Screening and characterization of Lipopeptide Biosurfactant Producing Paenibacilus Dendritiformis and its applicability for enhanced oil recovery

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Abstract

The biosurfactants produced by microorganisms have high demand from microbial-enhanced oil recovery (MEOR) and they have focused on a chemical surfactant for the past few decades for degrading petro-based pollutants and oil spills due to its non-toxicity and increasing bioavailability. The study aims to identify and screen potential lipopeptide biosurfactant produced by Paenibacillus species employing a design experiment based on RSM. The bacterial culture was isolated from Chilika Lake, India. The data generated from the biosurfactant stability experiments were used to fit a regression model using the parameters such as ph, temp, and salinity to predict the E24 index. R-squared value 0.91 obtained from the ANOVA model explains that the regression model was significant, and the model p-value obtained was < 0.05 and was also statistically significant. Therefore the statistical regression model obtained in the present investigation can predict the E24 index by using any combination of ph, temp, and salinity parameters. The novel isolates obtained in this research were further named Paenibacillus dendritiformis ANSKLAB02 and deposited in GenBank with accession number KU518891. The growth of this species under controlled conditions has a high potential to help in environmental clean-up and is suitable for use in MEOR applications.

1. Introduction

The most prevalent environmental hazard is petroleum pollution, which shows harmful effects on all marine ecosystems due to rapid industrialization leading to an increase in aromatic hydrocarbons over the years. The Environmental Protection Agency, USA, reported the contamination sources are massive: leakages from underground storage tanks, accidents in fuel transportation by ships; which are subject to corrosion, oil extraction, and processing; and inadequate release of oily waste generated by industries that use oil byproducts in the production of plastics, solvents, pharmaceuticals, and cosmetics, which cannot be reused or recycled, as they are toxic, flammable, pathogenic or corrosive. Moreover, for humans, hydrocarbons are considered highly toxic, carcinogenic, and mutagenic [1]. The advancement of sustainable technologies tends towards the natural methods for the remediation of soil and water contaminants has increased based on different biological activities. Microorganisms most prominently have a great diversity of bacteria such as *Actinobacteria*, *Mycobacterium*, *Penicillium*, *Mycococcus*, *Pseudomonas*, *Nitrosomonas*, *Flavobacterium*, etc. that promote the cracking of hydrocarbons molecules (dioxins, benzene, polychlorinated biphenyls, toluene, etc.) by micelle formation, increasing their mobility, bioavailability and exposure to bacteria, thus favoring bioremediation[2-3]. Some microorganisms have started producing several classes of surface-active compounds such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids, and polymeric biosurfactants [4-6]. The surfactant-enhanced bioremediation emerges as a promising technology for the remediation of PAH or hydrophobic organic compounds contaminated from water and soil surface. In contrast to chemical surfactants, biosurfactant's utilization for the bioremediation of contaminated water surface and oil spills is not yet well established. Interestingly, the attention of the scientific and industrial community recently focuses on determining potential biosurfactants that can be produced at a large scale because of their

lower toxicity, optimal activity at extreme conditions of temperatures, pH levels, and salinity, a higher degree of biodegradability, and higher foaming capacity [7-8].

Biosurfactants contain both the hydrophilic and hydrophobic domains capable of interfacial tension and decreasing surface. Biosurfactant has various compounds like lipopeptides, neutral lipids, fatty acids, and glycolipids. These are non-toxic, biodegradable biomolecules that show the emulsification of hydrophobic compounds [9]. Based on the biosurfactant domain, the hydrophilic domain comprises a carbohydrate, an amino acid, a phosphate group, or similar compounds. In contrast, the hydrophobic domain is most commonly comprised of the carboxylic acid chain. The present property of biosurfactant sustains to cut down the interfacial tensions and surface tensions and make them a potential candidate for enhancing oil degradation [10 - 11]. The biosurfactant production can be affected by various factors such as the nature of carbon and nitrogen sources used; phosphorus, iron, manganese, and magnesium may also be present. Besides, pH, temperature, agitation and mode of operation are the other essential factor that directly affects the quantity and quality of produced biosurfactant [12]. Therefore, the development of more multifunctional biosurfactants is required to broaden the spectrum of properties available. The biosurfactant production on an industrial scale makes it more worthy for better and efficient bioprocesses to make them competitive because of the chemically synthesized compound's market due to inefficient bio-processing methodology and poor strain productivity [13]. If the biosurfactants are produced from cheap substrates like agro-industrial wastes, which reduces the production cost. Therefore, it is beneficial to identify, isolate and characterize new strains producing biosurfactants from various natural sources like water bodies or contaminated soil. The research anticipated analyzing the stability and efficiency of a new lipopeptide biosurfactant produced by *Paenibacillus* sp. and the feasibility of its use in bioremediation.

In a previous study, we reported the biosurfactant production by *Bacillus subtilis* novel strain ANSKLAB03 that can yield 0.324 g of BS in 100mL of the medium [9]. The phenotypic divergence and phylogenetic discreteness suggested that the mentioned *Bacillus subtilis* strain ANSKLAB03 was novel and submitted to the GenBank database. The identified *Bacillus subtilis* strain ANSKLAB03 was thermodynamically stable and had -236.20kcal/mol of the free energy (ΔG) of the anticipated RNA structure [9]. In a study, *Bacillus tequilensis* ZSB10 isolated from Mexican brines, the author was apt to produce extra-cellular as well as cell-bound biosurfactant employing nine broth cultures composed from hydrolyzates procure from the cellulosic and hemicellulosic fragments of wine-trimming wastes [14]. Similarly, *Paenibacillus macerans* strain TKU029 can yield exopolysaccharides and the biosurfactant in a medium with 2 % (w/v) of squid pen powder as the ace source of carbon/nitrogen. At a concentration of 2.76 g/L, biosurfactant can reduce water surface tension from 72.30 to 35.34 mN/m and can reach an emulsification index of up to 56% in the presence of machine oil with 24h incubation. This biosurfactant was found stable at 121°C for 20 min and a varying pH range of 3 to 11. There were no observable structural changes even in<5 % salt solutions [15].

The present study aims to examine the effect of the lipopeptide biosurfactant produced by novel gram-positive *Paenibacillus* sp. strain, isolated from brackish water lagoon, on oil spill degradation by a

microbial consortium. The biosurfactant produced by isolated *Paenibacillus* sp. strain was screened, purified, quantified, and characterized by physicochemical properties. Its potential application for enhancing bioremediation was evaluated. The optimum dosage for the maximal output was evaluated. The study describes the location, isolation, screening, and characterization of biosurfactant-producing strain. Besides, extracellularly produced metabolites were extracted for further analysis. Series of assay confirmatory assays were performed for analysis metabolic action to determine the potential of *Paenibacillus* species in degrading the oil.

Subsequently, it is essential to determine the stability in the harsh environment as the biosurfactant activities are affected by physicochemical factors such as temperature, pH and salinity. Therefore the interactive to identify the effect of pH, temperature, and salinity on biosurfactant stability was tested using a design experiment-based response surface methodology (RSM) with the Box–Behnken experimental design using R programming [16].

2. Materials And Method

2.1 Sample Collection

For the present investigation, the samples were collected from an oil-contaminated site in *Chilika Lake*, Odisha, 19° 46' 30.3780" N and 85° 25' 4.8540" E *coordinates* of India and great genetic diversity). The samples were collected in sterile 50ml tubes and were immediately stored at 4˚C until usage. This was done to preserve the microbial consortium in the water sample.

2.2 Isolation of Microbial Consortium

The water samples were enriched in sterile Mineral Salt Medium (MSM). 1 ml of sample was inoculated in 100 ml MSM containing (in g/L): 15g $NaNO_3$, 1.1g KCl, 1.1g NaCl, 0.00028g $FeSO_4.7H_2O$, 3.4g KH_2PO_4, 4.4g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g yeast extract. The culture was incubated at 37°C in the shaker at 100 rpm. After 48 h of incubation, morphologically different colonies were isolated and screened for biosurfactant production [17].

2.3 Screening of Biosurfactant producing Isolates

Isolates were grown aerobically in a 500 ml Erlenmeyer flask containing 100 ml MSM. The medium consisted of (gl-1) 1.0g K2HPO4, 0.2g MgSO4.7H2O, 0.05g FeSO4.7H2O, 0.1g CaCl2.2H2O, 0.001g Na2MoO4.2H2O, 30g NaCl and crude oil (1.0%, w/v). Loopful of isolated bacterial cultures were inoculated in mineral salt medium and incubated in a shaker at 200 rpm and 30oCfor 7 days. After incubation, the broth was centrifuged at 6000 rpm and 4oC for 15 minutes. The obtained supernatant was filtered using a 0.45µm pore size filter paper (Millipore). The cell-free culture broth was further used to perform drop collapse assay, emulsification assay, oil spreading assay, and surface tension measurement. The bacterial cells were used for the BATH assay. The screening tests were performed in triplicates, and the results were presented as the mean values [18].

2.3.1 Oil spread assay

The assay was performed by the method described by Morikawa et al., 2000 [19]. To 20 ml of distilled water, 20 µl of crude oil was added to the plastic Petri dish surface. 10 µl of cell-free culture broth was added to this oil surface on the Petri plate. The presence of biosurfactant in the cell-free culture broth displaces the oil around it, forming an oil-free clearing zone whose diameter indicates the surfactant activity, called oil displacement activity. Distilled water was used as a negative control and Triton X-100 as a positive control.

2.3.2 Drop collapse test

In 1998, Bodour and Maier established a qualitative test named Drop-collapse test [20-21]. Two microlitres of crude oil were applied to the wells of 96 well microplates and equilibrated 24h. 5µl of the 48h grown culture was transferred to oil-coated well drop by drop. The wells were observed after a minute with a magnifying glass. The same procedure was performed with a centrifuged sample at 15,000 rpm for 5 min to remove cells. The flat drop was indicative of positive biosurfactant production, and the rounded drop indicates negative biosurfactant production [22].

2.3.3 Hydrocarbon overlay agar

ZMA plates were used to perform hydrocarbon overlay agar test; each plate was coated individually with 40µl of kerosene, hexadecane, benzene, and toluene. Pure isolates were inoculated on coated plates and incubated for 7-10 days at 28°C. The presence of emulsified halo was considered positive for biosurfactant production.

