Production and characterization of surfactin-like biosurfactant produced by novel strain *Bacillus nealsonii* S2MT and it's potential for oil contaminated soil remediation

**CURRENT STATUS:** UNDER REVIEW

Microbial Cell Factories  
BMC

Irfan Ali Phulpoto  
University of the Chinese Academy of Sciences

Zhisheng Yu  
yuzs@ucas.ac.cn  
University of the Chinese Academy of Sciences

*Corresponding Author*  
**ORCiD:** 0000-0001-6080-8544

Hu Bowen  
University of the Chinese Academy of Sciences

Fabrice Ndayisenga  
University of the Chinese Academy of Sciences

Li Jinmei  
University of the Chinese Academy of Sciences

Hongxia Liang  
University of the Chinese Academy of Sciences

Muneer Ahmed Qazi  
Shah Abdul Latif University

**DOI:**  
10.21203/rs.2.22091/v1

**SUBJECT AREAS**  
Applied & Industrial Microbiology

**KEYWORDS**  
*Bacillus nealsonii*, Biosurfactant, Surfactin, Response surface methodology, Bioremediation
Abstract

Background Biosurfactants, being highly biodegradable, ecofriendly and multifunctional compounds have wide applications in various industrial sectors including environmental bioremediation. Surfactin a member of lipopeptide family which considered as one of the most powerful biosurfactant due to its environmental applications, emulsification activities as well as therapeutic properties. Therefore, the aim of this study to investigate the surfactin like biosurfactants produced by newly strain S2MT and their potential applications for soil remediation. Results In this study, a novel biosurfactant producing strain Bacillus nealsonii S2MT was investigated from the lake sediment that reduced the surface tension 34.15± 0.6 mN/m -1 with excellent emulsifying potential 55± 0.3% in kerosene oil. Additionally, the highest biosurfactant product 1300 mg/L -1 was achieved during which the composition of the culture medium was optimized through a response surface methodology (RSM). Results showed that 2% glycerol and 0.1% NH 4 NO 3 were the best carbon/nitrogen substrates for biosurfactant production. The most significant parameters such as temperature (30 °C), pH (8), agitation (100 rpm), NH 4 NO 3 (0.1%), yeast extract (0%) and NaCl (0.5%) contributed to the surface tension reduction and therefore enhancing the biosurfactant yield. Moreover, the obtained product was found to be highly stable at environmental factors such as salinity, pH and temperature variations. In addition, the biosurfactant product was chemically characterized as cyclic lipopeptide relating to surfactin-like isoforms (C 13 -C 15 ) with a thin-layer chromatography (TLC) and liquid chromatography and mass spectroscopy (LC/MS) respectively. The corresponding biosurfactant displayed 43.6± 0.08 and 46.7± 0.01% remediation of heavy engine-oil contaminated soil at 10 and 40 mg/L concentrations respectively. Conclusion This study, therefore, confirmed that the strain S2MT was not only a potential
biosurfactant producer but also an efficient and environmentally acceptable candidate for soil remediation compared to synthetic compounds.

1 Background

The development of sustainable technology has driven the search for natural and biodegradable compounds to remediate sites contaminated with hydrocarbons. This has led to the discovery of surfactants of natural sources. The compounds have surface-active properties and one produced by microbes is termed as biosurfactant [1]. These biomolecules are produced by various microorganisms like bacteria, fungi, and yeast [2, 3]. The biomolecules consist of hydrophobic as well as hydrophilic moieties [4]. Hydrophobic ‘tail’ is a hydrocarbon chain contains saturated/unsaturated and hydroxylated fatty alcohols or, fatty acids and the hydrophilic ‘head’ is a polar group, contains of mono, oligo- or polysaccharides and peptides [5].

Moreover, biosurfactants are also valued better than synthetic counterparts for their low-toxicity, higher biodegradability, environment-friendly nature, increased surface activity, low critical micelles concentration (CMC), active at very low concentrations and stability at extreme conditions like pH, salinity and high or low temperature [6-8]. Due to such unique functional properties and eco-friendly nature, these biological surfactants are expected to become multi-functional constituents of the 21st century. Additionally, they have more applications in industry sectors such pharmaceutical, textile processing, agricultural, cosmetics, personal care, food industries, and environmental applications like soil remediation, hydrocarbon degradation and oil recovery [4, 9-11]. Biosurfactant can be produced by numerous bacterial species. Among them, Bacillus species are well known for lipopeptide types of biosurfactants [12]. Lipopeptide are one of the most powerful biosurfactants due to their therapeutic properties such as broad-spectrum antimicrobial activity, antiviral and antitumor activity [12, 13].
In general, surfactin, iturin, lichenysin, and fengycin are the most important members of the lipopeptide family [13, 14]. Amongst, surfactin is considered the most effective member because of its capability of surface tension reduction of water from 72 mN/m to 27mN/m, efficient emulsification activity, and potential environmental applications such as soil remediation [12]. Despite the countless benefits of lipopeptide biosurfactant over synthetic agents, it is still hindered, and not yet employed on a large scale, mainly due to the high cost of raw material, low product and extensive purification processes [7, 12, 15]. A number of efforts have been made recently to overcome the above factors by using cheap or cost-effective material, media optimization, and selection of potential microbes for biosurfactant production [7, 12, 15]. Although, these efforts still remained unsuccessful to generate commercially feasible and profitable lipopeptide production, and will be unable to do so unless the yield of the final product is significantly high from naturally producer microbes [16]. However, very limited reports have been highlighted related to biosurfactant producing microbes from the sediment of lakes. Literature suggested that, in an aquatic ecosystem, the lakes provide diverse ecological habitats, [17] and are considered as most important reservoirs for industrial as well as biotechnological molecules for example antibiotics, extracellular compounds i.e. enzymes and exopolysaccharides and their use in environmental applications like bioremediation [17, 18].

