by a wide variety of diverse microorganisms are of low ecotoxicity, good biodegradability, and non-toxic to the environment, and have great outcome potentials in scientific research and industrial applications (Ogru and Olannye, 2021). Biosurfactants (BS) are active substances produced by living microorganisms through fermentation, extraction or other biological processes. They have the ability to reduce the surface tensions or interfacial tensions at low concentration. These compounds exhibit a wide range of structural diversity, covering glycolipids, lipopeptides, phospholipids and neutral lipids (Sankhyan et al., 2024). They are typically present in microbial cell walls, plant leaves, and animal body fluids.

Biosurfactants are a kind of natural amphiphilic molecules produced by microbial metabolism, consisting of both hydrophilic and hydrophobic components. Compared to synthetic surfactants, biosurfactants offer greater advantages such as lower toxicity, higher biodegradability, better environmental compatibility, increased foaming, wide temperature and pH tolerance, and the ability to be produced from renewable resources. Therefore, it plays a significant role in the relevant industries and have various applications in daily life, including cosmetics, food processing, microbial enhanced oil recovery (MEOR), agriculture, and environmental bioremediation (Singh et al., 2022; Thraeib et al., 2022; Raouf et al., 2024; Guillén-Navarro et al., 2023; Sarkar et al., 2017). Furthermore, it may serve as antifungal, germicidal, antibacterial skincare products, and tumor growth inhibitor (Rincón-Fontán et al., 2018; Vecino et al., 2017; Wicke et al., 2000).

Biosurfactants are generated by various microorganisms such as bacteria, fungi, and yeasts (Balan et al., 2017; Handa et al., 2022; Kubicki et al., 2019). Microorganisms that exhibit effective biosurfactant production are often discov-

ered in oil polluted soils, oilfield produced water, lipid-rich wastewater, seawater, and marine sediments, where they can thrive on substrates and produces surfactants due to the environmental pressure. The growth of these organisms in specific environment is affected by various conditions like pH, temperature, composition of nutrients, etc. (Geetha et al., 2018). In 1968, *Bacillus Subtilis* strains were first reported to produce the lipopeptide surfactant (Arima et al., 1968). Subsequently, in 1985, the ability of *Bacillus licheniformis* JF-2 was found to produce a variety of lipopeptides that lowered the surface tension of the medium (< 30 mN·m^{-1}) under conditions of strict anoxia (Javaheri et al., 1985). Currently,there have been many studies on screening biosurfactant-producing strains, such as three biosurfactant-producing bacteria were isolated from a marine environment with better emulsifying ability (Wu et al., 2019), *Bacillus licheniformis* DS1 from the crude oi could degrade a wide variety of crude oil (Purwasena et al., 2019), eleven strains from processing wastewater showing significant solubilization and emulsification activities (Eldos et al., 2024). In addition, microorganisms such as *Pseudomonas aeruginosa*, *Corynebacterium*, *Rhodococcus* spp., *Arthrobacter parafneus*, *Aspergillus* spp., *Torulopsis* spp., and *Candida* spp. have been used to produce biosurfactants through fermentation (Dos Santos et al., 2022). The current research is mainly focused on screening and modification of new high-yield strains, analysis and optimization of synthetic pathways, and the development of multifunctionalized biosurfactants. With the continuous progress of biotechnology and the increasing awareness of environmental protection, it is anticipated that biosurfactants will be commercially applied in a wider range of fields, becoming a key force to promote green chemistry and sustainable development.

In this study, a new lipopeptide-producing strain *Bacillus Subtilis* R1-2 was isolated from the production water of the Daqing oilfield and identified using 16S rRNA gene sequences in GenBank, the optimal conditions for fermentation were determined through the optimization of culture and incubation parameters using orthogonal experimental methods, and chemical structures were identified using ESI-MS and FT-IR. Meanwhile, its surface activity, environmental tolerance, ability to change wettability and oil washing ability were evaluated.

2 Materials and Methods

2.1 Sample collection and strain screening

The bacterial strain was isolated from the enrichment culture of formation water samples of the Daqing oilfield in China. The Luria Bertani (LB) solid medium contained 5.0 g/L of yeast extract, 10.0g/L of tryptone, 10.0 g/L of NaCl and 15.0% of agar. The culture medium was a mineral salt medium contained 1.1 g/L of NaCl, 1.0g/L NH_4Cl, 5.0 g/L of $NaNO_3$, 0.5 g/L of KH_2PO_4, 0.7 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4{\cdot}7H_2O$, which was supplemented with yeast extract (4.0 g/L) and crude oil (50.0 g/L), using 1 mol/L of HCl or 1 mol/L of NaOH to adjust the medium pH which was maintained at 7.0. Enrichment cultures were performed in 250 mL flasks containing 100 mL of medium at 37°C with rotary shaking at 130-150 rpm for 7-15 d. Thereafter, the enrichment culture as inoculum was inoculated into the mineral salt medium containing crude oil (5.0 g/100 mL) as the sole carbon source to observe the emulsification effect. Subsequently, enrichment cultures were obtained by transferring the culture 3-5 times. Once the crude oil had been fully emulsified, isolation of single bacteria from cultures on Petri dishes. The isolation and purification of the colonies and the determination of the emulsifying and reduced surface tension capacity. This process was repeated several times until pure bacterial isolates were obtained. Finally, the pure bacterial strains were preserved at 4°C for further analyses (Li, 2020).

2.2 Identification of the selected strains

Bacterial morphology was observed by scanning electron microscopy (Hitachi-SU8100). Amplification of the 16S rRNA gene and nucleotide sequencing. 16S rRNA gene sequence was amplification using the Primers 27F:5'-AGAGTTTGATCM TGGCTCAG-3' and 1492R:5'-GGTTACC TTGTTACGA CTT-3'. The obtained sequences were compared with sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI), The sequences were aligned using MEGA 7.0 software (Kumar et al., 2018).

2.3 Culture medium optimization

Seven parameters were studied for their relationships and effects on surface tension (ST), BS crude yield and emulsification index (E_{24}) of lipopeptide biosurfactant production using orthogonal test method. The factors and levels orthogonal experiment are showed in Table S1. Surface tension, E_{24} index and cell growth curve were detected. The detection methods is as described in Section 2.4 below.

2.4 Measurement of surface tensions and emulsification index

The surface tension was measured on a fully automatic tensiometer JK99C (Shanghai Wang Xu Electric Co., Ltd., China) according to the plate method, using fermentation broth was adjusted to a pH > 8.0 using 1.00 mol/L NaOH at 25°C, and the fermentation broth was diluted with de-ionized (DI) water to determination of dilutions. E_{24} was measured with cell-free supernatant, the culture fluid was centrifuged at 5000 rpm for 10 min, then supernatant was added to an equal volume of liquid paraffin in a 10 mL test tube with plug (1:1, v/v). The mixtures were vortexed by maximum for 3 min, and the stability of the emulsion was determined after 24 h at room temperature. The emulsification index (E_{24}) was calculated as the height of the emulsion layer/total height (Silva et al., 2022). The cell growth curve was analyzed by measuring OD_{600} with a spectrophotometer (UV1901PC, China), and the culture broth was added 1.0 mol/L NaOH until the final

pH reached at 12.0, then centrifuged at 5000 rpm for 10 min to remove the bacterial precipitation. Thereafter, the supernatant was added 6.0 mol/L HCl until the final pH reached at 2.0 and then settled at 4°C overnight. The acid precipitation was collected by centrifuging at 5000 rpm for 10 min, and dried to achieve equilibrium weight, and the weight of the BS crude yield was determined (Qin et al., 2023).