2.3.4 Bacterial adhesion to hydrocarbon (BATH) assay

Cell hydrophobicity can be measured by its adherence to hydrocarbons in a method described by Rosenberg et al., 1980 [23]. Washed cell pellets were suspended in a buffer salt solution (g/L, 16.9 K_2HPO_4, and 7.3 KH_2PO_4) diluted to an optical density (OD) of ~ 0.5 at 610 nm. To 2 ml of cell suspension, 100 µl of crude oil was added and vortexed for 3 min. After shaking, it was allowed to stand for 1 hour that separated the oil and the aqueous phases. OD of the aqueous phase was measured at 610 nm in a spectrophotometer. With this, the percentage of cells that were attached to the oil surface was calculated using the following formula:

$$\%\ bacterial\ cell\ adherence = 1 - \frac{OD_{shaken\ with\ oil}}{OD_{original}}\ X\ 100$$

Where, OD_{shaken} with oil is OD of the cells vortexed with crude oil, and $OD_{original}$ is OD of the cell suspension in the buffer solution, pre-mixing. A few drops of INT solution (2-(4-iodophenyl)-3-(4-nitrophenyl)-5- phenyltetrazolium chloride) were added to the BATH assay solution and observed under a

light microscope. The INT turns red to reduction reaction inside the cells that indicate the cell's viability attached to the crude oil droplets [24].

2.3.5 Surface tension analysis

Surface tension measurement of cell-free culture broth was determined with a tensiometer, using the du Nouy ring method [25]. Triton X-100 solution was prepared at 1mg/ml concentration and used as the standard.

2.4 Biochemical Characterization

Biochemical characterization of the microbial consortium was performed for the best four organisms. 48 hour activated culture was used to perform the characterization.

2.5 Optimization of Biosurfactant production by *Paenibacillus*(Isolate 3)

2.5.1 Effect of pH

The range of pH; 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, was selected for the optimization of pH. 1% glucose as sole carbon source was added in MSM medium, and pH was adjusted by 0.1N HCl and 0.1N NaOH solutions using pH meter. The MSM medium was sterilized in an autoclave at 121°C for 15 min. Activated culture of *Paenibacillus*(4.28×10^8 CFU ml^{-1}) was inoculated and incubated at 37°C for seven days in an orbital shaker at 150 rpm [26].

2.5.2 Effect of Temperature

In addition to the previous method stated in section 2.5.1, the temperature was optimized by carrying out the study at different temperatures;20°C, 30°C, 40°C, 50°C and 60°C. 1% glucose as the sole carbon source was added during the preparation of Mineral Salt Medium (MSM), and the pH were adjusted at 7.0 MSM medium were dispensed in the different flask to be incubated at distinctive temperatures for seven days [27].

2.5.3 Effect of Carbon

In addition to the previous method stated in section 2.5.1, to study the effect of carbon source on the isolated *Paenibacillus* strain, 8 carbon sources mainly, crude oil, coconut oil, diesel oil, sucrose ($C_{12}H_{22}O_{11}$), starch $(C_6H_{10}O_5)_n$, maltose, mannitol, and glycerol were used. 1% of each carbon source was added in different flasks containing MSM medium [27-28]. And the medium was incubated as per the standard protocol mentioned in the previous section [28].

2.5.4 Effect of Nitrogen

In the present study, we have selected the 8 nitrogen sources specifically are ammonium nitrate (NH_4NO_3), ammonium phosphate $(NH_4)_3PO_4$, ammonium sulphate $(NH_4)_2SO_4$, ammonium

chloride (NH_4Cl), peptone, potassium nitrate (KNO_3), yeast extract, and urea ($CO(NH_2)_2$). In addition, MSM was prepared with 1% sucrose ($C_{12}H_{22}O_{11}$) as a carbon source, and 1g/l concentration of one by one nitrogen source, followed by the processor stated in section 2.5.1 [28]. The pH was adjusted at 7.0, and the medium was sterilized at 121°C for 15 min. Activated culture of *Paenibacillus* (4.28×10^8 CFU ml^{-1}) was inoculated, and it is incubated for 7 days at 37°C in an orbital shaker at 150 rpm [28].

2.5.5 Effect of the Carbon and Nitrogen Concentration

The optimized concentration of carbon and nitrogen is essential for maximum yield. In the MSM different concentrations of carbon and nitrogen were added which include: 1%, 2%, 3%, 4%, and 5% separately. The medium's pH was adjusted to 7.0 and was sterilized in an autoclave at 121°C for 15 min. Inoculated culture of *Paenibacillus* (4.28×10^8 CFU ml^{-1}) was incubated in an orbital shaker (150 rpm) at 37°C for 7 days [28].

2.5.6 Analysis for Optimization Conditions and Biosurfactant Extraction

The bacterial cells were centrifuged for 20 min at 13, 500Xg at 4°C and the supernatant was collected for emulsification activity at the end of each optimization process. The optimal growth conditions were confirmed by emulsification activity and bacterial biomass in each parameter[27-29].

2.6 Biosurfactant Extraction

For the inoculation of *Paenibacillus,* 100 ml of optimized medium were used from the pure extracted culture and were incubated in a shaking incubator at 120rpm for the incubation period of 7 days at 25°C. The cells were then removed by centrifugation at 5000rpm, 4°C for 20 minutes was performed to remove cell debris. 1MH_2SO_4 were used to adjust the pH of the supernatant at pH 2.0. An equivalent volume of chloroform: methanol in the ratio 2:1 was added. After being shaken well, the mixture was left overnight to get evaporated. The biosurfactant obtained were acquired in the form of white colour sediment [28-29].

2.7 Dry weight of biosurfactant

The sediment was taken out in weighed sterile Petri plate for weight the dry residue and kept in a hot air oven at 115°C for drying for 25 minutes. After drying, the plate was weighted [28-29]. The following formula calculated the dry weight of the biosurfactant:

$$Dry\ weight\ of\ biosurfactant = (weight\ of\ white\ sediment + dish) - weight\ of\ the\ empty\ dish$$

2.8 Biosurfactant Characterization

The purified *Paenibacillus dendritiformis* was dissolved in chloroform for thin-layer chromatography (TLC) by using CHCl3: CH3OH: H2O in the solvent system (elute) in the ratio of 65:15:2. 10 µl of the activated bacterial sample was spotted 2.5 cm above the TLC plate's bottom (20×20 cm, Silica gel 60, Fisher Scientific). Then, the TLC plate is placed in the developing chamber 90 degrees from the plate's bottom edge to the solvent, and the chamber was sealed. 0.19 % of Orcinol solution, 1 % of ninhydrin solution and Bromothymol blue were sprayed on TLC plate in UV light(366nm) for the development of spot on the plate, followed by heating at 110 °C for 15 minutes in a hot oven to detect red spots of the peptide. Iodine vapour is exposed on the TLC plate to observe yellow spots after development that indicates lipid presence [30-31].

2.9 Fourier Transform Infrared Spectroscopy (FTIR)

IR Spectrum GX (PerkinElmer, USA) was used to determine the distinct chemical structure, bonds, and functional groups present in the biosurfactant applying IR spectral analysis. Spectral lines were observed in the vicinity of 4,000- to 400-cm−1 for resolution of spectrum applying FTIR. 1 mg of *Paenibacillus dendritiformis* were used in this analysis to alloyed with 100 mg of KBr and crush against to obtain a pellet. An infrared absorption frequencies database was used to interpret the peaks [32-34].

2.10 Stability Test Calculation of Emulsification index (E_{24})

2ml of MSM was inoculated with the pure culture colonies and incubated for 48h. After incubation, 2 mL of crude oil was added. It is then vortexed at high speed for 1 min and then allowed to stand for 24 h. The emulsion index (E24) can be calculated as the percent ratio of the emulsified zone layer (mm) to the total height (mm), as described in Eq.[25]

$$Emulsification\ index = \frac{Height\ of\ the\ emulsification\ layer}{Total\ height}\ X\ 100$$

In contrast, an Experimental algorithm designed using RSM based on the Box–Behnken model was developed with three variables at three levels (+1, 0, −1): pH (5, 7, and 10); temperature (30, 40, and 60 °C); and salinity (4%, 6% and 8%). R programming was used to create a 3-factor Box–Behnken design. The response variable was Emulsification Index (E24)[35-37].

2.11 Statistical analysis

The effect of temperature, pH, and salinity on lipopeptide biosurfactant stability was measured in independent triplicates. The responses to the E_{24} of the Biosurfactant produced by *Paenibacillus* dendritiformis were statistically analyzed by means of variance analysis (ANOVA), including Fisher's text and Student's t test, with a significant level (α) of 0.05. All statistical analyses were conducted using R programming[38-39].

2.12 DNA isolation

The purified 5 ml of overnight isolated *Paenibacillus* species culture was centrifuged at 10,000 rpm for 10 minutes. In the cell pellet, 875µl of Tris EDTA (ethylene-diamine-tetraacetic-acid) buffer was added to preserve the DNA from degradation. 5µl of Proteinase K and 100µl of 10% sodium dodecyl sulfate in lysis buffer were added to the extracted *Paenibacillus* cells. Further for 1hr solution was incubated at 37°C after the proper mixing. A specific ratio of 1 ml of phenol and *trichloromethane* was added in a vial and incubated at room temperature for 5 minutes. At 10,000 rpm, vials were centrifuged for 10 minutes. For collecting an adequate amount of supernatant, this process is repeated thrice. 100µl / 5M sodium acetate was added to the supernatant followed by gentle mixing. For DNA precipitation, 2 ml of isopropanol were added slowly to the vial walls (dropwise) until DNA gets precipitated at 5000 rpm for vials were again centrifuged for 10 minutes. After removing the supernatant, 70% ethanol was added, and at 5000 rpm, it is centrifuged for 10 minutes. It is air-dried, and TE buffer is used for storage. The extracted DNA was run on 1% agarose gel to check band's purity and integrity. The extracted DNA was amplified using PCR[40-43].

2.13 16S rRNA Sequencing

Amplified products were isolated from 1% agarose gels in 1x TAE (Tris-acetate-EDTA) buffer at 10 Vmm-1 for 90 minutes and examined with a UV transilluminator and recorded with Bio-Rad's Gel Doc XR. The amplified by-product was elutriated using the thermofisher's Gene JET PCR Purification and Gel extraction kit as instructed in the manufacturer's guidelines. Sanger dideoxy sequencing technique by ABI (Applied Biosystems) was used to sequenced elutriated PCR by-product. For the assembly of both the forward as well as reverse trace files acquired from the sequencing DNA, the DNA Baser tool was utilized [44-47]. Clean traces (fragments) were determined in both the assemble (forward and reverse). Furthermore, for the elaborated bioinformatics analysis, the assembled sequences were stored in FASTA file format [48-52].