In this work, an efficient biosurfactant producing strain Bacillus nealsonii S2MT was investigated from the sediment of Lake. Initially, the strain was characterized by morpho-microscopic and molecular typing, and then validated for biosurfactant production and medium optimization using design experiment (Design Expert® (Version 12)) RSM. Furthermore, the obtained biosurfactant product of Bacillus nealsonii S2MT was chemically characterized by TLC and LC/MS techniques respectively. The product stability to different
environmental factors such as pH, temperature and salinity as well as the potential of crude biosurfactants for oil contaminated soil remediation was also evaluated.

2 Materials And Methods

2.1 Chemicals, reagents, and solvents

For the present study, chemicals, reagents, and solvents were used of analytical grade. Kerosene, Penzz oil (Pennzoil Products Company, Houston, TX) was purchased from the local auto workshop market in Beijing, China.

2.2 Isolation, identification, and maintaining of isolate S2MT

The isolate S2MT was isolated from the sediment of Yanqi Lake, Huairou district Beijing, China. Strain S2MT was sent for molecular identification using 16S rDNA gene sequencing to the company (Majorbio Sanger Bio-pharmTechnology Co., Ltd, Beijing, China). Obtained nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis in the Gen-Bank sequence database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Phylogenetic analysis was conducted using MEGA X [19]. The obtained 16S rDNA gene sequences of the present study were submitted to NCBI Gen-Bank for getting accession number. While identified bacterial strain S2MT has routinely maintained on Luria Bertani (LB) agar before each screening experiment. The pure culture stock was maintained on LB agar slants and kept at 4 °C for routine use. For long term storage, the pure bacterial culture was maintained in glycerol broth (≈ 18% v/v) and kept at -20 °C.

2.3 Media preparation and cultivation for biosurfactant production

For the inoculum preparation, a loop full pure bacterial culture was inoculated into a 50 ml LB broth followed by incubation period 14–18 hours in shaking environment 140 rpm at 28 °C. Afterwards, 2% inoculum (OD at 600 nm ~ 1.00) was inoculated into a 100 ml
sterilized minimal salt medium (MSM) broth in 250 ml conical flask contained, Na₂HPO₄ 2.2 g/L, KH₂PO₄ 1.4 g/L, MgSO₄·7H₂O 0.6 g/L, FeSO₄·7H₂O 0.01 g/L, NaCl 0.3 g/L, CaCl₂ 0.02 g/L. And 0.1% trace elements solution containing ZnSO₄·7H₂O 2.32 g/L, MnSO₄·4H₂O 1.78 g/L, H₃BO₃ 0.56 g/L, CuSO₄·5H₂O 1.0 g/L, NH₄MoO₄·2H₂O 0.39 g/L and KI 0.66 g/L according to the [20]. Glycerol 2% (v/v) and NH₄NO₃ 0.1% were used as carbon/nitrogen substrates for growth and biosurfactant production. Medium pH was adjusted 7.02 ± 02 using 1 M HCl and NaOH. After that, the culture broth was incubated into a shaking environment 140 rpm at 28 °C for 5 days. Every day the bacterial density (OD at 600 nm) was determined using UV-Spectrophotometer (Unico Instrument Co., Ltd., Shanghai, China). During the late stationary phase, the culture broths were taken out and centrifuged at 10,000 rpm for 15 minutes at 4 °C to collect the cell-free supernatants for the screening of biosurfactant production using different functional screening methods.

2.4 Determination of biosurfactant production

2.4.1 Oil displacement activity (ODA)

The ODA test was performed according to the method of [21] with minor modifications. 100 µl of used engine oil was placed on the surface of the Petri plate containing 40 ml of distilled water (Milli-Q). After that, 10 µl of cell-free broth was dropped on oil-coated thin film, and the zone of oil displaced was determined.

2.4.2 Drop collapse technique (DCT)

Pennzoil (Pennzoil Products Company, Houston, TX), 2 µl was placed on the lid of 96 well plates. The plate was left for equilibration for 1-2 hours. After that, the 5 µl cell-free supernatants were applied to check the potential of biosurfactant. After one minute, the positive result indicates that the drop of supernatants became collapsed [22].
2.4.3 Emulsification index (E24\%) 

This is another screening method for biosurfactant-producing microorganisms. The cell-free supernatants (2 ml) with an equal amount of kerosene were mixed on the vertex mixture for 2 minutes and allowed for 24 hours undisturbed at room temperature. The results were observed as described [21, 23].

\[ E24 = \frac{\text{height of emulsified layer}}{\text{total height of liquid}} \times 100 \] (1)

2.4.4 Tensio-activity measurement

The tensio-activity of the cell-free culture containing biosurfactants was determined with the digital surface tensio-meter (JYW-200B., China) using Du Nouy ring method [24]. Distilled water was used as a standard in this method. All the evaluations were taken in triplicates.

2.5 Medium optimization on various carbon and nitrogen substrates

Biosurfactant producing strain S2MT was subjected to different substrates as sole carbon sources such as glucose, glycerol (water-soluble) and kerosene (water-insoluble) with the combination of NH\(_4\)NO\(_3\), urea and yeast extract as nitrogen sources for the medium optimization. The medium (MSM) was prepared with 2 and 0.1% carbon and nitrogen sources respectively, and pH was maintained 7.0 followed by sterilization process at 121 °C for 15 minutes. The medium was subjected with 2% prepared inoculum (OD = 1.0 at 600 nm), the culture broths were then incubated in a shaking environment (140 rpm at 28 °C) for 5 days.