2.5 Extraction and purification of biosurfactants

The crude biosurfactant was precipitated and obtained from the cell-free supernatant by adding 6.0 mol/L of hydrochloric acid to adjust the value of pH to 2.0 and kept for 24 h for precipitation at 4°C. Then, the acid precipitate was generated, separated by centrifugation (5000 rpm at 4°C for 10 min), and extracted several times by ethyl acetate for more than 2 h each time (2:1 v/v), The extracts were obtained by rotary evaporation under the condition of 55°C-65°C to obtain paste solid, lyophilized, and weighed (Zhao et al., 2012).

2.6 Determination of critical micelle concentration (CMC)

The CMC was determined by measuring the decrease in surface tensions at 25°C with the plate method on a JK99C tensiometer. Solutions of purified biosurfactant in water of concentrations between 0 to 100 mg/L were tested. When an abrupt decrease of the ST was reached, the CMC was attained. It corresponds to the concentration at which biosurfactant associates into micelles. The value of surface tension was the average of at least triplicate measurements (de Araújo et al., 2019).

2.7 Structural analyses

2.7.1 Molecular weight determination

The molecular weights of the biosurfactant samples were determined by the electrospray ionization mass spectrometry (XEVO G2 TOF, Waters Corporation USA, temperature 80°C electrolyte voltage 200 V and spray inlet temperature 120°C). The samples were prepared as described in section 2.5, and sample of 1.0 mg in methanol 1.0 mL was supplied to electrospray ionization mass spectrometry for positive and negative m/z 100-2000 of the biosurfactant. The molecular weight of the biosurfactant could be identified based on the relationship between the positive and negative ratios of mass to charge (Liu et al., 2009).

2.7.2 Functional groups

The functional groups of chemical structures of the biosurfactants were investigated through Fourier-transform infrared (FT-IR) spectroscopy (Platinum KBr, INVENIO S-Hyperion 3000, Bruker, Germany). The samples were diluted in methanol and then scanned 64 times in the wavenumber range between 4000 and 400 cm^{-1} at 25°C, with a resolution of 0.5 cm^{-1} (Haddad et al., 2009).

2.8 Measurement of contact angles

The wettability was determined by measuring the contact angle. The cleaned core slices, with permeability values of 0.8 to 1. 0mD and thicknesses of 1 to 3 mm, were submerged in liquid paraffin and aged at 75°C for more than 14 d in order to produce oil-wet core pieces. The core slices were clean with filter paper, then dried in a vacuum oven. Subsequently, the prepared oil-wetted core pieces were submerged in different concentrations (0%, 0.2%, 0.4%, 0.5%, 0.8%, 1.0%) of the biosurfactant solutions for 24 h, allowing the surfactant molecules to be fully adsorbed onto the core slices, followed by measured with contact angle measuring instrument (HS-150A, V2.0, China) (Hou et al., 2016).

2.9 Measurement of the oil washing efficiency

The oil washing efficiency experiments were performed and modified from Li's method. Difference with Li's reported approach is that, he directly uses the differential weight method, this paper converts it to volume multiplied by density to get weight. Meanwhile, this test set up a control group. First, the crude oil was mixed with 60-80 mesh washed and dried quartz sand at a mass ratio of 1:5 in a bottle for aging at the reservoir temperature of 45°C for 1 week. Simultaneous determination of the density of crude oil (d). Afterward, the aged oil sands M1 (approximately 15.0 g in dry weight) were packed in flasks containing solutions (30mL) with different concentrations of surfactants (0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0%), respectively. Under the conditions of 90rpm and 45°C, the flask remained to be shaken for 2 h. Removed the oil and wash bottle and replenished the surfactant solution with the corresponding concentration to the highest mark of the oil wash bottle. Subsequently, the experiment was continued in an incubator at 45°C for 2 h. Then, the solution is poured out of the flask. The residual oil on the flask walls is carefully removed by dipping a skimmed cotton pad. The remaining surfactant solution in the flask is carefully rinsed with distilled water until the washout solution was clarified. After pouring the washing solution, the residual water was dried at 45°C. Read the volumetric of oil V_1. The mass of aged oil sands in the control group (distilled water) was M_2 and the volume of oil washed out was recorded as V_2. Oil washing efficiency was calculated using the following equation (Li et al., 2022).

$$X = Kd\left(\frac{V_1}{M_1} - \frac{V_2}{M_2}\right) \times 100\%$$

where X is the efficiency of oil washing, %; V_1 is the Volume of oil washed out by BS, mL; V_2 is the Volume of oil washed out by distilled water, mL; M_1 is the quantity of the oil sands in test group, g; M_2 is the quantity of the oil

sands in control group, g; K is the percentage of the oil in oil sands; and d is the density of crude oil, g/cm^3.

3 Results

3.1 Isolation and identification of strains

A total of nine morphologically distinct bacterial isolates, designated as R1-2, R3-3, R5-1, R5-4, R6-1, R8-2, R11-3, R13-4 and R14-4, were obtained in pure cultures from oil well produced water samples (Table 1). The bacterial strains were transferred into mineral salt medium with 10% glucose as the carbon source and incubated for 48 h. The population density of these bacterial strains in the cultures (OD), pH, surface tensions (ST), and the resistance of the fermentation broth to dilution were determined. Nine of these strains were able to grow in the mineral salt medium. Out of the nine bacterial strains, five strains were able to reduce the surface tension to 27-28 mN/m of the media in 48 h, demonstrating that they possess a strong ability to produce biosurfactants. The emulsification index revealed that all the bacteria were able to emulsify paraffin to a degree between 40-55%, except for R14-4, which exhibited a lower level of emulsification (10%). After diluting the fermentation broth of strain R1-2 to 60 times of its original concentration, its surface tension was approximately 35 mN/m, indicating that the strain has a higher production of biosurfactants than other strains. Therefore, strain R1-2 was selected for further studies.

Table 1. Surface tension reduction, OD, pH and emulsification index of the isolates

Number	OD	pH	Surface tension (mN/m)		E_{24} (%)
			Undiluted	ST/Dilution	
R1-2	2.28	7.34	27.30	35.10/ 60	55
R3-3	3.17	6.66	47.40	/	45
R5-1	5.44	7.07	56.75	/	40
R5-4	4.36	7.66	28.70	35.80/10	55
R6-1	1.13	7.24	27.70	37.90/10	55
R8-2	2.37	7.46	27.12	34.50/10	50
R11-3	3.06	7.22	53.73	/	45
R13-4	1.04	5.02	55.45	/	45
R14-4	6.58	5.85	27.91	35.19/60	10