2.14 Phylogenetic tree construction

The sequence obtained from the Sanger method was further analyzed using EMBOSS software. The sequence further performed a Blastn against 16S ribosomal rRNA sequence (Bacteria and Archaea) database using a cut-off value of >95% [44][50-51]. All the similar sequence was further downloaded from GenBank, and the phylogenetic tree was constructed to identify the bacteria's genus and species. Also, to study the evolutionary significance of the query bacteria and similar bacteria obtained from the GenBank. The evolutionary antiquity was rooted in employing the (NJ)Neighbor-Joining algorithm [52-54]. The most favorable tree amidst the sum of branches shows the length = 71.76000000. The tree was drawn to calibrate the evolutionary distances in the same unit concerning the branch's length toward determining the phylogenetic tree[55-56]. The evolutionary gaps have been measured applying the various methods [51] and were expressed in the number of base differences per sequence unit. The overall analysis consists of twenty nucleotide sequences. Codon was arranged in the order, 1st codon+2nd codon +3rd codon +Noncoding respectively. All positions encompassed gaps and lacking

data were eliminated. A total of 1319 positions were present in the final dataset. Evolutionary studies were overseen using MEGA X [50-51].

2.15 Elucidation of rRNA Secondary Structure

The thermodynamics of the RNA structure has been examined successfully through the approaches like absorbance micro-calorimetry and melting curves that entail isothermal titration calorimetry and differential scanning calorimetry. The RNA 3-D structure estimates to have a confounding number of shapes which demonstrates that predicting native structure in the equilibrium to be a difficult task [51]. Also, there are various distinct motifs in RNA structure, and the quantity of feasible sequence consolidation for utmost motifs is massive. However, by the tenets of thermodynamics, it is possible to predict the stable RNA structure. The main objective of thermodynamic principles is to provide a core establishment to predict and foresee the rRNA structure from the sequence. Despite this, it is unlikely to create the models of all the established thermodynamic specifications for all inclined helices with Watson-Crick base pairs. As consequence, potent and robust models have been created to foresee the thermodynamics of helix development from a restricted set of estimated measurements. A few of the prominent endeavors include the neighbor model foreseeing strong stabilities of RNA duplexes with Watson-Crick pairs by Borer et al. proposed algorithms. Likewise, a substitute investigation for thermodynamic with thermodynamic properties has been proposed by Gray et al. [50-51]. However, in another methodology, thermodynamic principles and phylogenetic information have been unified for nearby prediction of RNA duplex formation using its structural properties that encompass the identification of RNA spectroscopic properties.

3. Results And Discussion

3.1 Isolation and biochemical characterization

A total of 23 different species were found in the collected samples. On screening the cultures in the crude oil, four organisms were found to survive on the MSM plates. These were pure cultured and characterized using biochemical tests. The screened microorganisms belong to 4 different species of bacteria, which includes *Bacillus tequilensis* species (Isolate 1 - 4.32x108 CFU/ml), *Bacillus subtilis* species (Isolate 2 - 4.07x108 CFU/ml), *Paenibacillus* species (Isolate 3 - 4.28x108CFU/ml), and *Microbacterium* species (Isolate 4 - 4.16x108 CFU/ml) illustrated in Table 1 that were identified and distinguished employing Bergey's protocol as a source of reference. *Bacillus tequilensis* is biochemically analogous to Bacillus subtilis; however, it can be discriminated by positive production of 3 rhamnose compounds, namely ornithine decarboxylase, lysine decarboxylase, and arginine dihydrolase, as well as acid production. The biochemical test results for each isolate were verified with the literature to identify the genus. The detailed list has been mentioned in table 1. The gram staining and bacterial colonies of Isolate 3 have been depicted in figure 1.

Table 1: Biochemical characteristics of the four isolates

Biochemical characterization					
S.No	*Parameters*	*Microorganisms*			
		Isolate 1 (Bacillus subtilis)	*Isolate 2 (Microbacterium)*	*Isolate 3 (Bacillus tequilensis)*	*Isolate 4 (Paenibacillus)*
1	*Colonial Characters*	*Opaque, Off-white colonies, Large, Circular, Smooth, Uneven, Slightly Raised.*	Yellow colonies, translucent, smooth, Medium, circular, slightly raised.	*Opaque, Uneven, Large, Circular, Smooth, yellowish colonies, Slightly Raised.*	*Thin, spreading colonies, Circular Large, , smooth, Opaque, flat.*
2	Microscopic characters	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped
3	*Gram's Staining*	*Gram Positive*	*Gram Positive*	*Gram Positive*	*Gram positive*
4	*Pigmentation*	*Pigmentation was off - white*	*Pigmentation was Yellow*	*pigmentation was Yellowish*	*Pigmentation of off - white*
5	*Sucrose Fermentation*	*+ve*	*+ve*	*+ve*	*+ve*
6	*Casein Hydrolysis*	*+ve*	*+ve*	*+ve*	*-ve*
7	*Lipid Hydrolysis*	*+ve*	*+ve*	*+ve*	*+ve*
8	*Citrate Utilization*	*+ve*	*+ve*	*+ve*	*+ve*
9	*Starch Hydrolysis*	*+ve*	*+ve*	*+ve*	*+ve*
10	*Oxidase Activity*	*-ve*	*-ve*	*+ve*	*-ve*
11	*Methyl Red test*	*+ve*	*+ve*	*+ve*	*+ve*
12	*Lactose Fermentation*	*-ve*	*-ve*	*+ve*	*-ve*
13	*Glucose fermentation*	*+ve*	*+ve*	*+ve*	*+ve*
14	*Voges Proskeur*	*+ve*	*-ve*	*+ve*	*-ve*
15	*Motility*	*+ve*	*-ve*	*+ve*	*+ve*
16	*Indole*	*-ve*	*-ve*	*+ve*	*-ve*

	Production				
17	*Gas Production from Glucose*	*+ve*	*-ve*	*+ve*	*+ve*
18	*Catalase Activity*	*+ve*	*+ve*	*+ve*	*+ve*
19	*Nitrate Reduction*	*+ve*	*-ve*	*+ve*	*+ve*
20	*Gelatin Hydrolysis*	*+ve*	*+ve*	*+ve*	*+ve*
21	*H_2S Production*	*-ve*	*-ve*	*-ve*	*-ve*
22	*Spores*	*+ve*	*-ve*	*+ve*	*+ve*
23	*Urease Activity*	*-ve*	*-ve*	*-ve*	*-ve*

3.2 Screening for biosurfactant production

The biosurfactant production was confirmed by performing various assays on the four isolates. The section discusses the respective efficiencies for each of the screened isolates.

3.2.1 BATH assay

The cell hydrophobicity was estimated by the BATH assay**.** Table 2 shows the affinity of the positive strains of bacterial cells towards the hydrophobic substrate. Isolate 3 (87.23±0.71) shows the highest cell adherence property, whereas Isolate 4 (62.54±1.42) was observed with the least cell adherence. Furthermore, Isolate 1 shows 84.77±0.56 adherence, whereas Isolate 2 (79.46±0.23) shows cell adherence. Positive hydrophobicity of the cells is proclaimed as a signal for the production of biosurfactant. The visualization of bacterial cells adhered to crude oil confirmed the affinity of cells in the crude oil droplet's vicinity.Table2 discusses the BATH results for each isolate.

3.2.2 Hydrocarbon overlay agar plate

The HOA plate method identifies hydrocarbon elastic *Paenibacillus* species as well as shows the hydrocarbon-degrading activity of the isolated bacteria. In Table 2, Quantitative assessment of the bioemulsifiers were demonstrating the zone of clearance that Isolate 3 gave 1.7 mm, 1.6 mm, 1.5 mm, and 1.4 mm with benzene plated medium, kerosene, toluene, and hexadecane, respectively. Isolate 1 demonstrated the development across the hydrocarbon plated media with 1.4 mm diameter, 1.5 mm diameter, 1.3 mm diameter, and 0.2 mm diameter of clearance zone for toluene, benzene, hexadecane, and kerosene plated media, respectively. Isolate 3 and isolate 1 gave negative outcomes with benzene and kerosene but, however, gave great outcomes with hexadecane(Isolate 3 was 1.3mm diameter, Isolate 1 was 1.4mm diameter) and toluene(Isolate 3 was 0.6mm diameter, Isolate 1 was 1.5mm diameter).

Isolate 2 was 1.3 mm diameter, 1.5 mm diameter, 1.4 mm diameter, and 0.3 mm diameter zone of clearance for toluene, benzene, hexadecane and kerosene plated media. Figure 2 shows the clearance zone observed for hexadecane in the case of Isolate 3.

Table 2: BATH assay and Hydrocarbon overlay agar plate

Microorganism	*BATH assay*[a]	*Hydrocarbon overlay agar plate*[b]			
		Kerosene	*Hexadecane*	*Benzene*	*Toluene*
Isolate 1	++	*Nil*	*++*	*Nil*	*+*
Isolate 2	++	*Nil*	*++*	*Nil*	*++*
Isolate 3	++	*++*	*++*	*++*	*++*
Isolate 4	++	*+*	*++*	*++*	*++*

BATH assay[a]: '+++' - cell adhesion > 90%, '++' - 60 to 89% cell adhesion, '+' – 40 to 59% cell adhesion.

Hydrocarbon overlay agar plate[b]***:****'+' - clearance zone of 0.1-1 mm, '++' - clearance zone of 1.1 to 2 mm, '+++' - clearance zone of 2.1 to 3.5 mm.*

3.2.3 Drop collapse assay

Drop collapse assay is a hypersensitive test and can give a result with even a very small measure of biosurfactant produced. A few strains give a positive outcome with BATH assay; however, the strains test negative for drop collapse assay as their cultures with high cell hydrophobicity were act as biosurfactants themselves but were unable to produce the biosurfactant extracellularly. The analysis was performed in the duplicate set. And for conducting the analysis, 10µl of surfactant solution, and the cell-free culture broth were used. The positive outcomes with a flat droplet, as confirmed by the oil spread assay were shown by all microorganisms' strains. The drop of Isolate 1, Isolate 2, Isolate 3, and Isolate 4 collapsed in 1 min 3 sec, 56 sec, 1 min 6 sec, and 1 min 52 sec, respectively (Table 3).In order to assure the production of biosurfactants, oil spreading and surface tension experiments were performed on the microorganism's cell-free culture broths. Figure 3 shows the drop collapse test for the four isolates.

