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Diversity and Distribution of Potential Biosurfactant Producing Bacillus Sp MN 243657, GC-MS Analysis and its Antimicrobial Study

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Biosurfactants are microbially produced surface-active compounds. They are amphiphilic molecules with hydrophilic and hydrophobic regions. The demand for biosurfactants has been exponentially growing as they are nontoxic and biodegradable. They have different applications in several industrial sectors. The objective of this study was to isolate and characterize the native bacteria which produce biosurfactants from oil contaminated soil of different places in Kerala and Tamil Nadu, India. The soil samples were collected from petrol pumps and workshops where the soil is contaminated with petrol, diesel and oil. The bacteria were isolated from contaminated soil samples and confirmed as Bacillus sp.The cultures were screened for biosurfactant production by different screening techniques such as blood hemolysis, oil spreading assay, emulsification ability assay, bacterial adherence to hydrocarbons activity. The potential biosurfactant producing culture was selected and identified using molecular techniques and submitted to NCBI Gene Bank (MN 243657 – Bacillus sp). The selected bacterial culture was used for biosurfactant production and these were characterized by UV, TLC, FTIR and GC -MS analysis. The derived biosurfactant's Rf value was 0.68 as determined by a TLC chromatogram. In a UV-visible spectroscopy study, the isolated biosurfactant displayed a highest peak at 415 nm. According to FTIR analysis, the isolated biosurfactant displayed an intense peak at 3340 cm -1. The large peaks of the biosurfactant were observed at various retention times of 12.75, 10.22, 4.98, and 3.87, respectively, after GC-MS analysis. Antibacterial and antifungal activity of the biosurfactant was identified against pathogenic bacteria such as P.aeruginosa, E. coli, K. pneumoniae, S. aureus and fungi Aspergillus niger, Aspergillus terreus, Aspergillus flavus.

Keywords: Antimicrobial Study; Bacillus sp; biosurfactant; FTIR; GC-MS.

There is a world-wide concern about the liberation of hydrocarbon in the environment of both from industrial activity and accidental spills of oils. Most of the hydrocarbons are insoluble in water. Presence of these hydrocarbons in the environment is of considerable public health and ecological concern due to their persistence and toxicity. Different physical and chemical technologies are employed to clean the contaminated site such as excavation, thermal evaporation, soil flushing and soil vapour extraction[1]. Surfactants are surface active substances which reduces the surface tension

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and increase spreading and wetting properties. Surfactants includes synthetic surfactants and biosurfactants[2]. Majority of the synthetic surfactant production is from petrochemicals. These synthetic surfactants increase the level of environmental pollutions as they are hardly degraded by microorganisms and toxic in nature. Biosurfactants are surface active agents that are amphiphilic in nature which has hydrophilic region that consists of carbohydrate, phosphate, amino acid, alcohol or carboxylic acid and hydrophobic region that contain long fatty acid chain or hydroxyl fatty acids which reduce the surface and interfacial tensions between two immiscible liquids to form emulsions[3]. Biosurfactants can breakdown the hydrocarbon into other less toxic compounds and eventually to water and carbon dioxide. They have many advantages over synthetic surfactants such as lower toxicity, specificity, biodegradability and they can function in wide and extreme conditions and their bio compatibility[4]. Biosurfactants are synthesized by different microorganisms such as bacteria, yeast and fungi by utilizing hydrocarbons as carbon source.

Chemical composition and microbiological origin can be used to categorise different types of biosurfactants. High molecular weight biosurfactants, which show higher surface and interfacial tensions, and low molecular weight molecules, which show lower surface and interfacial tensions, are divided into two classes. Glycolipids like rhamnolipids, sophorolipids, lipopeptides, and phospholipids like gramicidens and subtilysin are examples of low-molecular-weight surfactants, whereas polymeric and particulate surfactants like emulisan, liposan, vesicles, and fimbriae are examples of high-molecular-weight surfactants.