The morphology of bacterial strain showed moist, milky

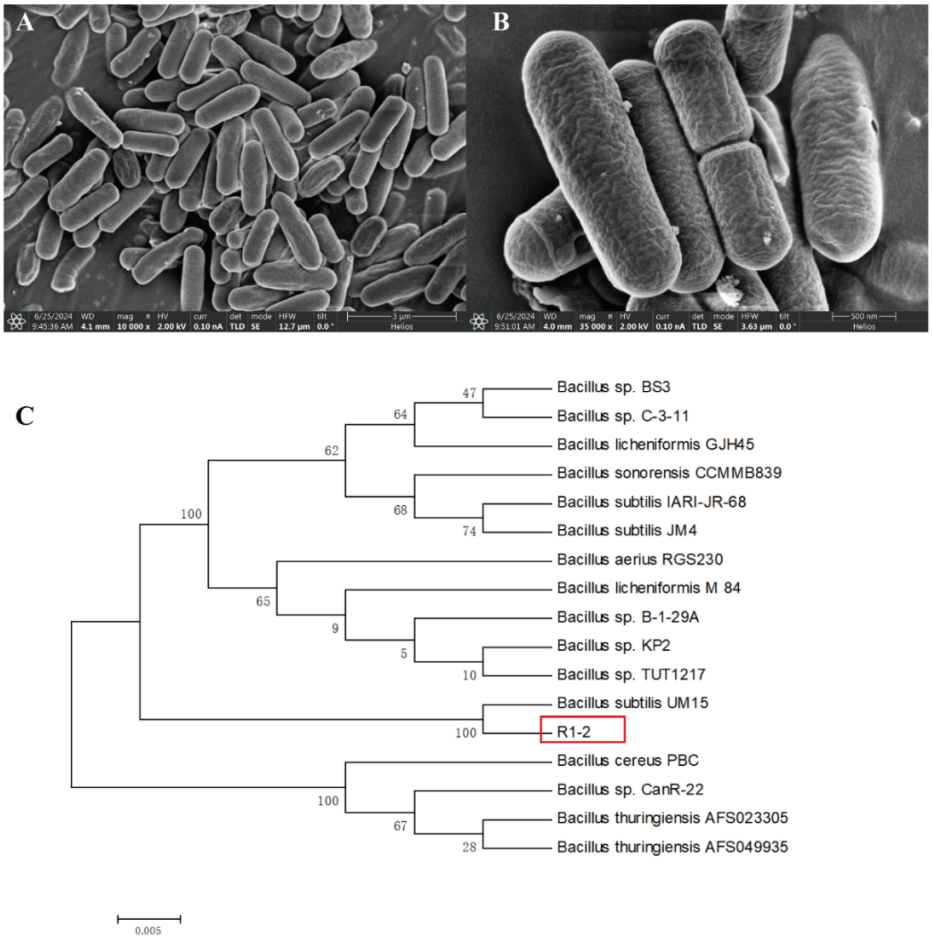

Figure 1. Isolation and identification of strain R1-2. (A) Scanning electron microscope of R1-2 strain magnified 10000×. (B) Scanning electron microscope image of R1-2 strain magnified 35000×. (C) Phylogenetic tree based on 16S rRNA gene sequences of strain R1-2 and related bacteria constructed using the neighbor-joining method in MEGA software of strain R1-2.

white and round with smooth edges on the flat dish. A group of short-rod shaped bacteria can be observed in close proximity to one another, exhibiting a high degree of spatial organization (Figure 1A). The dimensions of each bacterium are approximately 1.5-2.0μm in length and 0.5μm in width. They possess a smooth surface, though some fine texture is also evident; and the bacterial strain lacked endospores or flagella (Figure 1B). Meanwhile, it was observed that some bacteria exhibited the presence of minute cellular debris and particulate matter attached to their surfaces, which may be indicative of their interaction with their surrounding environment, and some cells remain in the division phase. Analysis of 16S rRNA gene sequences in GenBank indicated that the strain was 100% similarity to *Bacillus Subtilis* UM15. Based on the morphological and 16 S rRNA sequencing results, the strain R1-2 is identified as *Bacillus Subtilis* (Figure 1C).

3.2 Optimization of the culture media

To optimize the culture media and culture conditions, an L_{18} (21×3^7) orthogonal experiment was devised using data processing system (DPS 9.01) with constant additions of KH_2PO_4, K_2HPO_4, and $MgSO_4$ $7H_2O$ (Table S1 to Table S4), the surface tensions of the cell free broths under different conditions and emulsification index E_{24} values on liquid paraffin (1:1, v/v) were evaluated. As showed in Figure 2A, the surface tensions of the 18 groups ranged from 25.20 mN/m to 48.85 mN/m, and the emulsification indices (E_{24}) varied from 0% to 65%, respectively. Obviously, the group 13 (sucrose, 10.0 g/L, $NaNO_3$ 1.0 g/L, NaC_1 2.0 g/L, NH_4C_1, 2.0 g/L, pH 7.5 and volume of liquid 15 0 mL/500 mL) appeared the lowest surface tension at 25.20 mN/m, and the emulsification index of 60%. Followed by the group 5 (glucose 10.0 g/L, $NaNO_3$ 3.0 g/L, NaC_1 2.0 g/L, NH_4C_1, 2.0 g/L, pH 6.5 and volume of liquid 100 mL/50mL) appeared a surface tension at 25.8 mN/m, and the emulsification index of 52.5%.

Further analyses using direct calculation of the k-value and the analysis of variance (ANOVA) (Table S3 and Table S4) showed that there were significant differences in the carbon source content, NaCl, NH4Cl and pH. The sucrose 10.0 g/L, NaNO3 3.0 g/L, NaC_1 1.5 g/L, NH_4C_1 1.0 g/L, pH 7.0 and volume of liquid 150 mL/500 mL was the optimal combination. The groups 5, 13 and the combination of new formulations obtained after K-value calculation (New) were tested and the resulting group with the best surface tension and emulsification results was the new group (ST 25.60 mN/m and E_{24} 62.5%) as showed in Figure 2B. Overall, the optimal medium composition for strain R1-2 was sucrose 10.0 g/L, $NaNO_3$ 3.0 g/L, NaC_1 1.5 g/L, NH_4C_1 1.0 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.7 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, pH 7.0, and volume of liquid 150 mL/500 mL.

Under this condition, the pattern between bacterial growth curve and the emulsifying activity and BS yield were subsequently investigated. The strain R1-2 entered the logarithmic growth phase after 8h, and at this time the lipopeptide production also increased with a large amount (1.0 g/L). When the bacteria grew to the later stage of the logarithmic growth phase (26 h), the OD_{600} reached the maximum value (3.73), and the BS production (1.4 g/L) also reached the maximum value (Figure 2C). It indicates that BS production is closely related to the bacterial growth stage. The emulsifying ac-

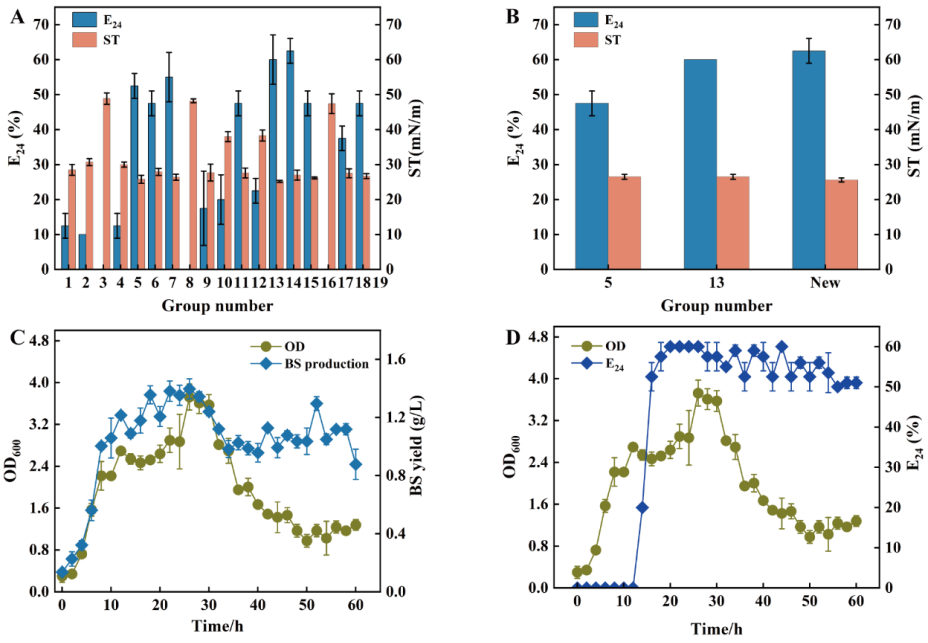

Figure 2. Appropriate growth and metabolic conditions for R1-2. (A) Emulsification index and surface tension were measured for the eighteen media. (B) Results of formulation validation tests. (C) The growth curve OD_{600} and BS yield of strain R1-2. (D) The growth curve OD_{600} and emulsion index of strain R1-2.