2.6 Experiment design for the statistical screening of critical factors for biosurfactant production

After screening the best carbon and nitrogen substrates, the most significant process parameters contributing towards biosurfactants production by the test bacterial strain
S2MT was determined. The screening experiments were designed using a statistical approach, a regular 2-level factorial model, for analyzing the main effects of multiple variables in an experiment. The experimental design was comprised of six most critical independent variables (factors) viz. temperature, pH, agitation, NH₄NO₃ con., NaCl conc., and yeast extract and two dependent variables (responses), i.e. surface tension measurement (mN/m) and production yield as shown in (Table 1).

Table 1
Design Summary used in the regular 2-level factorial model of experiments for biosurfactant production

| Factor | Name             | Units | Type   | Level          |
|--------|------------------|-------|--------|----------------|
| A      | Temperature      | °C    | Numeric| 25             |
| B      | pH               | ---   | Numeric| 6              |
| C      | Agitation        | RPM   | Numeric| 100            |
| D      | NH₄NO₃ conc.     | %     | Numeric| 0.1            |
| E      | Yeast Extract    | %     | Numeric| 0              |
| F      | NaCl conc.       | %     | Numeric| 0.1            |

2.7 Recovery of biosurfactant

For extraction of crude biosurfactant, the extracts were obtained by acid precipitation [25]. Cell-free supernatants were obtained from MSM culture broths (as mentioned above) by centrifuging at 10,000 rpm for 15 minutes at 4 °C. Supernatants were then acidified by adjusting the pH 2.0 with concentrated hydrochloric acid (HCl) followed by overnight refrigeration at 4 °C. Precipitated cell-free supernatants were again centrifuged at 10,000 rpm for 20 minutes to collect the crude biosurfactant and maintained the pH 7.0. Crude biosurfactants were then extracted with methanol followed by rotary evaporation at 40 °C.

2.8 Product stability to various environmental factors

Biosurfactants stability was monitored at different ranges of temperature, salt concentration, and pH [26]. Thermal stability of crude biosurfactant (40 mg/L dissolved in distilled water) was determined at varying temperatures such as (4- 121 °C), each for 1 hour, followed by cooling at room temperature. To examine the effect of NaCl on the
stability of biosurfactant, at a concentration of (1-9%, w/v). Similarly, the effect of pH was
determined at different ranges (3-10) by using 1 M HCl and NaOH to adjust the pH. All the
stabilities were determined by surface tension measurements and performed in triplicates.

2.9 Critical micelles concentration (CMC) determination
To examine the CMC of obtained crude biosurfactant, different concentrations (0-
100 mg/L) of biosurfactants solution were prepared, and the surface-tension was
measured subsequently [21].

2.10 Characterization of obtained biosurfactant product

2.10.1 Product analysis by thin-layer chromatography (TLC)
Extracted crude biosurfactants from bacterial strain were analyzed by TLC, on silica gel 60
plates (Merck CO., Inc., Darmstadt, Germany). Chloroform, methanol and water (65:15:2
(v/v/v)) solvent system were used. Various color developing reagent used to visualize the
type of biosurfactants such as ninhydrin 0.2% in ethanol for lipopeptide with red-pinkish
spots, 1-5% H$_2$SO$_4$ followed by heating at 110 °C for 20 minutes for glycolipids with brown
spots and iodine vapors for lipids [7].

2.10.2 Product analysis by liquid chromatography- mass spectroscopy (LC- MS)
Biosurfactant product was dissolved in methanol 1 mg/ml and filtered (0.22 µm). The
electrospray ionization (ESI) mass spectra of the product were analyzed on an LC- MS
system with high-performance liquid chromatography (Ultimate 3000, Dionex) coupled
with TSQ Endura™ Triple Quadrupole Mass Spectrometer (Thermo Scientific, USA). The
separation was performed on a Hypersil Gold C$_{18}$, 1.9 µm, 100 x 2.1 mm column. The
mobile phase was water, 1% formic acid and acetonitrile. The linear gradient system: 0-
3.5 min, 60-93% acetonitrile; 3-20 min, keeping 93% acetonitrile and 7% water (1%
formic acid); injection volume 5 µl; flow rate 0.300 ml/min. The LC- MS was performed on
positive modes with full scans ranging from m/z 200 to 2000.

2.11 Potential of obtained biosurfactant in heavy engine oil polluted soil remediation
The efficiency of crude biosurfactants was examined for heavy engine-oil polluted soil remediation. Twenty grams of de-moisturized soil was contaminated with 10% oil in a conical flask. The concentration of 10 and 40 mg/L of crude biosurfactant, and 10 and 40 mg/L of sodium dodecyl sulfate (SDS) as chemical surfactants and distilled water was used as control. All the solutions were 60 ml individually. The flasks were incubated in a shaking environment (130 rpm for 24 hours at 28 °C). After that, the samples were centrifuged at 5000 rpm for 15 minutes and supernatants were extracted with n-hexane followed by solvent evaporation, and residual oil was measured gravimetrically [12].

2.12 Statistical analysis of experiments
All the experiments related to biosurfactants functional screening i.e. emulsification, surface tension, and biosurfactant production were performed in triplicates. The mean ± and standard deviation (SD) were evaluated. The statistical software package, Design Expert® (Version 12), State-Ease, Minneapolis, MN, USA, was used to generate design, for the screening of various influential factors on biosurfactant production.