3.2.4 Oil spreading assay

The organisms with a positive drop collapse assay were found to give positive results for the oil spreading assay. It is observed in the present analysis that the surface-active compound in the *Paenibacillus* isolate solution is directly proportional to the oil displacement area. Furthermore, it is a qualitative study to check the presence of surfactants. Isolate 1, Isolate 2, Isolate 3, and Isolate 4 show the clearance zone of 1.4mm, 2.4 mm, 2.1 mm, and 1.8 mm. Figure 4 depicts the oil spread assay for Isolate 3.

3.2.5 Surface tension measurement

Surface tension was measured in the cell-free culture broth, and it showed a reduction in surface tension. A direct correlation was observed between drop collapse, oil spreading, and surface tension assays. Microorganisms with slight activity in any one of the assays were active in the other two. Isolate1 to Isolate 4 gave positive results with a reduction in surface tension to 41mN/m, 38 mN/m, 36mN/m, and 42mN/m surface tension, respectively, given in Table 3.

Table 3: Oil spreading assay, Drop collapse assay, and Surface tension measurement

Microorganism	*Drop collapse assay[b]*	*Oil spreading assay[a]*	*Surface tension measurement[d]*
Isolate 3	*++*	*+*	*++*
Isolate 1	*+++*	*++*	*+++*
Isolate 4	*++*	*++*	*+++*
Isolate 3	*++*	*++*	*++*

***Surface tension[d]**: '+++'- indicates surface tension which is less than 40 mN/m, '++'- indicates surface tension between 40 to 50mN/m,'+'- indicates surface tension between 51 to 70mN/m.*

Oil spreading assay[a]: '+' - indicates a clear zone of oil spread with a zone of 0.5-1.5 mm diameter, '++' – indicates a clear zone of oil spread with a zone of 1.6 to 2.5 mm diameter, '+++' – indicates a clear zone of oil spread with a zone of 2.6 to 3.5 mm diameter.

***Drop collapse assay[b]**: '+++'- indicates adrop collapse within 1 minute, '++'- indicates a drop collapse after 1minute and '+' – indicates a drop collapse after 3 minutes of biosurfactant addition.*

3.3. Optimization of Biosurfactant production for *Paenibacillus* (Isolate 3)

The pH was optimized based on the E24 index. At pH 7.0, the E24 value was 75% that decreased gradually at pH 8.0, showing a substantial effect (Figure 5(a)). Similarly, the optimum temperature was found to be35°C (E24: 73.37%) (Figure 5(b)). Isolate 3 is mesophilic and exhibits effective production at moderate temperature. Starch was settled up as the most favorable (E24: 82%) amidst the 8 different carbon sources, followed by sucrose (E24: 72%) (Figure 5(c)). Subsequently, yeast extract exhibits the maximal E24 value (82%) amidst 8 different nitrogen sources followed by Peptone (E24: 60%) (Figure 5(d)). For the production medium preparation, all optimized condition plays an essential role in increasing the yield. On varying the carbon and nitrogen sources from 1–5%, 2% of the starch (E24:84%) (Figure5 (e)) as well as 3% of the yeast extract (E24: 83%) (Figure5 (f)) gave maximum production efficiency.

3.4 Extraction of biosurfactant and dry weight

The culture inoculated in a mineral salt medium with oil was centrifuged to collect the supernatant. The collected supernatant was mixed with chloroform and methanol in the aforementioned ratio. White sediment was retained in an empty Petri plate and was weighed as mentioned in Table 4. The maximum amount of biosurfactant was produced by *Paenibacillus* (Isolate 3), amounting to 0.426 g per 100 mL of medium.

Table 4: Dry weight of biosurfactant produced by the organisms

Microorganism	Empty plate weight (g)	Biosurfactant containing plate weight (g)	Dry weight of biosurfactant (g/100ml)
Paenibacillus(Isolate 3)	23.785	24.211	0.426

3.5 Thin layer Chromatography

Paenibacillus dendritiformis producing biosurfactant characterization was executed using Thin Layer Chromatography. TLC plate is exposed to different reagents after the solvent completely traveled until the top of the TLC plate and affirmed unique characteristics of the spots. The color of the developed spot depends on the reaction between the developing reagent and the sample compound. The identified Rf value for the appeared spot on the TLC plate is 0.72 indicates that the compound is non-polar. Also, the resulting Rf value was nearby to the Rf values of lipopeptide previously reported by researchers for the lipopeptides produced by *Paenibacillus* sp. The yellow spot in the iodine vapour indicates the organic compound's presence and the lipid moiety in the structure (Figure 6). In the present analysis, we found the purple spot on treatment with Ninhydrin reagent, which indicates the peptides group's presence in the present organic lipid compound, whereas, the absence of a colored spot on treatment with Orcinol reagent assures the absence of carbohydrate compounds within the structure. Therefore, considering the entire observations ensure the presence of lipopeptide residues in the current structure of biosurfactant originated from *Paenibacillus dendritiformis*.

3.6 Fourier Transform Infrared Spectroscopy

The absorbance intensity will correlate proportionally to the quantity of functionality present in the activated TLC-purified biosurfactant isolate of *Paenibacillus* species. FTIR spectral peaks observed at 2,943, 2,911, 2,862, and 1,454 cm−1 wavenumbers confirm the –C–H stretching (–CH3, –CH2) of the long hydrophobic R chain of the lipid as the similar stretching pattern for –C–H of lipid in observed in several previously completed studies of *Bacillus* species producing biosurfactant (Figure 7). C=O bending of esters was indicated by the peaks at 1,739 and 1,742 cm−1 and C–O by 1,090 cm−1. Besides, 977–840 cm−1 wave number peaks indicate the presence of C=C in the fatty acid chain of the lipid fraction of the biosurfactant. Furthermore, the presence of amide and hydroxyl groups of protein in the structure was identified. Furthermore, the alimentation of the amide bond, i.e., –C=O (1630 cm−1), the carbonyl group (C=O) of amide (1,672 cm−1), and the N–H bending of primary or secondary amides (1,630 cm−1) illustrate the presence of the peptide fragments of the biosurfactant in the structure. The obtained peaks

of biosurfactant indicate that lipopeptide biosurfactants were produced by *P. dendritiformis;* in a fatty acid chain, we found the presence of a double bond of the lipopeptide.

3.7 Stability test of biosurfactant

Optimization of biosurfactant production for Paenibacillus (Isolate 3) was carried out using RSM (response surface methodology) based on the Box–Behnken design of experiments. The R package RSM was used to carry out the analysis. The three factors considered in the analysis were pH (ph), temperature (temp), and salinity (sal). The three levels for each factor included in the design were pH 5, 7, and 10, temperature 30, 40, and 60, and salinity 4%, 6%, and 8%, encoded as -1, 0, and 1 respectively in the design. The experiment was conducted as per the Box–Behnken design and the E24 index was measured for each combination of the parameters. Table 5 shows the E24 values obtained from the design experiment.

Table 5: Results of Box-Behnken experimental design.

Experiment number	pH	Temp(°C)	Salinity (%)	E24 (%)
1	-1	-1	0	69.58858859
2	0	0	0	42.78678679
3	0	-1	1	32.57657658
4	1	0	1	52.53753754
5	1	0	-1	26.5015015
6	-1	0	-1	71.57957958
7	0	0	0	40.08108108
8	0	1	-1	49.32132132
9	1	-1	0	24.66366366
10	0	0	0	44.52252252
11	-1	1	0	55.3963964
12	0	-1	-1	73.36636637
13	-1	0	1	40.13213213
14	0	0	0	34.92492492
15	1	1	0	56.87687688
16	0	1	1	65.91291291

Experimental data was used to fit a linear model with a response-surface component using the R package's RSM function. While fitting the model, first-order, two-way interactions and pure quadratic

terms were included. The regression equation obtained is shown below. In the equation, the first-order terms are pH, temperature (temp), and salinity (sal), while the two-way terms are ph*temp, temp*sal, and ph*sal, and the quadratic terms are ph^2 (ph square), temp^2 (temp square) and sal^2 (sal square).

E24 = 40.58 - 9.51pH + 3.41temp - 3.70sal + 11.60ph:temp + 14.37ph:sal + 14.35temp:sal + 1.72ph^2 + 9.33temp^2 + 5.39sal^2

The significance of the coefficients of the model is shown in Table 6. The *** mark in the pval column indicates that the p-value is < 0.001 while ** indicates p-value < 0.01 and * indicates p-value < 0.05, indicating statistical significance of the corresponding parameter (Table 6). The intercept, ph, ph*temp, ph*sal, temp*sal, temp^2 terms were statistically significant, and therefore they influence the E24 values. The E24 values varied from 24.6% to 73.3%, and the highest E24 was observed for pH 7, temperature 30°C, and 4% salinity (Table 7). Overall the model was found to be statistically significant (p-value 0.0008478) with F value as 19.82 (9 and 6 degrees of freedom), adjusted R-squared value as 0.91.

Table 6: Statistical significance of model parameters

Estimate or Coefficient	Standard error	Student t-test test statistics	pval
(Intercept)	40.57882883	2.243416351	1.84E-06***
ph	-9.51463964	1.586334915	0.00097***
temp	3.414039039	1.586334915	0.07488
sal	-3.701201201	1.586334915	0.05839
ph:temp	11.60135135	2.243416351	0.00207**
ph:sal	14.37087087	2.243416351	0.00068***
temp:sal	14.34534535	2.243416351	0.00069***
ph^2	1.722972973	2.243416351	0.47161
temp^2	9.32957958	2.243416351	0.00595**
sal^2	5.385885886	2.243416351	0.05324

Table 7: The results of ANOVA and lack-of-fit test of the model

Term	Df	Sum Sq	Mean Sq	F value	Pr(>F)
First order terms	3	927.0633629	309.021121	15.35000108	0.0032**
Two way interaction terms	3	2187.608863	729.2029545	36.22168641	0.0003***
Pure quadratic terms	3	476.0698311	158.6899437	7.882602973	0.0166*
Lack of fit	3	68.14782575	22.71594192	1.29454793	0.4185
Residuals	6	120.7900062	20.1316677		
Pure error	3	52.64218047	17.54739349		

The E24 values obtained from the design experiment are shown in two-dimensional contour plots. The x and y-axis of each contour plot indicate the parameter considered while keeping the third remaining parameter constant. The top left contour plot represents the variation in the E24 value as a pH and temperature function. In contrast, the top-right plot indicates the variation in E24 as a function of pH and salinity. The bottom contour plot indicates the variation in E24 values as a function of temp and salinity. The colour scales from green to yellow, where green indicates low E24 values while yellow indicates high E24 values. In the contour plot, the line with E24 values is also shown in figure 8.