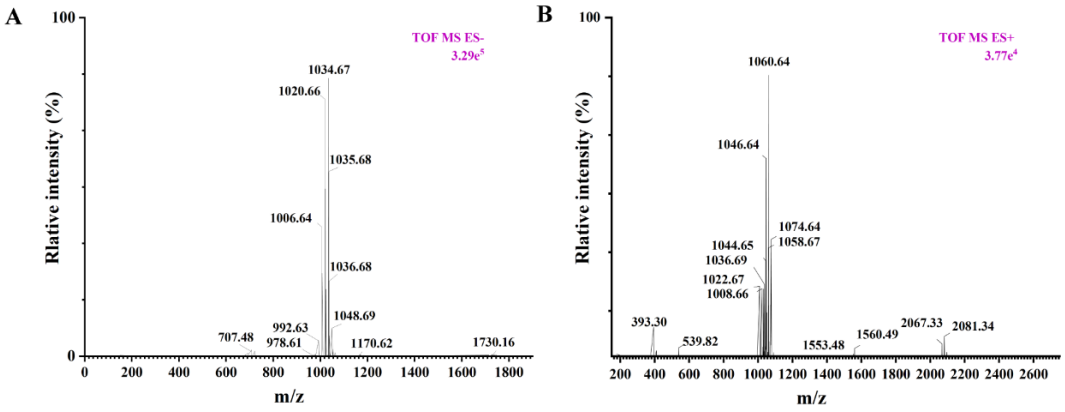

Figure 3. Mass spectrometry of biosurfactants produced by *Bacillus Subtilis* R1-2. (A) Negative ionization mass spectrometry. (B) Positive ionization mass spectrometry.

tivity clearly increased during the exponential phase, which demonstrated a positive correlation between the emulsifying activity and the bacterial growth curve, with the maximum E_{24} value of 60% at 26 h and OD_{600} value of 3.73 (Figure 2D).

3.3 Characterization of the biosurfactant

Biosurfactants produced by *Bacillus Subtilis* were determined by mass spectrometry with negative and positive-ion modes (Figure 3). When negative ionization was used, the peaks of m/z 978.61, 992.63, 1006.64, 1020.66, 1034.67 and 1035.68 were detected with higher intensities from mass spectrum (Figure 3A). The clusters are composed of the ion series with difference of 14, which implied that the purified biosurfactant was a mixture and contained different homologues with difference of -CH2 groups, the smallest group in organic compounds. In addition to the negatively ionized m/z, the positively ionized m/z ratio come from natural molecules and attached positive ions such as H^+ or/and Na^+ (Figure 3B). The m/z 1008.66, 1022.67, 1036.69 are H^+-ionized molecules 1006.64, 1020.66, 1034.67, since their positively ionized and negatively ionized m/z ratios are different by 2. The m/z 1044.65 and 1058.67 are Na^+-ionized molecules 1020.66 and 1035.68, since their positively ionized and negatively ionized m/z ratios are different by 24. Therefore, the biosurfactant produced by *Bacillus Subtilis* R1-2 is determined as surfactin homologues.

FTIR spectroscopy was used to identify the principal functional groups of the biosurfactant. As showed Figure 4, the absorption bands at 3300.16 cm^{-1} corresponded to the stretching vibrations of hydroxyl or amine groups, typical the carbohydrates and peptides, respectively. The peaks 2923.51and 2869.24 suggested the symmetrical stretching (γC-H) of CH_2 and CH_3 groups of aliphatic chains. The presence of peptide bonds was indicated by the peaks at 1638.74 cm^{-1} and 1714.55cm^{-1}, which were obtained due to the stretching mode of –CO–N bond, and C=O stretching mode, respectively. The absorption band at 1204.63cm^{-1} was characteristic of amide groups C–N. Therefore, both aliphatic chains (hydrophobic domain) and peptide moiety (hydrophilic domain) existed in the produced biosurfactant.

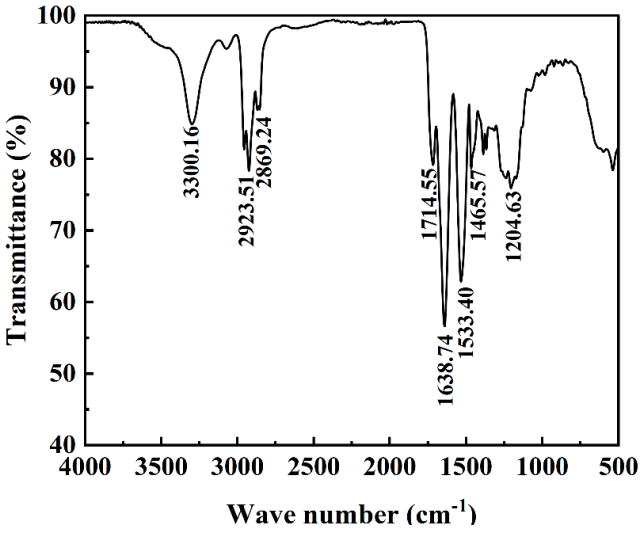

Figure 4. FT-IR spectrum of the purified biosurfactant produced from the *Bacillus Subtilis* R1-2. FT-IR spectrum analysis shows the spectrum of purified bioemulsifier at 400-4000 wave numbers (cm^{-1})

3.4 The surface and interfacial activity of the lipopeptide biosurfactant

3.4.1 The surface tensions and critical micelle concentration

Critical micelle concentration was determined by calculating the corresponding surface tensions of various concentrations of biosurfactant solutions up to 100 mg/L as shown in Figure 5. The surface tension first decreased sharply and then remained constant as biosurfactant concentrations increased. Ultimately, the critical micelle concentration (21.71 mg/L) was obtained, resulting in a decrease in surface tension from 71.99 mN/m to 28.79 mN/m. The lower CMC value indicates the less amount of biosurfactant required in applications. The results demonstrate that the biosurfactants produced by R1-2

possesses high surface activity at low concentrations.

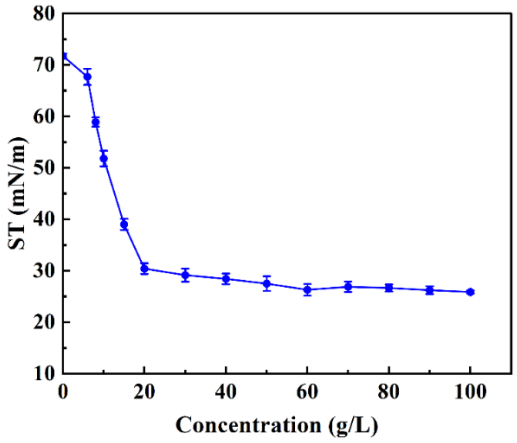

Figure 5. The surface tensions against the concentrations of the biosurfactant produced by the strain R1-2 in solutions.

3.4.2 Tolerance of the temperatures, pH and NaCl

The surface tensions of the cell-free broths at different temperatures, pH, NaCl concentrations were measured and analyzed (Figure 6). When temperatures ranged from 20°C to 90°C, the surface tensions remained almost constant and maintained around 25.0-28.8 mN/m, indicating a good thermal stability (Figure 6A). The effects of pH were investigated over a range of pH values from 1 to 14 at a temperature of 20°C for 24 h. When pH values ranged from 3 to 14, the surface tensions remained a little change and maintained around 24.4-28.7 mN/m, and only when pH values less than 2, the surface tensions increased significantly, indicating a good stability under neutral and alkaline conditions (Figure 6B). To further explore the salt tolerance, the surface tensions of the cell-free broths after adding different concentrations of NaCl was measured. When NaCl concentration was less than 120 mg/L, the surface tensions remained a little change and maintained around 24.6-28.4 mN/m, and only when the concentrations above 140 mg/L, the surface tensions increased significantly, indicating a NaCl tolerance up to 120mg/L (Figure 6C). Our results show that lipopeptide biosurfactants produced by the strain R1-2 have good surface activity under a wide range of temperatures, pH and NaCl concentrations.