3 Results And Discussion

3.1 Strain identification and biosurfactant production
The morphological and transmitted electron microscopic (TEM) characteristics of strain revealed the small, irregular, rough and white colonies that belong to the class Firmicutes and genus Bacillus (Additional file 1: Fig. S1). This S2MT strain was identified as Bacillus nealsonii, showing 99.93% similarity by 16S rRNA ribotyping, as illustrated in the phylogenetic tree (Additional file 1: Fig. S2). Nucleotide sequence of 16S rRNA of B. nealsonii S2MT strain was submitted in the NCBI Gen-Bank database with accession
For biosurfactant production, the strain S2MT exhibited positive activities in initial biosurfactant screening methods. The efficient activity of biosurfactant product in oil displacement test, $4.2 \pm 0.4$ cm clear zone, and drop collapse method (Additional file 1: Fig. S1). Moreover, the strain S2MT reduced the surface tension up to $34.15 \pm 0.6 \text{ mN/m}^1$, which is lower than the distilled water $81.3 \text{ mN/m}^1$ (Fig. 1A), and expressed the emulsification activity $55 \pm 0.3\%$ in kerosene oil after 24 hours. According to Cooper and Goldenberg [23] if bacterial isolate can reduce the surface tension ($40 \text{ mN/m}^1$) or less it could be a promising biosurfactant producer. The above results indicate that the strain Bacillus nealsonii S2MT is a potential biosurfactant producing strain. Numerous Bacillus sp. and related genera have been reported from different types of environments i.e. extreme, hydrocarbon-contaminated terrestrial or marine environment [7] for biosurfactants production by B.licheniformis, Aeribacillus sp. Bacillus subtilis [13, 27]. However, no previous report highlighted related to biosurfactant production by Bacillus nealsonii.

3.2 Medium optimization on different carbon/nitrogen substrates and product recovery

A number of different C/N sources (as mentioned above in methods) were provided to induce the biosurfactant product yield and the glycerol/NH$_4$NO$_3$, the best one, contributed to produce the highest biosurfactant quantity, showing the $34.15 \pm 0.6 \text{ mN/m}^1$ surface tension reduction and emulsification $55 \pm 0.3\%$ (Fig. 1A). Whereas, the total dry biomass and crude biosurfactant products were obtained $4140 \pm 70.71 \text{ mg/L}^{-1}$ and $1255 \pm 21.21 \text{ mg/L}^{-1}$ respectively (Fig. 1D). The glycerol/Urea preceded the yeast extract as
second important C/N sources (Fig. 1B, C), on which efficient surface tension reduction 36.7 ± 0.6 mN/m−1 and 46.6 ± 2% emulsification activity was noticed together with total dry biomass 1505 ± 205.0 mg/L−1 and crude biosurfactant 130 ± 13.43 mg/L−1 (Fig. 1D). However, other combinations of C/N sources such as glucose with yeast extract, urea, and even NH₄NO₃ did not alter biosurfactant production to significant level except increased in cellular biomass, while kerosene acted as an inhibitor due to its toxicity, as depicted in (Additional file 1: Figs. S3A- I, except D, F). All this together suggested that the 2% glycerol with 0.1% NH₄NO₃ was the most effective C/N source for biosurfactant production by strain B. nealsonii S2MT in comparison to glucose and kerosene substrates.

Previous findings also demonstrated that the NH₄NO₃ with various carbon substrates have been found an efficient nitrogen source for biosurfactant production by Bacillus species. Medeot et al. (2017) achieved the maximum biosurfactant concentration (1.7 mg/mL) by Bacillus amyloliquefaciens MEP218 upon using the glucose and NH₄NO₃ [28]. Likewise, Fernandes et al. (2016) reported NH₄NO₃ combined with sucrose to get the highest concentration of biosurfactant by Bacillus subtilis RI4914 (0.2 g/L) [29]. Also, Abdel-Mawgound et al. (2008) studied surfactin production by Bacillus subtilis BS5 using different carbon and nitrogen sources and concluded the highest biosurfactant production in NaNO₃ and NH₄NO₃ [30]. Evidently, nitrogen source play a crucial role in biosurfactant production [15], but it depends upon the carbon/nitrogen substrates combination.

3.3 Statistical screening of critical factors for biosurfactant production

The results of surface tension (SFT) reduction and product yield, showing the effects of six factors by combining them in different proportions as design by the model are mentioned in (Table 2). Minimum SFT values 33.7, 34.2, 34.3, and 34.5 mN/m−1 and product yield of
1300, 1110, 900, and 850 mg/L\(^{-1}\) were observed in Run number 10, 7, 13 and 19 respectively. Contrarily, the increased value of SFT was found 64, 49.5, 46.8 and 44.5 in Run no. 14, 20, 11 and 17 respectively. Whereas the product recovery was only 10, 15, 60, and 60 mg/L\(^{-1}\) by the same Run numbers. It was thus found that surface tension reduction has directly proportional to the product yield.

Table 2

Design layout of a regular 2-level factorial model showing different influential factors and their effect on Surface tension and biosurfactants product yield