Biosurfactants has many applications in cosmetics, food processing, pharmaceuticals, nanotechnology, textile industries and agriculture[5]. It has important role in pharmaceutical industry because antiviral, anti-adhesive, anti-microbial, spermicidal, hemolytic, anti-inflammatory and immune modulatory properties[6]. They have vital role in agriculture because it can be used for destroying plant pathogen and for increasing the availability of nutrients to the plants[7]. They have many applications in food industry such as solubilizing vegetable oils, improving the antimicrobial and antibiofilm properties and ensures emulsion of food during transport and storage[8]. In nanotechnology biosurfactants act as reducing agents, functionalization, emulsifier and provide self – assembly structures in encapsulation.

The aim of this research is to isolate and screen the native *Bacillus sp* that produces biosurfactants. The biosurfactant was further characterized using, UV - Vis, TLC, FTIR and GC - MS analysis and compared with standard surfactin.

MATERIALS AND METHOD

Collection of Soil Samples

Soil samples were collected from oil spilled surfaces of different automobile workshops and petrol pumps of Kerala and Tamil Nadu, India. Sample sites were dug around 5cm to collect the contaminated soil. Three samples were collected from Kalamassery, Aluva, Palakkad of Kerala, India. Another three samples were obtained from Eachnari,Coimbatore, Madukkarai of Tamil Nadu, India. The samples were collected in sterilized containers, well packed and were taken to the laboratory for analysis.

Isolation of Bacteria from Contaminated soil

The microorganisms of soil were isolated by serial dilution method and plated on nutrient agar and incubated at 37^0C for 24 hrs.[9].One gram of soil sample was suspended in 100mL. of distilled water and vortexed well. Each sample was serially diluted from 10^{-1} to 10^{-4}. After serial dilution, pour plate technique was carried out to isolate bacterial cells colony. The prominent colonies with different morphological characteristics were sub-cultured on nutrient broth (1.3g in 100mL distilled water) and incubated at 37^0C for 24 hrs. and stored at 4^0 C for further studies.

Morphological and Biochemical analysis of the isolates

The isolated bacterial cells were perceived with Gram staining under a compound microscope. (Unicon compound microscope). Bacterial cell's shape such as bacilliwas observed and selected. Biochemical analysis of the bacterial isolates such as Gram staining, Glucose Fermentation, Colonial Characters, Voges Proskauer, Starch Hydrolysis, Methyl Red Test, Catalase Activity, Lipid Hydrolysis, Indole Production, Casein Hydrolysis, Gelatin Hydrolysis, Spores were

carried out. All biochemical assays were performed according to Bergey's Manual of Systematic Bacteriology[10].

Confirmation of Bacteria Using Specific Medium

Screening of the *Bacillus sp* was done on Starch agar (25g in 100mL), Casein agar (6.3g in 100mL) and Bacillus agar (4.1g in 100mL) medium. Bacterial strains were inoculated to differential medium by streaking method and incubated at 37^0C for 24 hrs. Bacterial colonies which produced clear zone in starch agar and casein agar were inoculated in Hi Chrome Bacillus agar medium and incubated at 37^0C for 24 hrs. for further confirmation of bacteria. After incubation, based upon the morphology and pigment production, *Bacillus sp* was confirmed and used for further studies[11].

Screening of Bacteria for Biosurfactant Production

The bacterial isolates were inoculated into 100 mL of Minimal Salt Medium (MSM) [D-glucose 3g, $NaNo_3$ 0.4g, K_2HPo_4 0.34g, KH_2PO_4 0.06g, Yeast Extract 0.12g, (Himedia, Mumbai, India) Water 100mL] and supplemented with diesel (1%) w/v) as the carbon source and incubated at 37^0C for 7days. After 7 days incubation in an orbital shaker, the extraction of Biosurfactant was done by centrifugation. Minimal Salt Medium (MSM) and Zobell medium can be used for the production of biosurfactant by *Bacillus sp* has proved in the studies of Saritha et al[10]. The culture media were centrifuged at 8000 rpm for 10 min (Remi R-8C) and supernatant was collected for screening methods. This cell free culture broth was used for screening tests such as Hemolytic activity, Oil spreading assay, Emulsification ability assay, Bacterial Adherence to Hydrocarbon Activity, Drop collapse assay[12].