3.4.3 Wettability alteration and oil washing efficiency

The contact angles of core slice treated with liquid paraffin and aged at 75°C for 14 d (oil-wet, control) and the oil-wet core slices treated with different concentrations of the lipopeptide biosurfactant solutions were measured, respectively. As showed that Figure 7, the contact angle of the oil-wet core slice (control group) was 89.2°; while after treatment with the lipopeptide biosurfactant solutions of

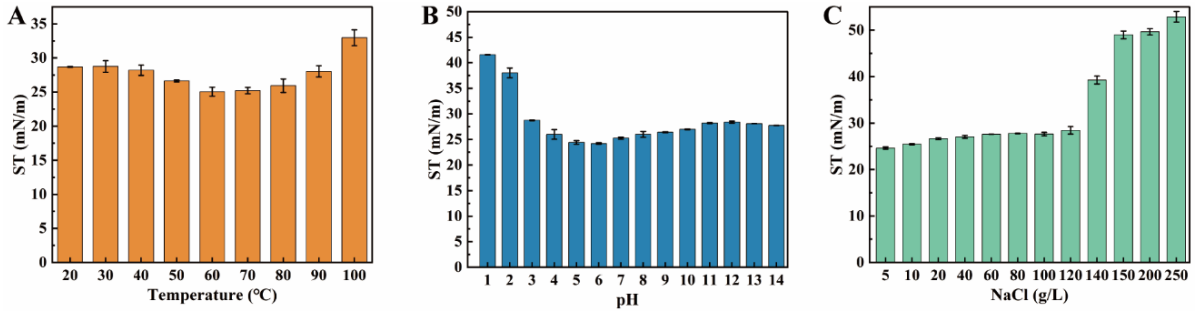

Figure 6. Surface tension of biosurfactant after the tolerance test upon different physicochemical conditions. (A) temperatures. (B) pH and (C) NaCl concentrations.

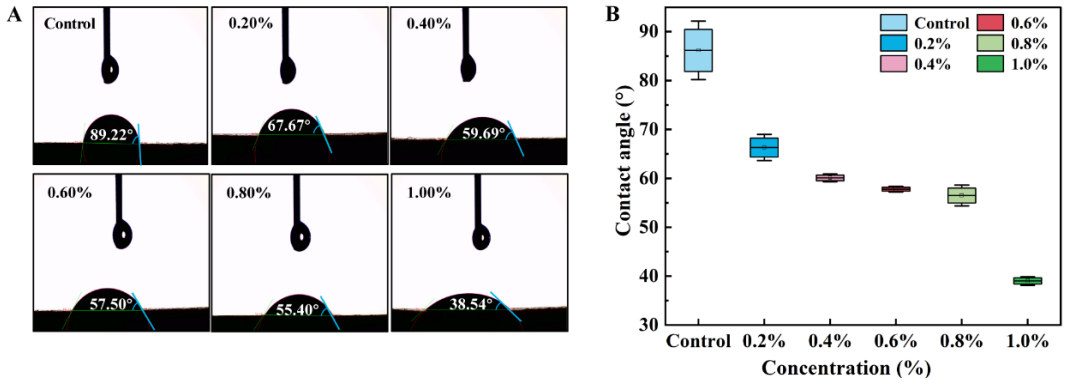

Figure 7. Determination of contact angle of R1-2 solutions at different concentrations over the hydrophobic surface. (A) Images of contact angles measured with a HS-150A pendant drop apparatus on core slice in aged crude oil. (B) Changes in wetting angle of core slices treated with different concentrations of surface.

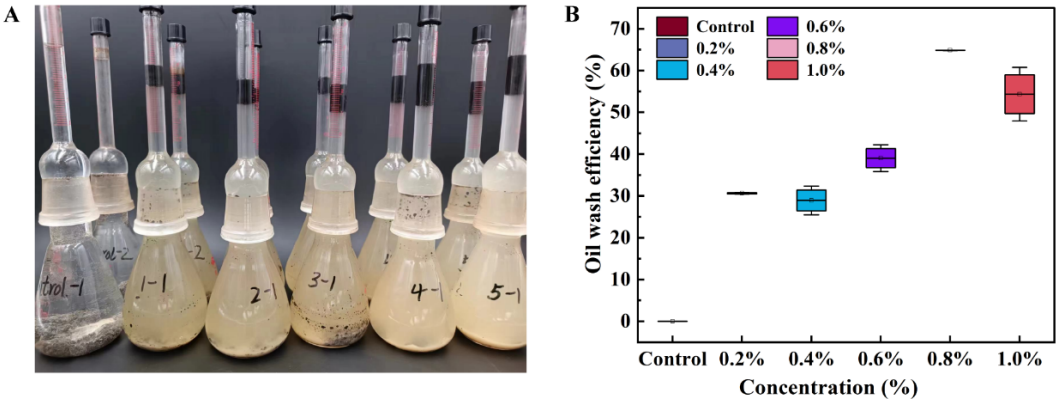

Figure 8. Efficiency of oil washing of biosurfactants produced by R1-2. (A) Images of the amount of oil washed out by different concentrations of BS. From left to right, represent the control, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% experimental group, respectively. (B) Variation of wash out the oil content with surfactant additions.

different concentrations of 0.2%, 0.4%, 0.5%, 0.8%, 1.0%, the contact angles were significantly decreased from 86.2° to 66.3°, 60.1°, 57.8°, 56.5°, and 39.0°, respectively (Figure 7A), which indicates that an increase in the concentration of the lipopeptide biosurfactant in solutions is associated with a reduction in the contact angles of the distilled water with the core slices. In comparison with the control, the contact angle exhibited 54.7% reduction after treatment of the lipopeptide biosurfactant solutions. The results suggest that the lipopeptide biosurfactants produced by the strain R1-2 have a strong capacity of the wettability alteration from oil wetting to water wetting (Figure 7B).

The oil washing efficiencies by lipopeptide biosurfactant solutions with different concentrations ranging from 0.2% to 1.0% were evaluated. As shown in Figure 8, compared with the control group, the oil washing efficiencies increased by 30.6% to 64.8%, and the solution with a concentration of 0.8% lipopeptide biosurfactant exhibited the highest oil washing efficiency (Figure 8B). These results suggest that lipopeptide biosurfactant produced by the strain R1-2 are highly surface-active at a very low dosage,and is potential applications in environmental bioremediation and enhanced oil recovery.

4 Discussion

The exploration of new biosurfactants and biosurfactant-producing strains have emerged as a pivotal area of focus within the realm of biotechnology research. Biosurfactants are the surface active metabolites secreted by various bacteria, yeast, and fungus. Compared to petro-based surfactants, biosurfactants offer advantages such as lower toxicity, higher biodegradability, biocompatible, environmentally friendly, wide temperature and pH tolerance, and the ability to be produced from renewable resources (Desai and Banat, 1997; Kuyukina et al., 2005). As a result, biosurfactants have potential applications in a number of industries, including petroleum, food, cosmetics, pharmaceuticals, agriculture and bioremediation. A number of different habitats have been explored for biosurfactant-producing bacteria, such as petroleum-contaminated soils, marine samples and wastewater discharge points. Petroleum reservoirs are extreme environments because of the high temperature, pressure and salinity. Microorganisms that are capable of surviving and metabolism in this extreme environment have developed a suite of unique adaptive mechanisms to cope with the specific challenges.