| Run | Factor1 A: Temp (°C) | Factor2B: pH | Factor3 C: Agitation | Factor4 D: NH4NO3 (%) | Factor5 E: Yeast extract (%) | Factor6 F: NaCl (%) | Response1: SFT (mN/m) | Response2: Yield (mg/L) |
|-----|----------------------|-------------|----------------------|----------------------|-----------------------------|---------------------|----------------------|----------------------|
| 1   | 25                   | 8           | 180                  | 1                    | 0                           | 0.5                 | 37.7                 | 80                   |
| 2   | 27.5                 | 7           | 180                  | 0.55                 | 0.1                         | 0.3                 | 37.4                 | 400                  |
| 3   | 25                   | 6           | 140                  | 0.2                  | 0.2                         | 0.5                 | 34.9                 | 380                  |
| 4   | 27.5                 | 7           | 100                  | 0.55                 | 0.1                         | 0.3                 | 37.6                 | 390                  |
| 5   | 30                   | 6           | 180                  | 0.1                  | 0                           | 0.5                 | 34.9                 | 380                  |
| 6   | 25                   | 8           | 180                  | 1                    | 0.2                         | 0.1                 | 36.5                 | 60                   |
| 7   | 25                   | 8           | 100                  | 0.1                  | 0.2                         | 0.5                 | 34.2                 | 1110                 |
| 8   | 27.5                 | 7           | 140                  | 0.55                 | 0.1                         | 0.3                 | 36.8                 | 440                  |
| 9   | 30                   | 8           | 180                  | 1                    | 0.2                         | 0.5                 | 37.6                 | 60                   |
| 10  | 30                   | 8           | 180                  | 0.1                  | 0                           | 0.5                 | 33.7                 | 1300                 |
| 11  | 25                   | 6           | 100                  | 1                    | 0                           | 0.5                 | 46.8                 | 60                   |
| 12  | 30                   | 6           | 100                  | 1                    | 0.2                         | 0.5                 | 37.6                 | 30                   |
| 13  | 30                   | 8           | 180                  | 0.1                  | 0.2                         | 0.1                 | 34.3                 | 900                  |
| 14  | 25                   | 6           | 100                  | 0.1                  | 0                           | 0.1                 | 64.6                 | 10                   |
| 15  | 27.5                 | 7           | 140                  | 0.55                 | 0.1                         | 0.3                 | 37.1                 | 380                  |
| 16  | 30                   | 6           | 100                  | 0.1                  | 0.2                         | 0.1                 | 35.4                 | 50                   |
| 17  | 30                   | 8           | 100                  | 1                    | 0                           | 0.1                 | 38.2                 | 60                   |
| 18  | 25                   | 6           | 180                  | 1                    | 0.2                         | 0.1                 | 38.1                 | 61.6                 |
| 19  | 25                   | 8           | 180                  | 0.1                  | 0                           | 0.1                 | 34.5                 | 830                  |
| 20  | 30                   | 6           | 180                  | 1                    | 0                           | 0.1                 | 49.5                 | 15                   |

The analysis of variance and regression of both responses (R1 = SFT (mN/m\(^{-1}\)) and (R2 = yield (mg/L\(^{-1}\)) gave details of the most significant terms of the model as shown in (Additional file 1: Table S3). The ANOVA summary of the model indicated the high signification P-value (P < 0.05) to elucidate the effect of significate model terms that affected SFT and yield. The most significant factors were pH, temperature, yeast extract and NaCl conc., with P-values (P = 0.0028), (P = 0.0036), (P = 0.0042) and (P = 0.0319) respectively. Whereas, the most significant factors with P-values on yield were NH\(_4\)NO\(_3\) (P = 0.0005), temperature (P = 0.0039) and pH (P = 0.0044). The normal probability chart
significantly contributed, and the Pareto chart indicates the rank wise positive and negative effects of factors on biosurfactant production based on the model (Additional file 1: Figs. S4). However, the effects of individual factors of significant model terms are depicted (Additional file 1: Figs. S5).

Response surface plot and wireframes show the effect of combine factors i.e. pH vs temperature, \(\text{NH}_4\text{NO}_3\) vs. temperature, \(\text{NaCl}\) vs. temperature and \(\text{NaCl}\) vs. pH on surface tension reduction and biosurfactants yield (Figs. 2A- F). Figures 2A, D shows the surface tension value was found decreased and observed maximum biosurfactant yield, when the pH and temperature were increased. The increased pH (8) and high temperature (30 °C) may have a significant effect on biosurfactants, whereas a decreased of both factors may have an insignificant effect on biosurfactant production [31]. The \(\text{NH}_4\text{NO}_3\) (0.1%) and high temperature (30 °C), and increased pH (8) and \(\text{NaCl}\) con. (0.5%) also showed a positive effect on both responses R1 and R2 (Fig. 2B, C, F). Similarly, the significant effect was found at the high \(\text{NaCl}\) conc. (0.5%) and high temperature (30 °C) on biosurfactant production (Fig. 2E), whereas, low \(\text{NaCl}\) conc. and high temperature may also have a negative effect on biosurfactants [31]. The design experiment (v. 12), based on the regular two-level factorial model suggested the equation for increase biosurfactant production as

\[
\text{Yield} = 349.83 + 12.71 (A) + 213.37 (B) + 1.66 (C) - 283.34 (D) - 5.21 (E) + 88.34 (F) \tag{2}
\]

Where, the A, B, C, D, E, and F coded value of the temperature, pH, agitation, \(\text{NH}_4\text{NO}_3\), Yeast extract, and \(\text{NaCl}\) respectively. After optimization conditions, the biosurfactant production was increased 3.58% more, than initial production by strain S2MT.

3.4 Stability of biosurfactant production

The effect of environmental factors on crude biosurfactants is shown in (Fig. 3A) and
As a result of temperature variation, the surface tension was found to be a decreased value of 31.5 ± 0.1 mN/m at 100°C, however, when the temperature decreased as low as 4 °C, the surface tension was found to be increased of 44.2 ± 2.5 mN/m. The biosurfactant product by S2MT strain was found highly stable at high temperature. In pH ranges, the surface tension was found to be increased at pH 3 as 40.8 ± 0.4 mN/m, whereas, the value was decreased 36.7 ± 0.4 mN/m at 6 pH. The acidic condition was not in the favor of biosurfactants stability but could be caused by the precipitation of biosurfactants [12, 32]. In the case of NaCl concentration, slight differences were found in surface tension measurement; since the elevated salt concentrations gradually increased the surface tension values from 37.6 ± 0.6 mN/m to 39.65 ± 0.06 mN/m in 3 and 9% of NaCl concentrations respectively. The reason for the increase in surface tension might be the ionic salts form ion-dipole interactions with water, which is stronger than the gaseous phase and salt interactions, and causing the solute molecules to avoid the interface [12].