Hemolytic activity

Hemolytic activity is the preliminary screening test to recognize the biosurfactant producing bacteria. Hemolytic activity was performed using the procedure by Ewida& Mohamed[13]. Human blood (5%) was added to nutrient agar medium for preparing blood agar plates. Isolated bacteria were streaked in blood agar plates and incubated for 24 hrs. at 37^0C. Then the bacterial colonies were visually analyzed for the presence of zone of inhibition. The presence of zones around the colonies indicated the presence of biosurfactants with hemolytic activity.

Oil spreading assay

Oil spreading assay was accomplished as narrated15. by Phulpoto et al[14]. with slight modifications. The hypothesis of this method is that biosurfactants can change the angle of reciprocal action of oil and water. 50 µL oil was added to the surface of 30mL of distilled water in a Petri plate (70x15mm) to form a thin oil layer. 5 µL of the culture supernatant are gently pipetted on the center of the oil coated thin film layer and after 5minutes, observations were recorded. If the biosurfactant activity is present, a clear zone will be produced by displacing oil. The diameter of zone corresponds the biosurfactant activity. The biosurfactant will produce a surface pressure to displace the oil. It is called as oil displacement activity. Distilled water without cell free supernatant was maintained as negative control, in which no clear zone was detected.

Emulsification ability assay

The Emulsification ability was assayed by calculating the emulsion index as described by Saritha et al.[12], 500 µL oil was added to 1mL cell free supernatant and vortexed at high speed for 3minutes. Then kept undisturbed at room temperature for 24hrs. After 24hrs the height of the stable emulsion layer and height of the liquid were determined. The emulsion index E was estimated as the percentage of the height of the emulsion layer after 24hours with the total height of liquid. Distilled water was maintained as negative control.

$$E = [\text{ Height of the emulsion layer after 24 hrs / Total height of the liquid }] \times 100$$

Bacterial Adherence to Hydrocarbon Activity

Bacterial adherence to hydrocarbons was assessed by measuring the hydrophobicity of cells as described by Ambrin et al[15]. The cell culture was centrifuged for 10000 rpm for 10min. (Remi R-8C). The cell pellets were collected and supernatant discarded. The pellets were washed twice with Phosphate buffer to eliminate the interfering solutes. Then cell pellets were resuspended in Phosphate buffer solution (pH 7.0). Optical Density of this suspension was measured at 600nm in a Spectro photometer. 50µL of oil was added to 1mL of cell suspension and vortexed for 3min.

After vortexing, crude oil and aqueous phase were allowed to separate for 2hours. OD of the aqueous phase was measured with a UVSpectro photometer at 600nm. Hydrophobicity was calculated by following formula.

% *Bacillus* adherence= [1- (OD of aqueous phase ÷ OD of the initial suspension)] x 100

Drop collapse assay

Drop collapse assay was carried out with a modified procedure of Rani et al[16]. Drop collapse assay explains about the destabilization of a liquid drop by biosurfactant containing cell free supernatant on the oil surface. Sterile Petri plates were coated with oil and stabilized for 1hr to make sure a consistent oil coating. 1 µL cell free supernatants were put onto the center of the petri plate. The reactions were observed visually with the help of a magnifying glass after 15min. If the drop of the supernatant was collapsed, the result was marked as positive. If the drop persisted beaded, the result was marked as negative. A negative control was kept with distilled water without biosurfactant.

Identification of Bacteria Using Molecular Techniques

After screening techniques, the potential biosurfactant producing cultures were selected for molecular techniques. Bacterial genomic DNA was isolated using Phenol Chloroform method[17] and used as template to amplifying the 16S rRNA gene by Polymerase Chain Reaction (PCR). The specific primer 16SF (5'–AGAGTTTGATCMTGGCTCA G -3') and 16SR (5'-ACGGYTACCTTGTTACGACTT-3') was used to amplify the 16S rRNA. The PCR was run with Applied Biosystems Thermal Cycler by following thermal cycle; initial denaturation for 5 min at 94° C, denaturation for 30 sec at 94° C, annealing for 30 sec at 55 ° C. and extension for 1.30 min at 72 ° C and the final extension for another 5 min. at 72 ° C. The cycles were repeated for 35 times. Amplified products were segregated in 1.5 % of agarose gel with 0.5 µg/ mL Ethidium bromide and was imaged using Biotech Submarine Gel System.