In this study, a new biosurfactant-producing strain R1-2 was isolated from production water of the Daqing oilfield in China and was identified using the 16S rRNA gene sequences in GenBank. The surface activity, emulsification index, wettability performance and oil washing efficiency of the lipopeptide biosurfactant produced by the strain R1-2 were evaluated. The lipopeptide biosurfactant produced by the strain R1-2 has good surface activity with a low surface tension of 27.30 mN/m, high emulsification index of 55%, high oil washing efficiency of 64.84%, and good wetting ability, suggesting a potential application in bioremediation and enhanced oil recovery. There are many reports that the strains of *Bacillus Subtilis* isolated from various environments can produce different kinds of biosurfactants, such as a strain of *Bacillus Subtilis* isolated from marine soil sediment producing biosurfactant with a yield of 74 mg/g substrate after 3 4h of incubation (Al-Dhabi et al., 2020); a strain of *Bacillus Subtilis* isolated from stock tank water produced biosurfactant with critical micelle concentration of 0.06 g/L (Veshareh et al., 2019) and another strain of *Bacillus Subtilis* 50499 from crude oil-contaminated soil showed the emulsification index against n-hexane, corn, and crude oil was as high as over 90% (Du et al., 2023).

The relationship between bacterial growth and biosurfactant production is a complex process involving multiple steps and factors. In order to achieve maximum biosurfactant production, reduced costs and increased product stability several factors such as the growth stage of the bacteria, the selection of carbon and nitrogen sources, and the growth

conditions must be taken into consideration. By optimizing these parameters, the yield and purity of biosurfactant can be significantly improved, providing high-quality raw materials and products for industrial production, drug development and other fields. The composition of the culture media, such as carbon and nitrogen sources, inorganic salts, and growth conditions, all have an impact on microbial growth as well as the synthesis of metabolic byproducts like BSs (Kumar et al., 2021). Yaraguppi et al., optimized the biosurfactant production employing Bacillus aryabhattai strain ZDY2 using Plackett Burman Design (PBD) and response surface approach (Yaraguppi et al., 2020). Rawat used Plackett-Burman design and screen 15 nutrients and 4 dummy factors affecting the production of biosurfactant,maximal biosurfactant yield up to 3.79g/L (Rawat et al., 2024). In our study, the optimal medium composition and culture conditions were evaluated by L_{18} ($2^1 \times 3^7$) orthogonal experiment and confirmation experiment (Figure 2A and 2B). Meanwhile, the optimized medium was employed to investigate the correlation between growth and metabolism of the strain R1-2 (Figure 2C and 2D). The optimal medium composition, incubation conditions, and the optimal time to produce the biosurfactant were determined through experimental procedures (sucrose 10.0 g/L, $NaNO_3$ 3.0 g/L, NaCl 1.5 g/L, NH_4Cl 1. 0 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.7 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, pH 7.0, and volume of liquid 150 mL/500 mL, incubation 26 h).

It is well established that the bacillus species can produce a variety of bioactive compounds, mainly including lipopeptides, peptides, and proteins. The majority of Bacillus spp. synthesized lipopeptides can be grouped into three primary families: surfactin, iturin, and fengycin (Ja'afaru et al., 2022). Different structures exhibit different properties. The biosurfactant produced by the strain R1-2 was classified using ESI-MS and FT-IR. ESI-MS negative and positive-ion modes (Figure 3A and 3B) and the results showed that a cluster of peaks with a 14 Da difference was present and the main signal was at m/z 1034.67and 1060 indicating that the biosurfactant produced by *Bacillus Subtilis* R1-2 would most probably be the lipopeptide surfactin isomer. The m/z values 978.61, 992.63, 1006.64, 1020.66, 1034.67 and 1035.68 are identical to those of C_{12}-surfactin $[M\text{-}H]^-$, C_{13}-surfactin $[M\text{-}H]^-$, C_{14}-surfactin $[M\text{-}H]^-$ and C_{15}-surfactin $[M\text{-}H]^-$ molecular weights. It could be inferred the biosurfactant produced by the strain R1-2 was surfactin homologues because they had the same molecular weight as reported (Wang et al., 2010). Infrared analysis of the biosurfactant produced by the strain R1-2 inherited a pattern similar to that of surfactin, indicating the presence of lipopeptide component, as showed in Figure 4. Wavenumber 3300.16 cm^{-1}, corresponding to the stretching vibrations of the hydroxyl or amine groups, wavenumber 1638.74 cm^{-1} and 1714.55 cm^{-1} which were obtained due to the stretching mode of –CO–N bond, and C=O stretching mode respectively. Wavenumber 2923.51and 2869.24 suggested the symmetrical stretching (γC-H) of CH_2 and CH_3 groups of aliphatic chains. Therefore, both aliphatic chains (hydrophobic domain) and peptide moiety (hydrophilic domain) exist in the biosurfactant samples. These patterns and bands are well matched to the surfactin family of the lipopeptide biosurfactant (Al-Wahaib et al., 2014).

Stability under harsh conditions is crucial for the application of surfactants. In this work, the effect of different temperatures, pH and NaCl concentrations on the surface activity of the lipopeptide biosurfactant produced by the strain R1-2 were investigated. The lipopeptide biosurfactant exhibited a good stability in the temperature range of 20°C to 100°C (Figure 6A) and the pH range of 3 to 14 (Figure 6B). Only in the pH less than 2, the surface tension increased. This may be attributed to the potential protonation of specific functional groups (e.g., amino groups) within the surfactant molecule at extremely low pH, which can result in alterations to the molecule's hydrophilic and hydrophobic balance, the formation of molecular chain breaks or conformational changes, and changes in the solubility of the surfactant (Kim et al., 2019). Meanwhile, the results also showed that biosurfactant was stable at NaCl concentrations up to 140 g/L (Figure 6C).

Contact angle and oil washing efficiency are two crucial indicators for evaluating the performance of surfactants. In this work, the both properties of lipopeptide biosurfactant produced by the strain R1-2 were evaluated at different concentrations of biosurfactant (Figure 7A). The contact angle of the un-treatment by lipopeptide biosurfactant solution was performed as the control group and measured to be 86.17°-89.22°, which are similar to the reported data (Alghamdi et al., 2019). Following the treatment of the surface of the oleophilic core slices with different concentrations of lipopeptide biosurfactant solutions(0.2%, 0.4%, 0.5%, 0.8%, 1.0%)for 24 h, the contact angles were found to decrease from 86.2° to 66.3°, 60.1°, 57.8°, 56.5°, and 39.0°, respectively (Figure 7B). This suggests that lipopeptide biosurfactant produced by R1-2 can rapidly adsorb to lipophilic surfaces and alter its wettability, which in turn is reported to enhance oil recovery (Ghasemi et al., 2019). Similar results were reported where contact angle decreased from 71° to 35° with the treatment of 0.1% biosurfactant produced by *Bacillus Subtilis* (Hadia et al., 2019). For the oil washing efficiency of lipopeptide biosurfactant produced by R1-2 (Figure 8), it is 64.84% at a concentration of 0.8%. All these results clearly highlighted the potential applications of the lipopeptide biosurfactant produced by the strain R1-2 in environmental bioremediation and enhanced oil recovery.

5 Conclusion

In summary, a lipopeptide-producing strain, *Bacillus Subtilis* R1-2, was isolated from the production water of the Daqing oilfield. A combination of the ESI-MS and FT-IR analyses show that the biosurfactant produced by the strain R1-2 is a surfactin family of the lipopeptide containing four members, C_{12}-surfactin, C_{13}-surfactin, C_{14}-surfactin and C_{15}-surfactin, respectively. The lipopeptide biosurfactant exhibits excel-

lent surface activity and a low CMC of 21.71 mg/L, a good thermal stability in a temperature range of 20°C to 100°C and pH range of 3 to 14, a high salt tolerance of NaCl up to 140 g/L, a significant effect in changing of the contact angles of core slices from 86.2° to 39.0°, and resulting wettability alteration from strong oil-wet to strong water-wet. Meanwhile, the lipopeptide biosurfactant demonstrates a good oil washing ability with an efficiency of 64.84%, which suggests that the lipopeptide-producing strain R1-2 is great potential applications across a diverse range of bioremediation and enhanced oil recovery.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Author Contributionts

BZM conceptualized and designed the study. SZY, LZ, YFL, JFL and HZG directed the data analysis. GJL and LHW performed the research. GNQ performed the research and drafted the paper. BZM was involved in the revision of the manuscript. All authors approved the final manuscript.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (42173076), the Natural Science Foundation of Shanghai (21ZR1417400) and the Fundamental Research Funds for the Central Universities of China (JKJ01241714). The authors thank the Research Centre of Analysis and Test of East China University of Science and Technology and Daqing Huali Biotechnology Co. Ltd for the help on ESI-MS and FT-IR analyses.