3.8 DNA isolation, amplification and phylogenetic assessment

The phylogeny assessment of Isolate 3 was performed. DNA was isolated and amplified using PCR. After the amplification, the DNA was quantified using a nanodrop and qubit fluorometer. Qubit fluorometer estimated DNA yield as 1281.6 µgl−1and had optimal purity. Thus, the obtained amplified DNA was further used for phylogenetic assessment [30-33]. 16S rRNA sequence was 1483 base pairs long and was single-stranded bearing a molecular weight of 450176.00 Daltons. GC% and AT% was 54.96% and 45.04%, respectively. Comparatively, G was the highest, and T was the lowest. The sequence was further analyzed by BLASTn against 16S ribosomal RNA sequences (16S ribosomal Bacteria and Archaea) database using a cut-off value of >95%. The first hit shows that the query sequence matches with *Paenibacillus dendritiformis* strain T168 16S ribosomal RNA gene, partial sequence by 98%, whereas 4 hits were seen in the next go that showed similarity with various strains of *Paenibacillus thiaminolyticus* 16S ribosomal RNA gene, partial sequence. All the similar sequences were further downloaded from GenBank, and a phylogenetic tree was constructed using the neighbor-joining algorithm in Mega software. The phylogenetic tree has been depicted in figure 9.

In contemporary research, the secondary structure of 16S rRNA of *Paenibacillus dendritiformis* strain ANSKLAB02 has demonstrated helical regions with the interior, hairpin, bulge and multi-branched loops that may bind to23S rRNA. The free energy (ΔG) of the rRNA secondary structure for *Paenibacillus dendritiformis* strain ANSKLAB02was calculated to be − 131.00 kcal mol−1 been elucidated using UNAFOLD software. The structure has been depicted in figure 10. The thermodynamics result from each base of the *Paenibacillus dendritiformis* strain ANSKLAB02dataset shows the average energy of external closing pair helix, stack, multi-loop, bulge loop, and hairpin loop, as,-299.80 kcal/mol, −2.03 kcal/mol,

0.11 kcal/mol,0.210 kcal/mol and −1.62 kcal/mol, respectively. The closing pair and interior loop had ΔG value = − 2.11 kcal/mol. Further, the two TNA structures entropy was estimated using the RNA fold server and is depicted as a hill plot in figure 11.

4. Conclusion

Petroleum pollution has been a threat to the environment that arises from various natural and anthropogenic sources. These complex toxic compounds have to degrade so that the by-products or the remnants are also intoxicated. The present study identifies the problem and introduces marine bacteria producing biosurfactant that was isolated from brackish water. The bacterial biosurfactant increases the surface area of hydrophobic water-insoluble substrates and hence, increases the solubility and bioavailability of hydrocarbons for their bioremediation. These can stimulate the growth of oil-degrading bacteria, thus, improving their ability to utilize hydrocarbons. The purified materials can be implemented in bioremediation processes or can even act as bioemulsifier-overproduced by the bacteria.

Further, studies need to execute on the genetic deviation or alteration of an expression vector, where the newly introduced *Paenibacillus* sp. ought to be promiscuous for the cause. In the current study, four isolates from coastal water surface extracellularly produced the biosurfactant. Different assays and tests evaluated all bacterial isolates to ascertain their capability to produce biosurfactants. The isolate with maximum biosurfactant yield belonging to *Paenibacillus* sp. (Isolate 3) was screened further by performing various assays. The isolate was biochemically characterized, and its phylogeny was estimated. The isolated *Paenibacillus dendritiformis* strain (ANSKLAB02) was grown on MSM medium using crude oil as the Carbon source to produce a biosurfactant producing lipopeptide, which has high stability at a wide range of pH levels, high temperature, and salinity. This bacterium had the most increased emulsification activity (E24 = 73.37%) in crude oil and was found to decrease the surface tension to 36mN/m, indicating its oil-reduction capacity. The probability of reducing the surface tension ultimately depends on the biosurfactant's molecular alignment from a broader perspective. Based on this study, RSM with Box–Behnken experimental design could be used as an effective tool for biosurfactant stability test analysis for bioremediation applications. The selected bacterial strain was modified by optimizing the various essential environmental parameters required for its biosurfactant production. The best outcome was attained in the medium at pH 7.0, 30°C temperature, 4% salinity (NaCl) with starch (2%), and yeast extract (3%). These enhanced settings are used to determine the dry weight of the biosurfactant produced by the microorganism. The novel strain of *Paenibacillus* species had 0.427g of biosurfactant in 100mL of the medium. The data generated from the biosurfactant stability experiments were used to fit a regression model using the parameters such as ph, temp, and salinity to predict the E24 index. R-squared value 0.91obtain from the ANOVA model explains that the regression model we fit was significant, explaining 91% of the data set, and the model p-value obtained was < 0.05, which is statistically significant. Not only the p-value but the individual coefficients of the data were also found to be significant. The most significant parameters are first-order, two-way interactions, and pure quadratic terms such as ph, ph*temp, ph*sal, temp*sal, temp^2, respectively, are significantly associated with the E24 index. Therefore the statistical model obtained in the present research can be used to predict the E24

index. The isolated *Paenibacillus* strain was found quite promiscuous. However, further investigation is required to increase the efficacy of lipopeptide biosurfactant activity.

Declarations

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6. Competing interests

The authors declare that they have no competing interests.

7. Author's Contribution

AN involved in Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. RK involved in Data curation, Formal analysis, Validation, Visualization. SKS involved in Investigation, Supervision, Writing – review & editing the MS.

8. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

9. Consent for publication

Not applicable.

10. Ethics approval and consent to participate

Not applicable.

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Figures

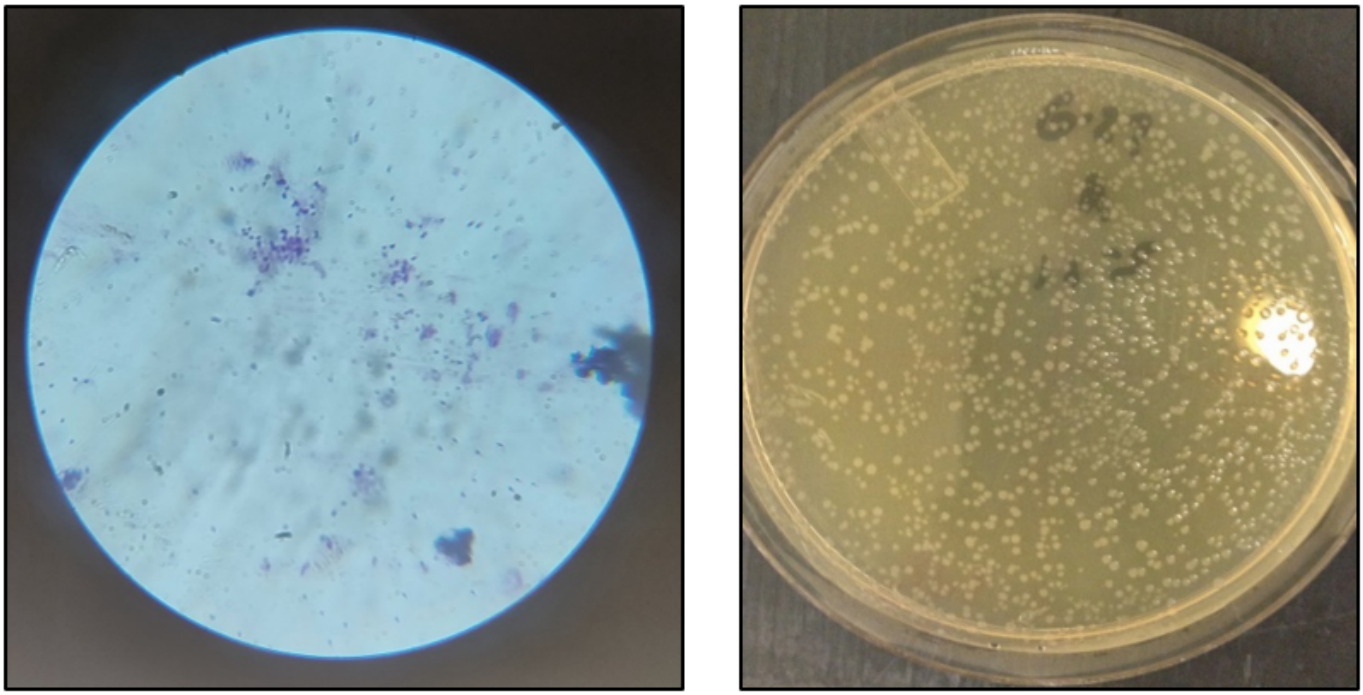

Figure 1

(A) *Bacillus tequilensis(***Isolate 3***)*shows the **gram positive thin rods shaped bacteria were observed on gram staining and (B) shows the yellow pigmented colonies of** *Bacillus tequilensis(***Isolate 3) grown on nutrient medium**