Amplified 16S rRNA fragments were purified and the products were sent to Chromus Biotech, Bangalore for sequencing analyses. The obtained16S rRNA gene sequences were uploaded in Basic Local Alignment Search Tool (BLAST) to determine the identity of the isolates based on gene sequence similarity. Phylogenetic tree and molecular evolutionary analyses were done using MEGA10 software. The obtained 16S rRNA gene sequences were submitted to NCBI Gene Bank for accession number.

Extraction and purification of biosurfactant

The extraction of biosurfactant was done using the acid precipitation procedure by Phulpoto[14]. The biosurfactant extraction was carried out by centrifugation (10,000rpm for 15minutes) of MSM broth culture. Cell free supernatant was precipitated by 6N HCl with pH 2.0. This was stored in refrigerator at 4^0C for overnight. Precipitated cell free supernatant was centrifuged at 10,000rpm for 15minutes (Remi R-8C) and obtained pellet was maintained at pH 7.0 by adding deionized water. The crude biosurfactant was retrieved from pelleted precipitates by adding methanol and chloroform (1:2 ratio) and vortexed for 5minutes. The mixture was kept undisturbed for 30minutes and collected the lower layer containing biosurfactants and evaporated to dry at 37^0C for 1hour. This was used for further characterization of biosurfactants.

Biomass Estimation

The biomass was estimated by dry mass method as explained by Araujo et al[3]. Weight of empty Eppendorf tube was taken initially. 1mL crude broth culture was added to the same Eppendorf tube. These tubes were centrifuged at 10,000 rpm for 15 minutes (Remi R-8C). Supernatant was discarded and pellet was washed with distilled water and evaporated to dry by heating at 50^0C. Then measured the weight of the Eppendorf tube with the pellet. Difference of empty tube's weight and Eppendorf tube with pellet were taken for estimation of the biomass. Cell biomass was estimated by the following formula

Cell biomass= [(mass of the tube with pellet – mass of empty tube) ÷2]x1000

Analytical Method

Thin Layer Chromatography

The preparatory characterization of the extracted biosurfactant was done with thin layer chromatography after calibrating the Retention factor value. The extracted biosurfactant (10µL)

was loaded on TLC plate using capillary tubes and the biosurfactant was separated with the mobile phase of chloroform: Methanol: Water (60:15:2 – v/v/v). After three quarter movement of the sample, the plate was dried in room temperature and sprayed 1% ninhydrin for the clear visualization of biosurfactants. Commercial Surfactin from Merck, Mumbai was used as a positive control in TLC biosurfactant characterization. Retention factor (Rf) values of each sample was calculated by the following formula.

Rf value = [Distance travelled by solute/ Distance travelled by solvent]

UV-Visible spectroscopy analysis

UV spectrophotometer is a widely used analytical technique to estimate the quantity of chemicals in the solution. The chemical's reflective properties are based on the wavelength of radiated light. The extracted biosurfactants was further characterized by UV-Visible analysis (Labtronics LT2204 Spectrophotometer) in the range of 200-800nm with the scanning interval of 100mm[18].

Fourier Transform Infrared Spectroscopy (FTIR)

Biosurfactants were analyzed using FTIR spectrum for the detection of various types of functional groups. It can be utilized to recognize the components of the unknown composition mixture. The FTIR spectrum was analyzed using dried sample with potassium bromide pellets by Shimadzu FTIR -8200 (Japan). About 1mg of dried biosurfactant was ground with 100mg of potassium bromide and pressed to produce transparent pellets. Biosurfactant samples were placed directly under the infrared beam which were analyzed by FTIR for detailed structural analysis with a resolution of 4cm $^{-1}$ in the spectral region of 4000 – 550 cm $^{-1}$. The comparison studies were carried out with the standard Surfactin (Merck Mumbai).