Conflict of Interest

The authors have no conflicts of interest to declare.

References

Al-Dhabi, N.A., Esmail, G.E., Mohammed, A.K., Valan, G.M., Karuppiah, A.V., 2020. Characterization and fermentation optimization of novel thermo stable alkaline protease from Streptomyces sp. Al-Dhabi-82 from the Saudi Arabian environment for eco-friendly and industrial applications. Journal of King Saud University-Science, 32(1): 1258-1264. https://doi.org/10.1016/j.jksus.2019.11.011

Al-Wahaibi, Y., Joshi, S., Al-Bahry, S., Elshafie, A., Al-Bemani, A., Shibulal, B., 2014. Biosurfactant production by *Bacillus Subtilis* B30 and its application in enhancing oil recovery. Colloids and Surfaces B: Biointerfaces, 114: 324-333. https://doi.org/10.1016/j.colsurfb.2013.09.022

Alghamdi, A.O., Abu-Al-Saud, M.O., Al-Otaibi, M.B., Ayirala, S.C., Alyousef, A., 2019. Electro-kinetic induced wettability alteration in carbonates: tailored water chemistry and alkali effects. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 583: 123887. https://doi.org/10.1016/j.colsurfa.2019.123887

Arima, K., Kakinuma, A. and Tamura, G., 1968. Surfactin acrystalline peptidelipid surfactant produced by *Bacillus Subtilis*: Isolation, characterization, and its inhibition of fibrin clot formation. Biochemical and Biophysical Research Communications, 31(3): 488-496. https://doi.org/10.1016/0006-291x (68)90503-2

Balan, S.S., Kumar, C.G. and Jayalakshmi, S., 2017. Aneurinifactin, a new lipopeptide biosurfactant produced by a marine Aneurinibacillus aneurinilyticus SBP-11 isolated from Gulf of Mannar: Purification, characterization and its biological evaluation. Microbiological Research, 194(1): 1-9. https://doi.org/10.1016/j.micres.2016.10.005

Dos Santos, R.A., Rodríguez, D.M., Ferreira, I.N. da S., de Almeida, S.M., Takaki, G.M. de C., de Lima, M.A.B., 2022. Novel production of biodispersant by Serratia marcescens UCP 1549 in solid-state fermentation and application for oil spill bioremediation. Environmental Technology, 43: 2956-2967. https://doi.org/10.1080/ 09593330.2021.1910733

de Araújo, L.L., Sodr´e, L.G., Brasil, L.R., Domingos, D.F., de Oliveira, V.M., da Cruz, G.F., 2019. Microbial enhanced oil recovery using a biosurfactant produced by Bacillus safensis isolated from mangrove microbiota-Part I biosurfactant character ization and oil displacement test. Journal of Petroleum Science and Engineering, 180: 950–957. https://doi.org/10.1016/j. petrol.2019.06.031

Desai, J.D. and Banat, I.M., 1997. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews, 61: 47-64. https://doi.org/10.1128/ mmbr.61.1.47-64.1997

Du, Y., Wang, Y., Cui, T.Y., Ge, L.P., Yu, F., Zhao, M.Q., Xu, Q.H., Jin, M.J., Chen, B., Long, X.W., 2023. Efficient production of biosurfactant surfactin by anewly isolated *Bacillus Subtilis* (sp.) 50499 strainfrom oil-contaminated soil. Food and Bioproducts Processing, 142: 40-49. https://doi.org/10.1016/j.fbp.2023.09.002

Eldos, H.I., Zouari, N., Saeed, S., Ashfaq, M.Y.M., Al-Ghouti, M.A., 2024. Isolation, identification, and characterization of potential biosurfactant-producing bacteria from processing wastewater for the development of eco-friendly green technology. Bioresource Technology Reports, 25: 101763. https://doi.org/10.1016/j.biteb.2024.101763

Geetha, S.J., Banat, I.M. and Joshi, S.J., 2018. Biosurfactants: Production and potential applications in microbial enhanced oil recovery (MEOR). Biocatalysis and Agricultural Biotechnology, 14: 23-32. https://doi.org/10.1016/j.bcab.2018.01.010

Ghasemi, A., Moosavi-Nasab, M., Setoodeh, P., Mesbahi, G., Yousefi, G., 2019. Biosurfactant production by lactic acid bacterium Pediococcus dextrinicus SHU1593 grown on different carbon sources: strain screening followed by product characterization. Scientific Reports, 9 (1): 1-12. https://doi.org/10.1038/s41598-019-41589-0

Guillén-Navarro, K., López-Gutiérrez, T., García-Fajardo, V., Gómez-Cornelio, S., Zarza, E., De, L., Rosa-García, S., Chan-Bacab, M., 2023. Broad-spectrum antifungal, biosurfactants and bioemulsifier activity of *Bacillus Subtilis* subsp. Spizizenii-A potential biocontrol and bioremediation agent in agriculture. Plants, 12: 1374. https://doi.org/10.3390/plants12061374

Haddad, N.I.A., Wang, J. and Mu, B.Z., 2009. Identification of a Biosurfactant Producing Strain: *Bacillus Subtilis* HOB2. Protein & Peptide Letters, 16: 7-13. https://doi.org/10.2174/092986609787049358

Hadia, N.J., Ottenheima, C., Lia, S.D., Hua, N.Q., Stubbs, L.P., Lau, H.C., 2019. Experimental investigation of biosurfactant mixtures of surfactin produced by *Bacillus Subtilis* for EOR application. Fuel, 251: 789-799. https://doi.org/10.1016/j.fuel.2019.03.111

Handa, S., Aggarwal, Y., Puri, S., Chatterjee, M., 2022. Pharmaceutical prospects of biosurfactants produced from fungal species. Journal of Basic Microbiology, 62: 1307-1318. https://doi.org/10.1002/jobm.202200327

Hou, B.F., Wang, Y.F., Cao, X.L., Zhang, J. Song, X.W., Ding, M.C., Chen, W.H., 2016. Surfactant-induced wettability alteration of oil-wet sandstone surface: Mechanisms and its effect on oil recovery. Journal of

Surfactants and Detergents, 19: 315-324.
https://doi.org/10.1007/s11743-015-1770-y

Javaheri, M., Jenneman, G.E., McInerney, M.J., Knapp, R.M., 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. Applied and Environmental Microbiology, 50(3): 698-700.
https://doi.org/10.1128/aem.50.3.698-700.1985

Ja'afaru, M.I., Abbas, T., Ajunwa, O.M., Olaifa, K., 2022. Characterization and statistical optimization of biosurfactant production using *Bacillus Subtilis* isolated from automotive oil-contaminated soil in Yola, Nigeria. Scientific African, 17: e01357.
https://doi.org/10.1016/j.sciaf.2022.e01357

Kubicki, S., Bollinger, A., Katzke, N., Jaeger, K.E., Loeschcke, A., Thies, S., 2019. Marine biosurfactants: biosynthesis, structural diversity and biotechnological applications. Marine Drugs, 17: 408.
https://doi.org/10.3390/md17070408

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35: 1547-1549.
https://doi.org/10.1093/molbev/msy096

Kuyukina, M.S., Ivshina, I.B., Makarov, S.O., Litvinenko, L.V., Cunningham, C.J., Philp, J.C., 2005. Effect of biosurfactants on crude oil desorption and mobilization in a soil system. Environment International, 31: 155-161.
https://doi.org/10.1016/j.envint.2004.09.009

Kumar, A., Singh, S.K., Kant, C., Verma, H., Kumar, D., Singh, P.P., 2021. Microbial biosurfactant: a new frontier for sustainable agriculture and pharmaceutical industries. Antioxidants, 10(9): 1472.
https://doi.org/10.3390/antiox10091472

Kim, C.H., Lee, D.W., Heo, Y.M., Lee, H., Yoo, Y., Kim, G.-H., Kim, J.-J., 2019. Desorption and solubilization of anthracene by a rhamnolipid biosurfactant from *Rhodococcus* fascians. Water Environment Research, 91: 739-747.
https://doi.org/10.1002/wer.1103

Li, H.N., 2020. Research on recovery enhancement technology of thick oil emulsifying functional bacteria, master's thesis, China University of Petroleum, 2020, China Digital Thesis database.