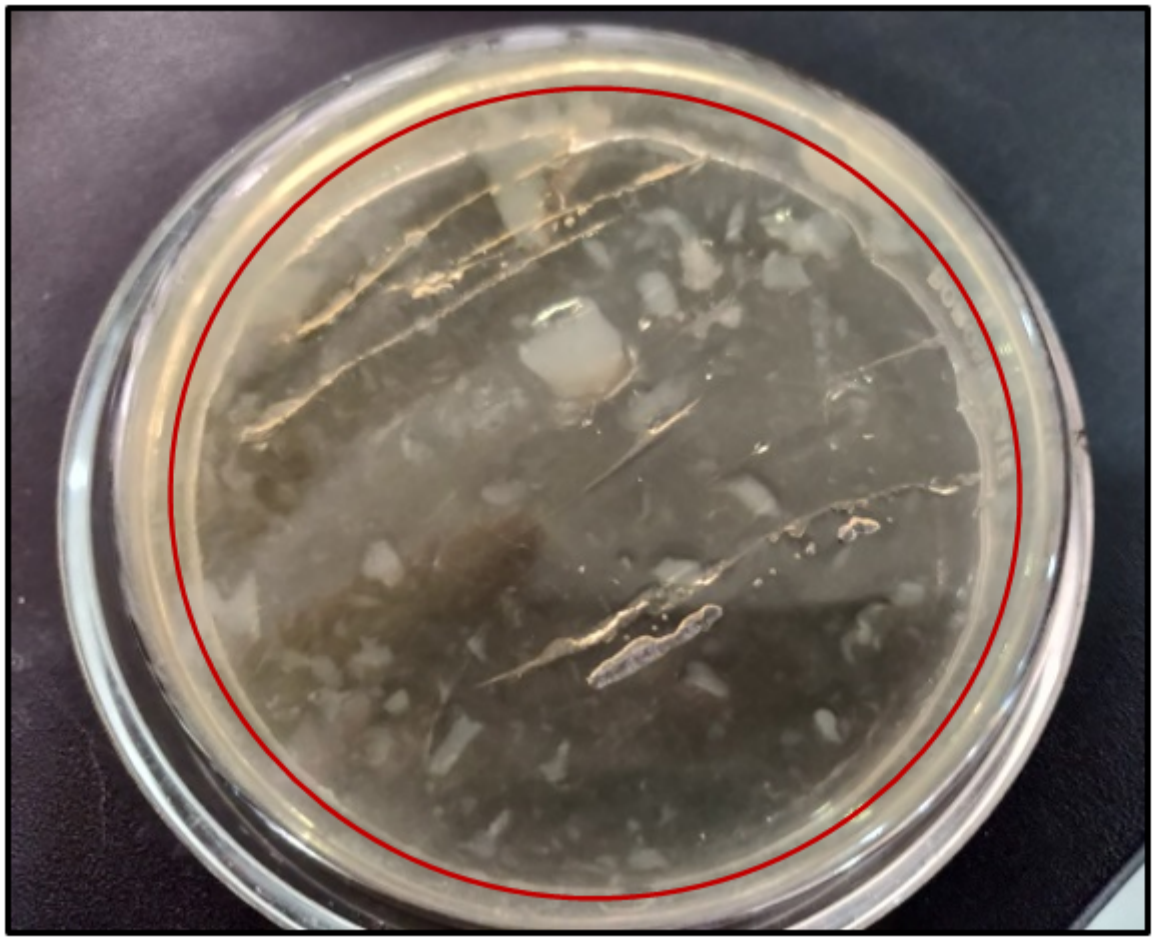

Figure 2

Hydrocarbon Overlay test in hexadecane plated medium. The zone of clearance marked with red circle is indicative of positive test result.

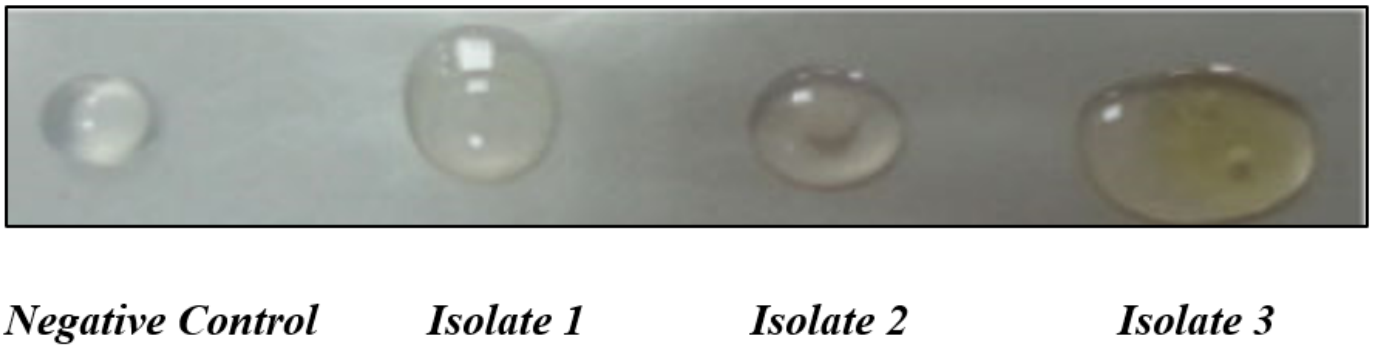

Figure 3

Drop collapse assay showcasing positive results for Isolate 1, 2 and 3, whereas the negative control having water without microorganism depicting the respective drops with surface tension, i.e. absence of biosurfactant

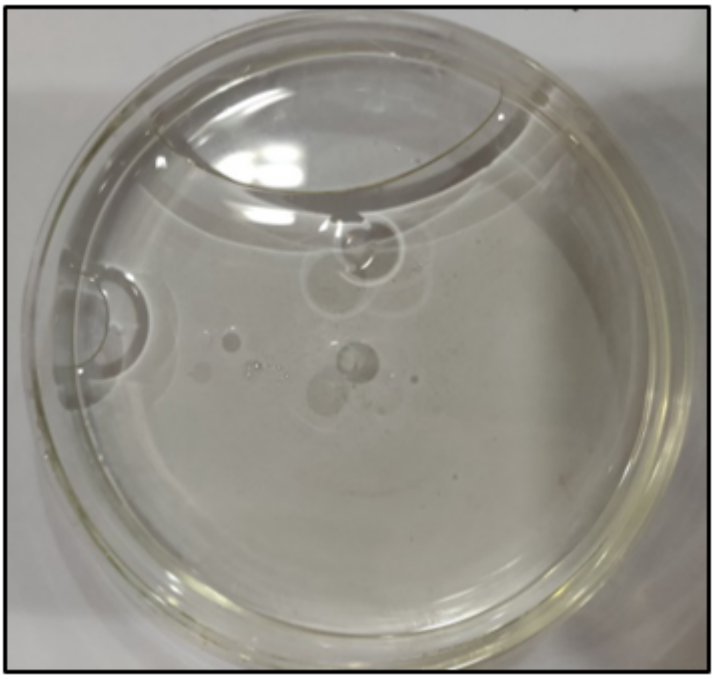

Figure 4

Oil spread test shown by Isolate 3 (*Paenibacillus*). The clear zone observed is indicative of positive test result.

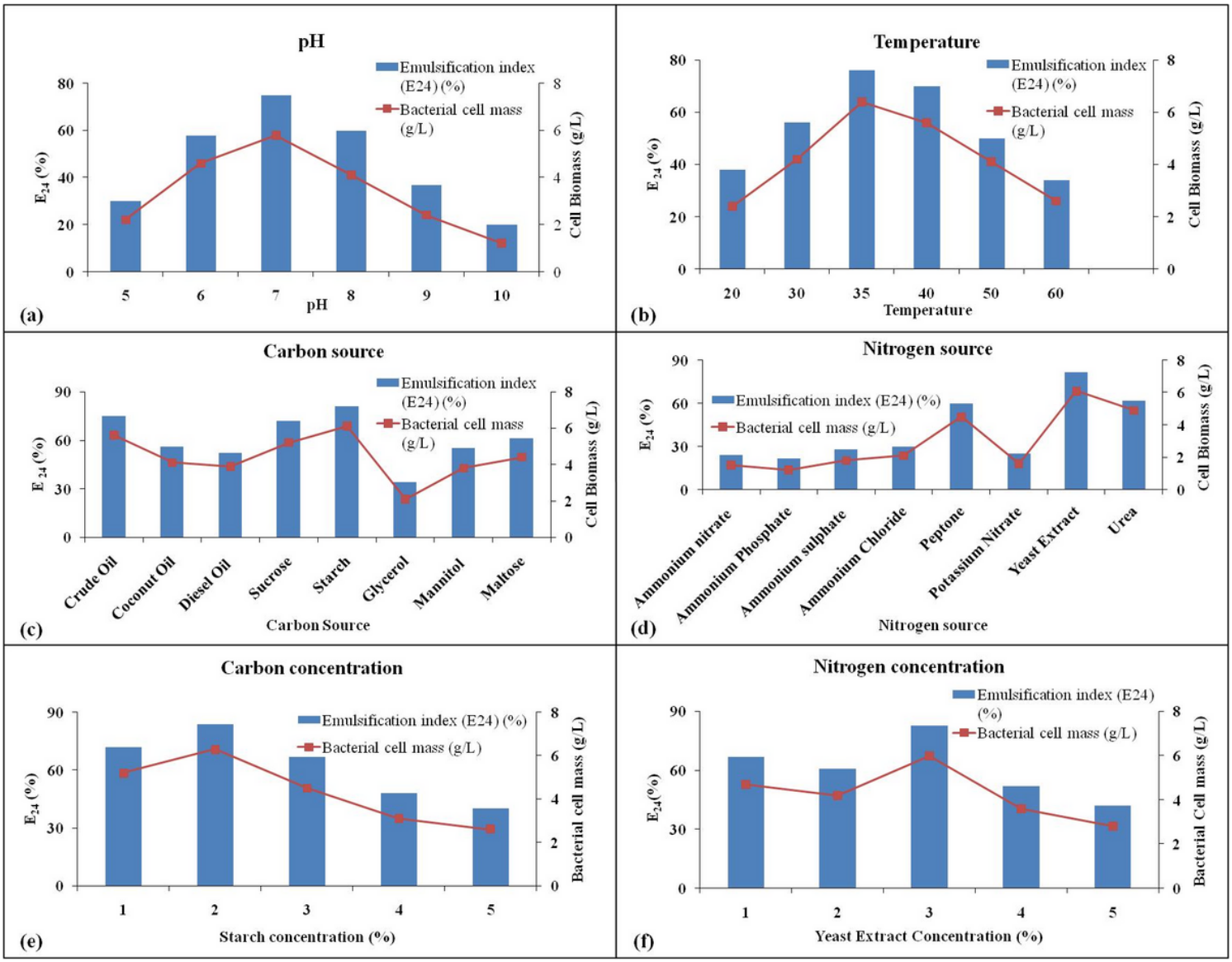

Figure 5

(a) pH, (b) temperature, (c) carbon source, (d) nitrogen source, (e) carbon concentration and (f) nitrogen source concentration optimization to maximize the yield of biosurfactant. Graph depicts the E_{24} index values and cell mass corresponding to varying physical and chemical parameters for Isolate 3