Gas Chromatography - Mass Spectrometry Method

Gas chromatography - mass spectrometry (GC – MS) is an analytical method which combines the properties of gas chromatography and mass spectrometry to recognize the various substances with in a sample. 5% HCl methanol was assorted with 10mg of sample biosurfactant. 1mL of sterile water added to put out the reaction. Methanol was added to retrieve the sample. 1µL of biosurfactant sample was placed into a gas chromatograph machine (Agilent). The carrier gas, Helium was utilized at the constant flow rate of 1.5mL min $^{-1}$ and the GC injector's functioning temperature was at 260 ^{0}C. The ion source temperature was synchronized at 280 ^{0}C. 90.67 minutes were the total GC running time which was scheduled by GC – MS analyst. The identifications of peaks in chromatographs by comparing with references of mass spectra and NIST08 mass spectral data base[19].

Antimicrobial assay

The antimicrobial activity of the biosurfactant was measured using well diffusion method[20]. The antibacterial activity was done against Gram positive and Gram-negative bacteria such as *Paeruginosa, E coli, K pneumoniae, S aureus* and antifungal activity against *Aspergillus niger, Aspergillus terreus, Aspergillus flavus*. Muller Hinton Agar medium (3.8g in 100mL) was prepared and 70µL of bacterial culture *P aeruginosa, E coli, K pneumoniae, S aureus* were spread. 5mm diameter agar well was prepared with the help of a sterilized stainless cork-borer. 10µL of extract was added to each well. Ampicillin disc (10mg) was kept as a positive control. DMSO (Dimethyl sulfoxide) was set as negative control and plates were incubated at 37^{0}C for 24hrs. Diameter of zoneof inhibition indicates the antibacterial activity.

Malt extract agar medium (5g in 100mL) was prepared according to the manufacturer's instructions (Hi media) and poured in petri plates. After solidification, 10mm diameter wells were made using sterilized stainless cork-borer. 80µL of the fungal spores were added to well and incubated at 30^{0}C for 2-5 days. 5µL Fluconazole (antifungal drug) was kept as standard and DMSO was set asnegative control. Antifungal activity was evaluated by measuring the zones of inhibition in mm[21].

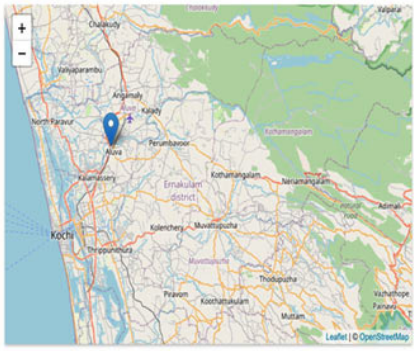
RESULTS AND DISCUSSION

Isolation and confirmation of bacteria from oil contaminated soil

Six bacterial strains were isolated by serial dilution plate procedure from oil contaminated soil of different places of Kerala and Tamil Nadu, India. The result showed highest bacterial colony

Aluva, Kerala, India Geographic Information

Country	India
Latitude	10.107796
Longitude	76.350693
DMS Lat	10° 6' 28.0656" N
DMS Long	76° 21' 2.4948" E
UTM Easting	6,47,993.79
UTM Northing	11,17,637.02
Category	Towns
Country Code	IN
Zoom Level	11



Palakkad, Kerala, India Geographic Information

Country	India
Latitude	10.784703
Longitude	76.653145
DMS Lat	10° 47' 4.9308" N
DMS Long	76° 39' 11.3220" E
UTM Easting	6,80,749.18
UTM Northing	11,92,662.14
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Country Code	IN
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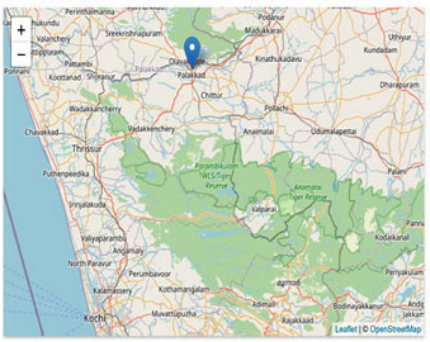