3.5 Critical micelles concentration (CMC) determination

The result of CMC is shown in (Fig. 3B). Surface-tension value of various biosurfactant solutions (0-100 mg/L) were recorded. The CMC value of an obtained product was found to be at 40 mg/L, where the surface-tension was measured 81.2 mN/m to 34.5 ± 0.56 mN/m, afterward, there were no much significant changes observed in surface-tension measurement. The present results of CMC were an agreement with Datta et al., (2018), who observed CMC (40 mg/L) by Bacillus subtilis MG495086 [21].

3.6 Product characterization

The crude biosurfactant was analyzed on the TLC plate by strain Bacillus nealsoneii (S2MT)
that indicated lipopeptide in nature. Different spots at Rf of 0.25, 0.35 and 0.75 were observed after sprayed of 0.2% ninhydrin as illustrated in (Additional file 1: Fig. S6). That might be most related to the surfactin family of the lipopeptide. Similar results were obtained by Ramyabharathi et al (2018) on Bacillus subtilis Bbv57 which produced surfactin and iturin that was confirmed on TLC with Rf value of 0.3 for surfactin and 0.7 for iturin family by comparing with standard (Sigma-Aldrich) [33]. Likewise, Yánez-Mendizábal et al. (2012) reported surfactin and iturin with Rf values 0.3 and 0.7 respectively [34]. Joy et al. (2017) also reported the pattern of TLC by Bacillus sp. (SB2) with an Rf value of 0.72 and 0.55 for lipopeptide biosurfactants [7]. Furthermore, the biosurfactant product was confirmed by LC-ESI/MS. The results of m/z peaks are summarized in (Additional file 1: Table S2). A total six different surfactin like isoforms from (C13-C15) of lipopeptide, at m/z 1008.76 and 1030.74 with retention time (Rt 12.48); 1022.78 and 1044.75 (Rt 14.26); and 1036.79 and 1058.78 with (Rt 15.70) were detected by LC/MS as shown in the chromatogram (Fig. 4). In this respect, the similar pattern was reported by Li et al. (2008) of different surfactin like homologs by B. licheniformis HSN221, when who cultivated in MSM medium with glucose, yeast extract, and ammonium nitrate [14]. Also Chen et al. (2017) reported surfactin homologs at m/z 994, 1008, 1022, 1036 with isoforms of C12, C13, C14, and C15 respectively, by B. licheniformis MB01 [13]. The above analyses revealed that the biosurfactant produced by Bacillus nealsongii S2MT strain is a lipopeptide in nature having the highest resemblance with the surfactin family. In general, surfactin like biosurfactants relating to the family of cyclic lipopeptide and mostly produced by Bacillus spp. [27].

3.7 Potential of crude biosurfactant in heavy engine oil polluted soil remediation
Petroleum hydrocarbon contaminants are major social and ecological issues. These contaminants bind to soil particles and are difficult to remove because of their strong sorption and hydrophobicity. Microbial surfactants in oil-polluted soil can emulsify these compounds and enhanced their solubility, decreased surface tension and increased oil displacement from soil particles [12, 25, 35, 36]. The potential of obtained crude biosurfactant by B. nealsonii S2MT in heavy oil-contaminated soil remediation resulted in $43.6 \pm 0.08$ and $46.7 \pm 0.01\%$ with the concentration 10 and 40 mg/L of crude biosurfactant respectively. However, with synthetic surfactants, sodium dodecyl sulfate (SDS) obtained $39.4 \pm 0.01$ and $45.3 \pm 0.14\%$ removal of contaminants with the same concentration of 10 and 40 mg/L respectively. Whereas $18.5 \pm 0.07\%$ removal of contaminants obtained with distilled water as shown in (Fig. 5).

In this respect, Souza et al. (2018) obtained 20% remediation of oil-contaminated sand by Wickerhamomyces anomalus CCMA 0358 [37], which was lower than the current study. Felix et al. (2019) used semi-purified biosurfactant by B. subtilis in diesel oil-contaminated soil remediation, and who obtained 78.5% and 81.8% removal with a concentration of 12.5 mg/L and 37.5 mg/L [12]. Another hand, 80% hydrocarbon removal from soil was achieved by using chemical surfactant SDS and Triton X-100 [38]. In contrast, the biosurfactants are natural compounds, eco-friendly, non-or-less toxic and biodegradable, and also more powerful than a synthetic one, and could be used as a crude product in bioremediation applications.

4 Conclusion

In the present investigation, a naturally potential biosurfactant producer bacterium Bacillus nealsonii S2MT was isolated from the sediment of the Lake (first report). The strain has effective surface tension reduction and emulsifying capabilities, and also well stability power to various environmental factors. Interestingly, the strain produced
1255 mg/L⁻¹ crude lipopeptide biosurfactant by utilizing cheap glycerol and NH₄NO₃ as carbon and nitrogen sources. The product was improved up to 1300 mg/L⁻¹ when the medium composition was optimized on various factors using RSM by design experiment statistical software. The CMC values of the obtained product were determined at 40 mg/L⁻¹. Additionally, the biosurfactant product was confirmed as surfactin a member of lipopeptide family through TLC and LC-MS analysis. Furthermore, the strain has effective heavy engine oil polluted soil remediation potential and could be used in many other environmental applications.