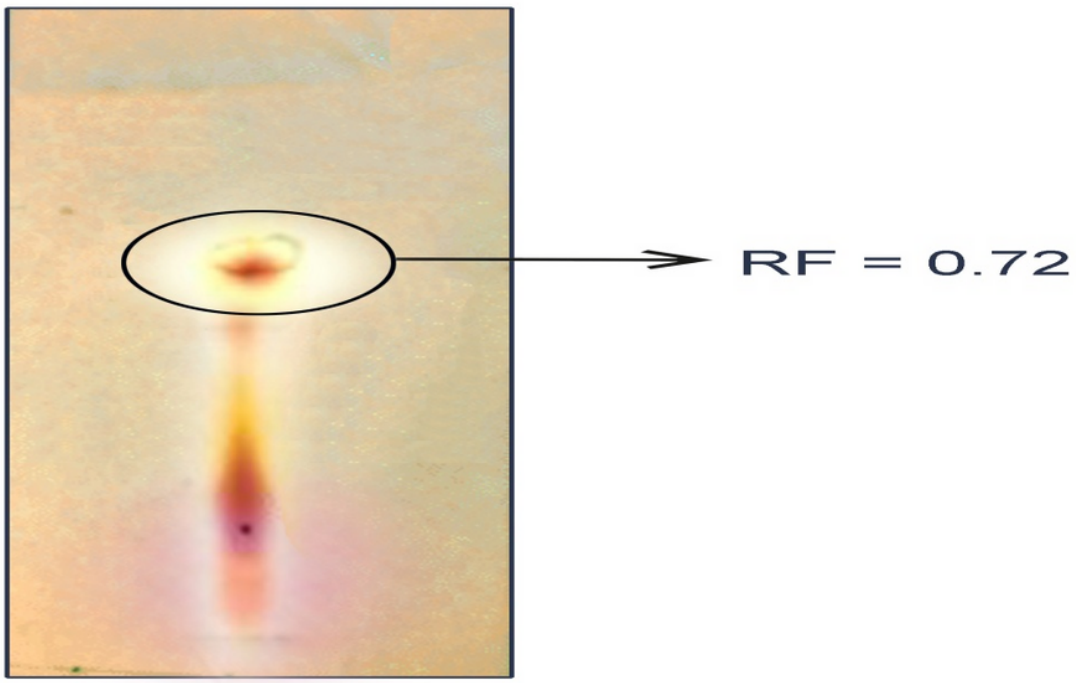

Figure 6

TLC of extracted biosurfactant produced by *Paenibacillus dendritiformis*

3.6 Fourier Transform Infrared Spectroscopy

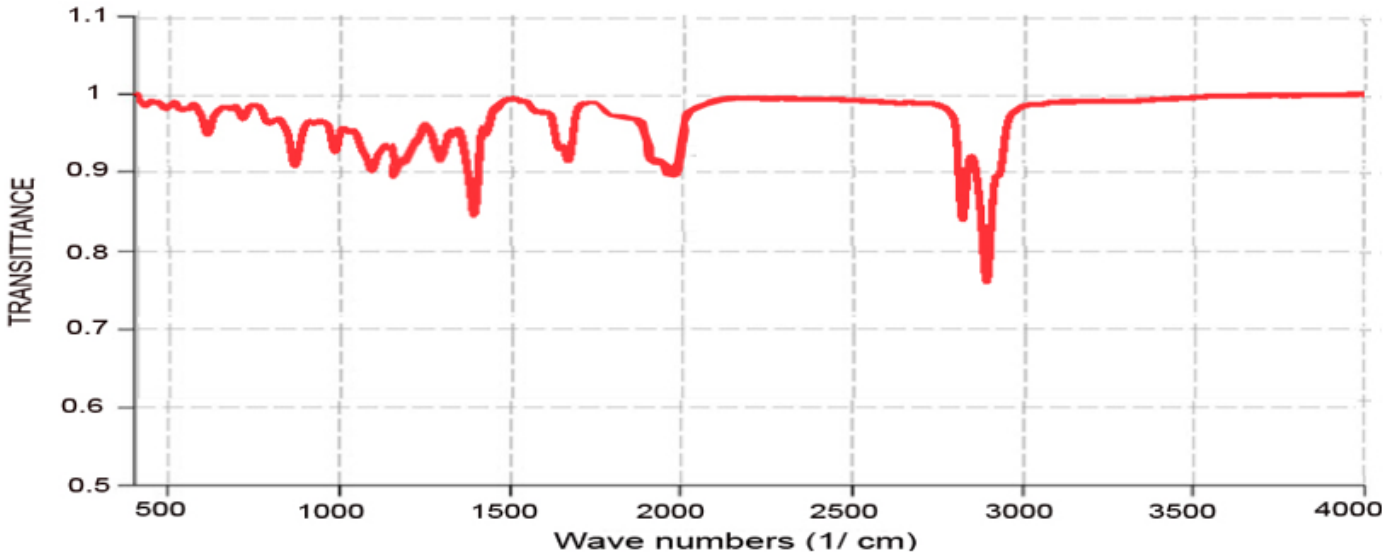

Figure 7

FTIR spectra of solvent extracted biosurfactant produced by *Paenibacillus dendritiformis*

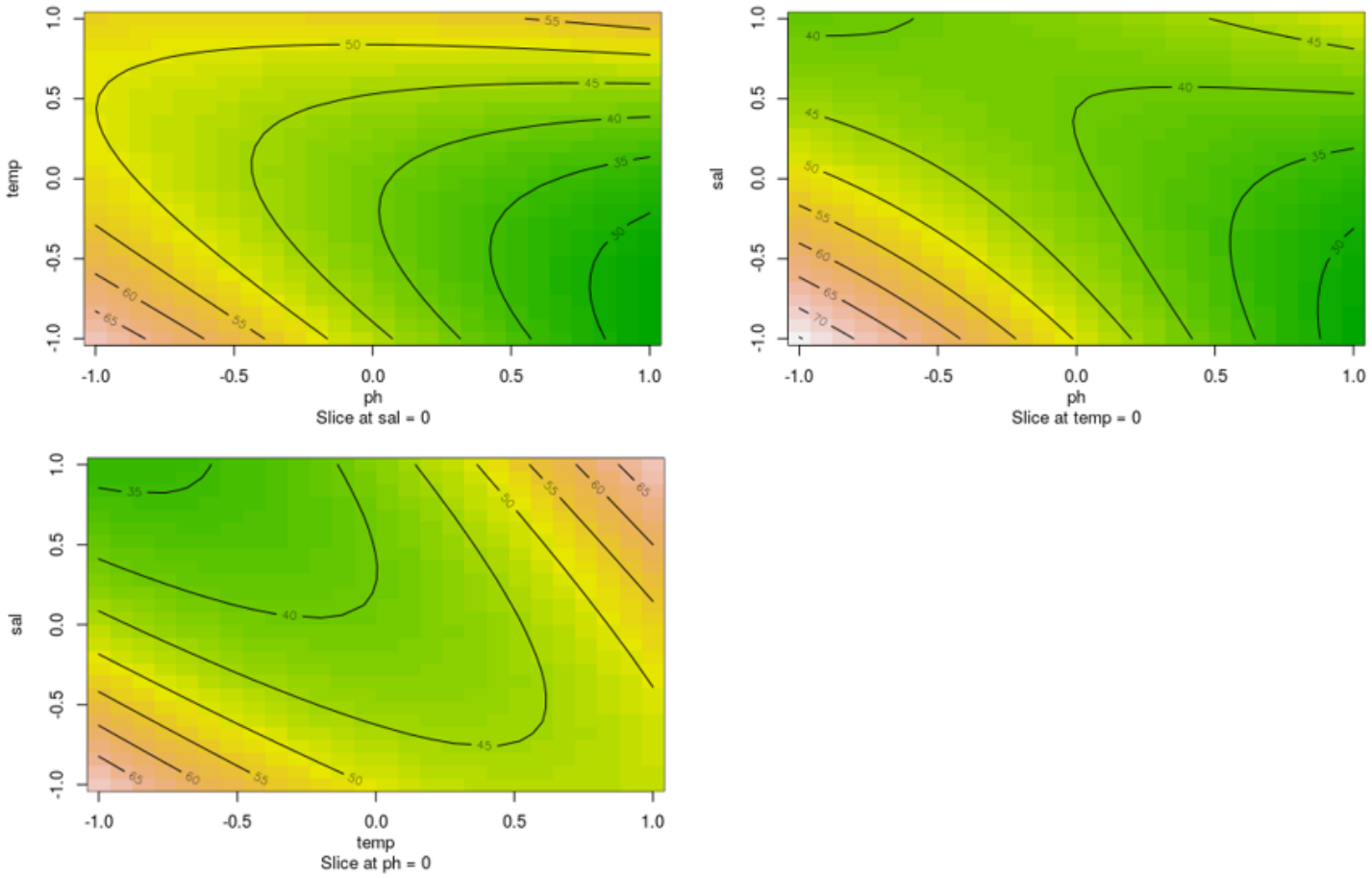

Figure 8

Contour plot of emulsification index (Ei24) of the biosurfactant produced by Paenibacilus dendritiformis against environmental factors (pH, temperature and salinity) after incubation for 24 h.