Li, Z.Z., Lin, J.Z., Wang, W.D., Huang, H., Yu, D.H., Li, S., 2022. Effect of Rhamnolipid Amidation on Biosurfactant Adsorption Loss and Oil-Washing Efficiency. Langmuir, 38,: 2435-2444.
https://doi.org/10.1021/acs.langmuir.1c02551

Liu, X.Y., Yang, S.Z., and Mu., B.Z., 2009. Production and characterization of a C15-surfactin-O-methyl ester by a lipopeptide producing strain *Bacillus Subtilis* HSO121. Process Biochemistry, 44: 1144-1151.
https://doi.org/10.1016/j.procbio.2009.06.014

Ogru, K.I. and Olannye, P.G., 2021. Microbial studies of biosurfactant producing bacteria from crude oil contaminated soil. Journal of Applied Sciences and Environmental Management, 25: 1729-1735.
https://doi.org/10.4314/jasem.v25i9.27

Purwasena, I.A., Astuti, D.I., Syukron, M., Amaniyah, M., Sugai, Y., 2019. Stability test of biosurfactant produced by *Bacillus licheniformis* DS1 using experimental design and its application for MEOR. Journal of Petroleum Science and Engineering, 183: 106382.
https://doi.org/ 10.1016/j.petrol.2019.106383

Qin, W.Q., Fei, D., Zhou, L., Guo, Y.J., An, S., Gong, O.H., Wu, Y.Y., Liu, J.F., Yang, S.Z., Mu, B.Z., 2023. A new surfactin-C17 produced by *Bacillus Subtilis* TD7 with a low critical micelle concentration and high biological activity. New Journal of Chemistry, 47: 7604-7612.
https://doi.org/ 10.1039/d3nj00123g

Raouf, S., Ismail, Y., Gamaleldin, N., Aboelkhair, H., Attia, A., 2024. Screening of sustainable biosurfactant produced by indigenous bacteria isolated from Egyptian oil fields for microbial enhanced oil recovery. Geoenergy Science and Engineering, 239: 212974.
https://doi.org/10.1016/j.geoen.2024.212974

Rawat, G., Kumar, V., Kumar, A., Naik, B., Saris, P.E.J., Khan, J.M., Kumar, V., 2024. Exploring the potential of novel Bacillus sp. G6: Isolation, characterization, and optimization of biosurfactant production from oil-contaminated soil. Journal of Molecular Liquids, 396: 124013.
https://doi.org/10.1016/j.molliq.2024.124013

Rincón-Fontán, M., Rodríguez-López L., Vecino, X., Cruz, J.M., Moldes, A.B., 2018. Design and characterization of greener sunscreen formulations based on mica powder and a biosurfactant extract. Powder Technolog, 327: 442-448.
https://doi.org/ 10.1016/j.powtec.2017.12.093

Sankhyan, S., Kumar, P., Pandit, S., Kumar, S., Ranjan, N., Ray, S., 2024. Biological machinery for the production of biosurfactant and their potential applications. Microbiological Research, 285,127765.
https://doi.org/10.1016/j.micres.2024.127765

Sarkar, P., Roy, A., Pal, S., Mohapatra, B., Kazy, S.K., Maiti, M.K., Sar, P., 2017. Enrichment and characterization of hydrocarbon-degrading bacteria from petroleum refinery waste as potent bioaugmentation agent for in situ bioremediation. Bioresource Technology, 242: 15-27.
https://doi.org/10.1016/j.biortech.2017.05.010

Silva, T.P., Paixão, S.M., Tavares, J., Gil, C.V., Torres, C.A., Freitas, F., Alves, L., 2022. A New biosurfactant/bioemulsifier from Gordonia alkanivorans strain 1B: Production and characterization. Processes, 10: 845.
https://doi.org/ 10.3390/pr10050845

Singh, R.D., Kapila, S., Ganesan, N.G., Rangarajan, V., 2022. A review on green nanoemulsions for cosmetic applications with special emphasis on microbial surfactants as impending emulsifying agents. Journal of Surfactants and Detergents, 25: 303–319.
https://doi.org/10.1002/jsde.12571

Thraeib, J.Z., Altemimi, A.B., Al-Manhel, A.J.A., Abedelmaksoud, T.G., El-Maksoud, A.A.A., Madankar, C.S., Cacciola, F., 2022. Production and characterization of a bioemulsifier derived from microorganisms with potential application in the food industry. Life, 12: 924.
https://doi.org/10.3390/life12060924

Vecino, X., Cruz, J.M., Moldes, A.B., Rodrigues, L.R., 2017. Biosurfactants in cosmetic formulations: trends and challenges. Critical Reviews in Biotechnology, 37: 911-923.
https://doi. org/10.1080/07388551.2016.1269053

Veshareh, M.J., Azad, E.G., Deihimi, T., Niazi, A., Ayatollahi, S., 2019. Isolation and screening of *Bacillus Subtilis* MJ01 for MEOR application: biosurfactant characterization, production optimization and wetting effect on carbonate surfaces. Journal of Petroleum Exploration and Production Technology, 9(1): 233-245.
https://doi.org/10.1007/s13202-018-0457-0

Wang, J., Haddad, N.I.A., Yang, S.Z., Mu, B.Z., 2010. Structural Characterization of Lipopeptides from Brevibacillus brevis HOB1. Applied Biochemistry and Biotechnology, 160: 812-821.
https://doi.org/10.1007/s12010-009-8536-9

Wicke, C., Hüners, M., Wray, V., Nimtz, M., Bilitewski, U., Lang, S., 2000. Production and structure elucidation of glycoglycerolipids from a marine sponge-associated Microbacterium species. Journal of Natural Products, 63: 621-626.
https://doi.org/10.1021/ np990313b

Wu, Y., Xu, M., Xue, J.L., Shi, K., Gu, M., 2019. Characterization and Enhanced Degradation Potentials of Biosurfactant-Producing Bacteria Isolated from a Marine Environment. ACS Omega, 4: 1645-1651.
https://doi.org/ 10.1021/acsomega.8b02653

Yaraguppi, D.A., Bagewadi, Z.K., Muddapur, U.M., Mulla, S.I., 2020. Response surface methodology-based optimization of biosurfactnt production from isolated Bacillus aryabhattai strain ZDY2, Journal of Petroleum Exploration and Production Technology, 10: 2483-2498.
https://doi.org/10.1007/s13202-020-00866-9

Zhao, Y., Yang, S.Z. and Mu, B.Z., 2012. Quantitative analyses of the isoforms of surfactin produced by *Bacillus Subtilis* HSO 121 using GC-MS. Analytical Sciences, 28: 789-793.
https://doi.org/10.2116/analsci.28.789