Declarations

Acknowledgments:
We thank financial support by a National Key Research and Development Program of China (2018YFD0800403). We would also like to highly acknowledge to Chinese Scholarship Council (CSC) for funding the scholar. We are also grateful to the Institute of Microbiology, University of Chinese Academy of Science (UCAS) for providing the TEM imaging facility.

Authors’ contributions
IAP and ZY conceived and designed the study. IAP, HB, LJ and HL carried out the majority of experiments. IAP, MAQ and HB analyzed the data and drafted the manuscript. IAP, FN, MAQ and ZY revised the manuscript. All authors read and approved the final manuscript.

Funding
This work was financially supported by a National Key Research and Development Program of China (2018YFD0800403).

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Aparna A, Srinikethan G, Hedge S: Effect of addition of biosurfactant produced by Pseudomonas ssp. on biodegradation of crude oil. *International Proceedings of Chemical, Biological & Environmental Engineering* 2011:71-75.

2. Sunde M, Pham CL, Kwan AH: Molecular characteristics and biological functions of surface-active and surfactant proteins. *Annual review of biochemistry* 2017, 86:585-608.

3. Gaur VK, Regar RK, Dhiman N, Gautam K, Srivastava JK, Patnaik S, Kamthan M, Manickam N: Biosynthesis and characterization of sophorolipid biosurfactant by Candida spp.: Application as food emulsifier and antibacterial agent. *Bioresource technology* 2019:121314.

4. Ghasemi A, Moosavi-Nasab M, Setoodeh P, Mesbahi G, Yousefi G: Biosurfactant Production by Lactic Acid Bacterium Pediococcus dextrinus SHU1593 Grown on Different Carbon Sources: Strain Screening Followed by Product Characterization. *Scientific reports* 2019, 9:5287.

5. Nayarisseri A, Singh P, Singh S: Screening, isolation and characterization of biosurfactant-producing Bacillus tequilensis strain ANSKLAB04 from brackish river water. *International Journal of Environmental Science and Technology* 2018:1-10.

6. Arora A, Cameotra SS, Kumar R, Balomajumder C, Singh AK, Santhakumari B, Kumar P, Laik S: Biosurfactant as a promoter of methane hydrate formation: thermodynamic and kinetic studies. *Scientific reports* 2016, 6:20893.

7. Joy S, Rahman PK, Sharma S: Biosurfactant production and concomitant hydrocarbon
degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments. *Chemical Engineering Journal* 2017, 317:232-241.

8. Sun S, Wang Y, Zang T, Wei J, Wu H, Wei C, Qiu G, Li F: A biosurfactant-producing *Pseudomonas aeruginosa* S5 isolated from coking wastewater and its application for bioremediation of polycyclic aromatic hydrocarbons. *Bioresource technology* 2019, 281:421-428.

9. Burch AY, Browne PJ, Dunlap CA, Price NP, Lindow SE: Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production. *Environmental microbiology* 2011, 13:2681-2691.

10. De Almeida DG, Soares Da Silva RdCF, Luna JM, Rufino RD, Santos VA, Banat IM, Sarubbo LA: Biosurfactants: promising molecules for petroleum biotechnology advances. *Frontiers in microbiology* 2016, 7:1718.

11. Das AJ, Kumar R: Utilization of agro-industrial waste for biosurfactant production under submerged fermentation and its application in oil recovery from sand matrix. *Bioresource technology* 2018, 260:233-240.

12. Felix AKN, Martins JJ, Almeida JGL, Giro MEA, Cavalcante KF, Melo VMM, Pessoa ODL, Rocha MVP, Gonçalves LRB, de Santiago Aguiar RS: Purification and characterization of a biosurfactant produced by *Bacillus subtilis* in cashew apple juice and its application in the remediation of oil-contaminated soil. *Colloids and Surfaces B: Biointerfaces* 2019, 175:256-263.

13. Chen Y, Liu SA, Mou H, Ma Y, Li M, Hu X: Characterization of Lipopeptide biosurfactants produced by *Bacillus licheniformis* MB01 from marine sediments. *Frontiers in microbiology* 2017, 8:871.

14. Li Y-M, Haddad NI, Yang S-Z, Mu B-Z: Variants of lipopeptides produced by *Bacillus licheniformis* HSN221 in different medium components evaluated by a rapid method
ESI-MS. *International Journal of Peptide Research and Therapeutics* 2008, 14:229-235.

15. Silva S, Farias C, Rufino R, Luna J, Sarubbo L: Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Colloids and Surfaces B: Biointerfaces* 2010, 79:174-183.

16. Zhi Y, Wu Q, Xu Y: Genome and transcriptome analysis of surfactin biosynthesis in *Bacillus amyloliquefaciens* MT45. *Scientific reports* 2017, 7:40976.

17. Joshi P, Pande V, Joshi P: Microbial Diversity of Aquatic Ecosystem and its Industrial Potential. *J Bacteriol Mycol Open Access* 2016, 3:00048.

18. Mwirichia R, Muigai A, Tindall B, Boga HI, Stackebrandt E: Isolation and characterisation of bacteria from the haloalkaline Lake Elmenteita, Kenya. *Extremophiles* 2010, 14:339-348.

19. Kumar S, Stecher G, Li M, Knyaz C, Tamura K: MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution* 2018, 35:1547-1549.

20. Qazi M, Malik Z, Qureshi G, Hameed A, Ahmed S: Yeast extract as the most preferable substrate for optimized biosurfactant production by rhlB gene positive *Pseudomonas putida* SOL-10 isolate. *Journal of Bioremediation & Biodegradation* 2013, 4:2.