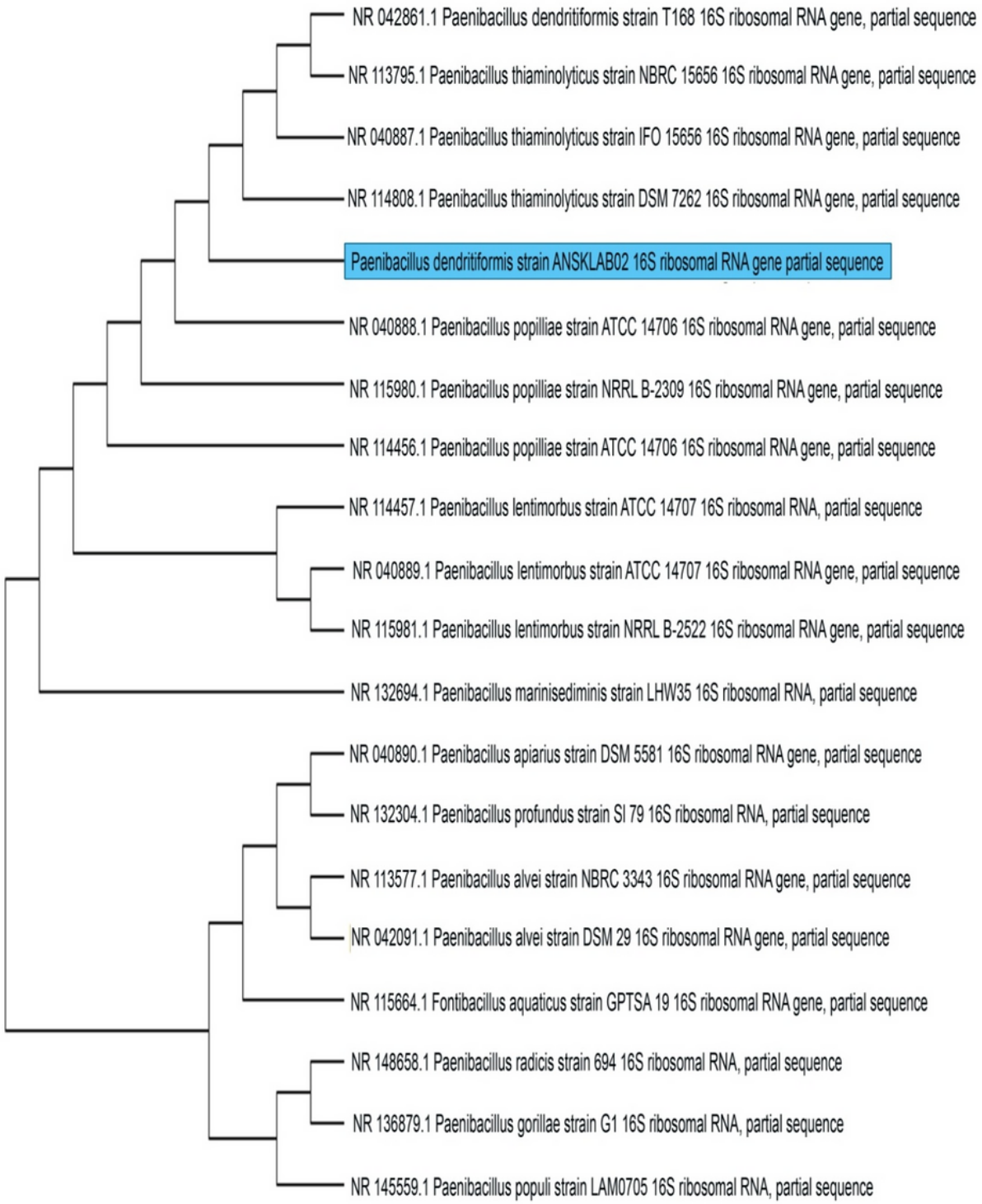

Figure 9

Evolutionary relationships of *Paenibacillus dendritiformis strain ANSKLAB02 against other species of Paenibacillus*

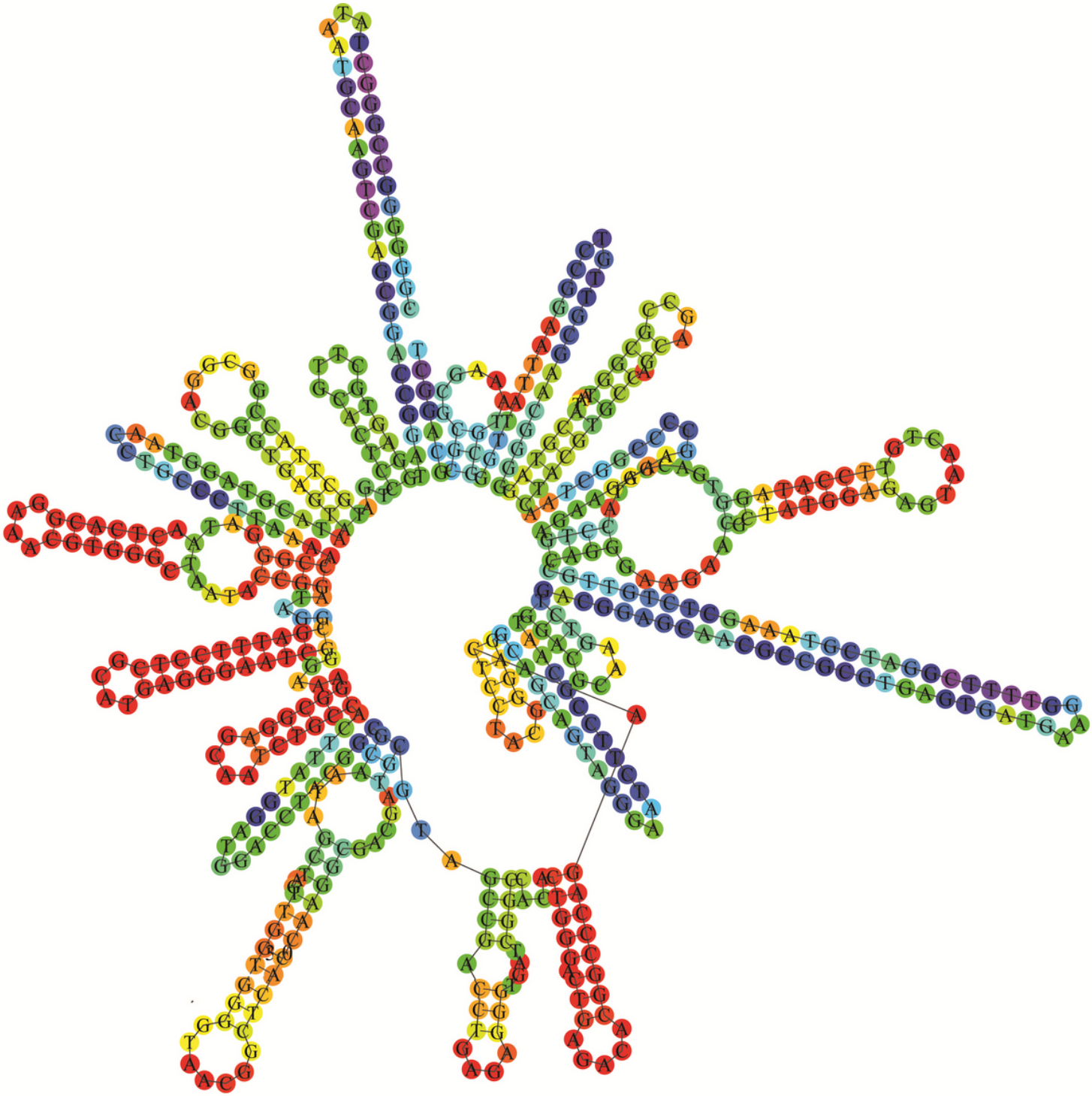

Figure 10

Predicted rRNA structure of the isolated strain through UNAFOLD server

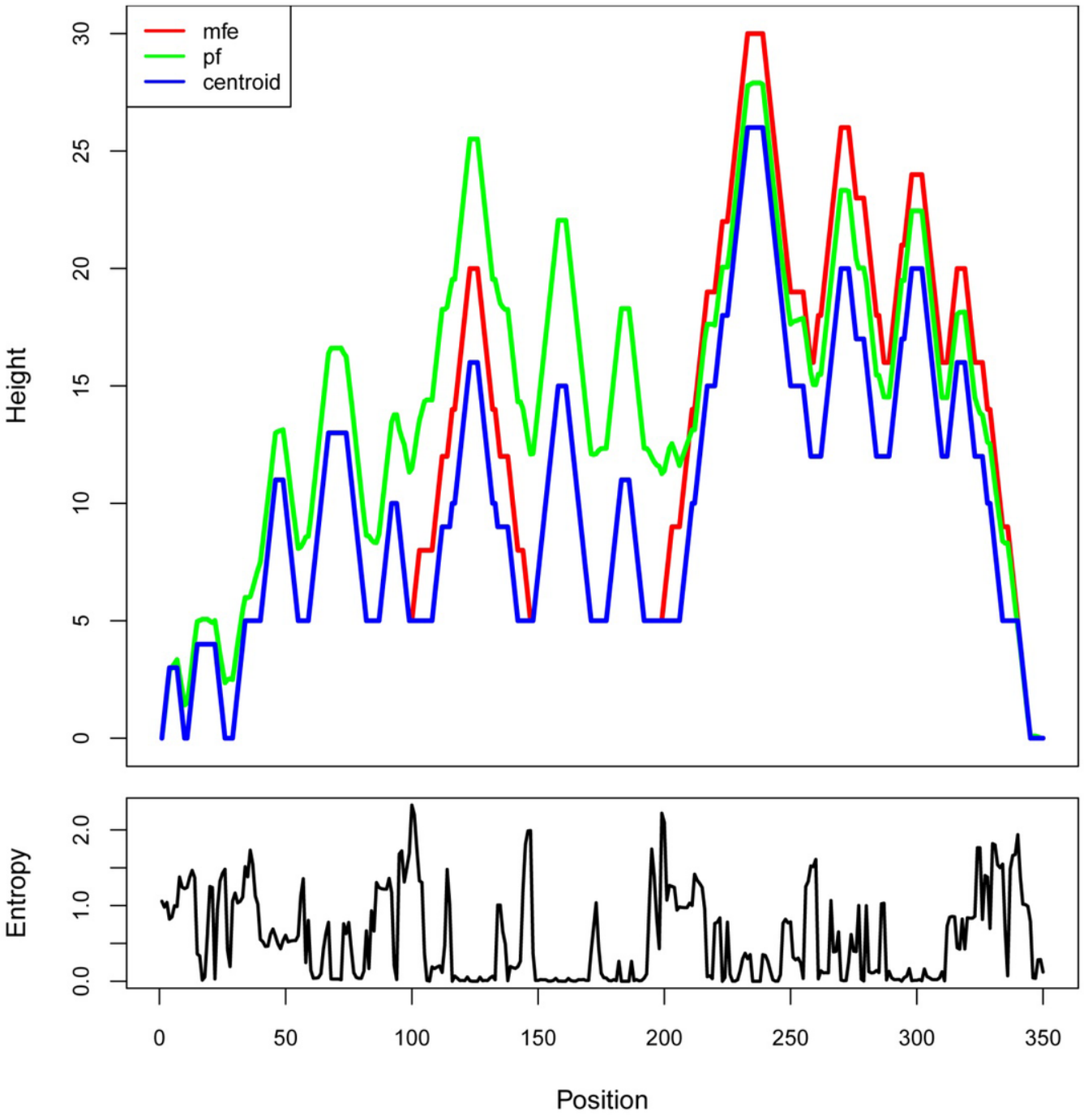

Figure 11

Hill plot representing sequence wise energy and entropy distribution of 16SrRNA for the isolated strain