Kalamassery, Kerala, India Geographic Information

Country	India
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Longitude	76.315773
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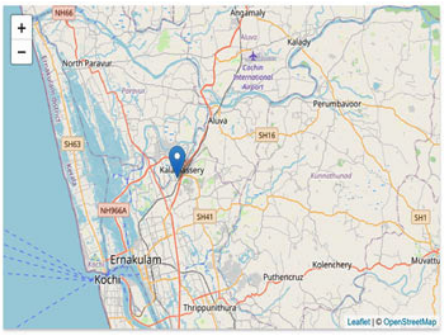
DMS Long	76° 18' 56.7828" E
UTM Easting	644191.82
UTM Northing	1111447.39
Category	Cities
Country Code	IN
Zoom Level	10



Madukkarai, Tamil Nadu, India
Geographic Information

Country	India
Latitude	10° 54' 0.00" N
Longitude	76° 58' 12.00" E
DMS Lat	10° 54' 49.3956" N
DMS Long	76° 57' 10.0224" E
UTM Easting	713429.6106
UTM Northing	1207128.253
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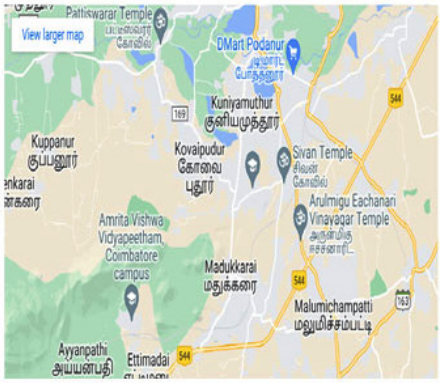
Country Code	IN
Zoom Level	10



Coimbatore, Tamil Nadu, India
Geographic Information

Country	India
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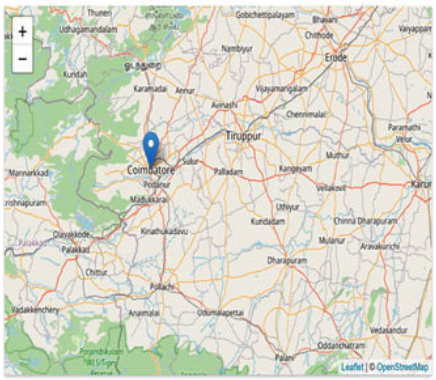
Longitude	76.961632
DMS Lat	11° 0' 16.4016" N
DMS Long	76° 57' 41.8752" E
UTM Easting	7,14,332.01
UTM Northing	12,17,183.77
Category	Cities
Country Code	IN
Zoom Level	11



Eachanari, Tamil Nadu, India
Geographic Information

Country	India
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Longitude:	76.9844677
Latitude DMS:	10°55'44.08"N
Longitude DMS:	76°59'4.08"E
UTM Easting:	716883.18
UTM Northing:	1208831.29
Category	Town
Country Code	IN
Zoom Level	11

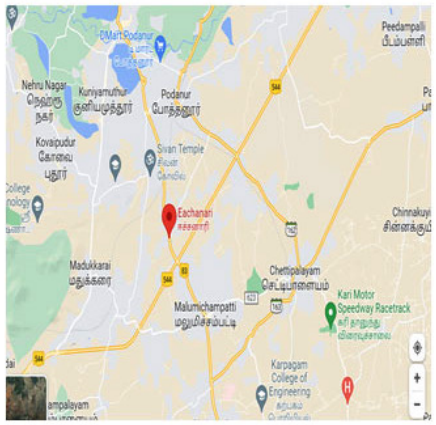

Fig. 1. Map of Sampling Site

count (10.6 x 10^5 CFU/mL) from Kalamassery, Kerala, India. and least bacterial colony count from Madukkarai (1 x 10^3 CFU /mL.) Tamil Nadu, India, as it was the soil of a new petrol pump. In various previous studies such as Deblina et al[22], Ambrin et al[15], nutrient agar was used for the isolation of bacteria. In this study also we used nutrient agar for the isolation of bacteria from contaminated soil. Many researchers have isolated bacteria from soil. Ndibe et al[23] has isolated bacteria which produces biosurfactants from the river. Various bacteria such as *Bacillus sp* and *Arthrobacter sp*. were secluded from clayey soil reported by Prashanthi et al[24]. Identical results were noted in the writings of Naif et al[25]. The sampling site location was depicted (Fig.1). The individual viable bacterial cells in the soil sample multiplied to form an easily visible colony. The number of colonies counted and reported in Colony Forming Units (CFUs). The results of Colony Forming Units were represented in bar graph to visualize the number of colonies of different sampling sites easily (Fig.2).