21. Datta P, Tiwari P, Pandey LM: Isolation and characterization of biosurfactant producing and oil degrading *Bacillus subtilis* MG495086 from formation water of Assam oil reservoir and its suitability for enhanced oil recovery. *Bioresource technology* 2018, 270:439-448.

22. Burch AY, Shimada BK, Browne PJ, Lindow SE: Novel high-throughput detection method to assess bacterial surfactant production. *Applied and Environmental Microbiology* 2010, 76:5363-5372.
23. Cooper DG, Goldenberg BG: Surface-active agents from two Bacillus species. *Applied and Environmental Microbiology* 1987, 53:224-229.

24. Du Noüy PL: A new apparatus for measuring surface tension. *The Journal of general physiology* 1919, 1:521.

25. Lee DW, Lee H, Kwon B-O, Khim JS, Yim UH, Kim BS, Kim J-J: Biosurfactant-assisted bioremediation of crude oil by indigenous bacteria isolated from Taean beach sediment. *Environmental pollution* 2018, 241:254-264.

26. Goswami M, Deka S: Biosurfactant production by a rhizosphere bacteria Bacillus altitudinis MS16 and its promising emulsification and antifungal activity. *Colloids and Surfaces B: Biointerfaces* 2019.

27. Mehetre GT, Dastager SG, Dharne MS: Biodegradation of mixed polycyclic aromatic hydrocarbons by pure and mixed cultures of biosurfactant producing thermophilic and thermo-tolerant bacteria. *Science of The Total Environment* 2019, 679:52-60.

28. Medeot DB, Bertorello-Cuenca M, Liaudat JP, Alvarez F, Flores-Cáceres ML, Jofré E: Improvement of biomass and cyclic lipopeptides production in Bacillus amyloliquefaciens MEP218 by modifying carbon and nitrogen sources and ratios of the culture media. *Biological control* 2017, 115:119-128.

29. Fernandes P, Rodrigues E, Paiva F, Ayupe B, McInerney M, Tótola M: Biosurfactant, solvents and polymer production by Bacillus subtilis RI4914 and their application for enhanced oil recovery. *Fuel* 2016, 180:551-557.

30. Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NA-H: Optimization of surfactin production by Bacillus subtilis isolate BS5. *Applied Biochemistry and Biotechnology* 2008, 150:305-325.

31. Najafi A, Rahimpour M, Jahanmiri A, Roostaazad R, Arabian D, Ghobadi Z: Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by
32. Rocha MVP, Barreto RVG, Melo VMM, Gonçalves LRB: Evaluation of cashew apple juice for surfactin production by Bacillus subtilis LAMI008. *Applied biochemistry and biotechnology* 2009, 155:63-75.

33. Ramyabharathi S, Meena KS, Rajendran L, Karthikeyan G, Jonathan E, Raguchander T: Biocontrol of wilt-nematode complex infecting gerbera by Bacillus subtilis under protected cultivation. *Egyptian Journal of Biological Pest Control* 2018, 28:21.

34. Yánez-Mendizábal V, Zeriouh H, Viñas I, Torres R, Usall J, de Vicente A, Pérez-García A, Teixidó N: Biological control of peach brown rot (Monilinia spp.) by Bacillus subtilis CPA-8 is based on production of fengycin-like lipopeptides. *European Journal of Plant Pathology* 2012, 132:609-619.

35. Sobrinho HB, Rufino RD, Luna JM, Salgueiro AA, Campos-Takaki GM, Leite LF, Sarubbo LA: Utilization of two agroindustrial by-products for the production of a surfactant by Candida sphaerica UCP0995. *Process Biochemistry* 2008, 43:912-917.

36. Bezza FA, Chirwa EMN: Biosurfactant from Paenibacillus dendritiformis and its application in assisting polycyclic aromatic hydrocarbon (PAH) and motor oil sludge removal from contaminated soil and sand media. *Process Safety and Environmental Protection* 2015, 98:354-364.

37. Souza KST, Gudiña EJ, Schwan RF, Rodrigues LR, Dias DR, Teixeira JA: Improvement of biosurfactant production by Wickerhamomyces anomalous CCMA 0358 and its potential application in bioremediation. *Journal of hazardous materials* 2018, 346:152-158.

38. Conte P, Agretto A, Spaccini R, Piccolo A: Soil remediation: humic acids as natural surfactants in the washings of highly contaminated soils. *Environmental pollution*
Figures

**Figure 1**

Biosurfactant production profile on fixed glycerol substrate with NH4NO3 (A), urea (B), and yeast extract (C). Total dry biomass and crude biosurfactant production after five days incubation period at 140 rpm in shaking environment at 28 °C (D).
Figure 2

Three dimensional (3D) response surface plots showing the interactive effects of various factors on the surface tension reduction (A- C) and biosurfactant product yield (mg/L) (D- E).
Figure 3

Stability of crude biosurfactant on various environmental factors i.e. temperature ranges 4-121 °C, NaCl conc. 1-9% (w/v), and pH ranges 3-10 (A). Critical micelles concentration profile of different concentrations of crude biosurfactant 0-100 mg/L-1 (B).
LC-ESI/MS spectra of lipopeptide biosurfactant (C13-15). Two isoforms 1008.76, and 1030.74 with retention time (Rt) 12.48 (A), two isoforms 1022.78 and 1044.76 with (Rt) 14.26 (B) and three isoforms 1022.77, 1036.78, and 1058.77 with (Rt) 15.7 (C) detected on positive scan mode.
Figure 5

Potential of biological surfactants for heavy engine oil polluted soil remediation by comparing with synthetic surfactants, the percent recovery of engine oil from contaminated soil.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Supplimentary data.docx