Biochemical analysis

The bacterial strains were identified as *Bacillus sp* using biochemical reactions and specific medium like Starch agar, Casein agar and Bacillus agar with the reference of Bergey's manual. For further confirmation 16S rRNA study was carried out. The colonial morphological characteristics of isolates were noticed as circular large slightly raised off white smooth colonies. According to cellular morphology, the isolates were Gram positive endospore forming rod shaped cells. Previous literatures such as Ndibe et al[23],

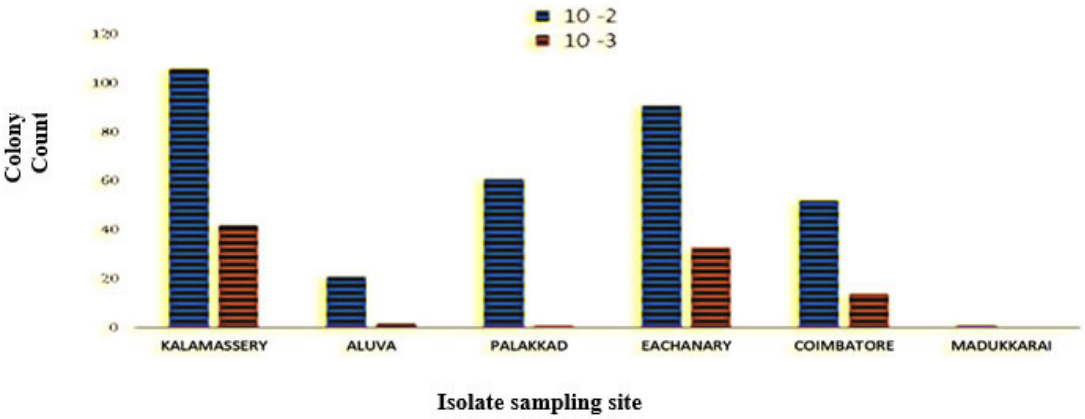

Fig. 2. Bacterial colony population study

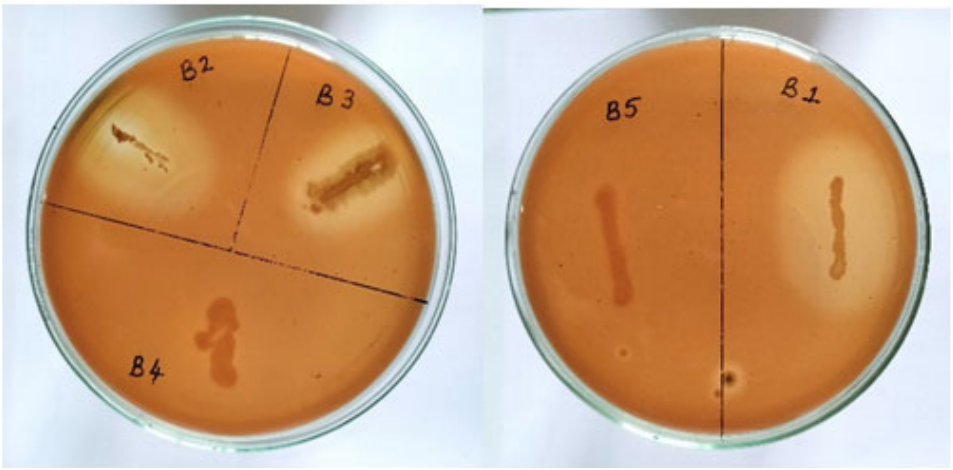

Fig. 3. Hemolytic activity

Table 1. Biochemical analysis of Microorganisms

Bacterial Isolates	Gram staining	Glucose Fermentation	Voges Proskeur	Starch Hydrolysis	Methyl Red Text	Catalase Activity	Lipid Hydrolysis	Indole Production	Casein Hydrolysis	Gelatin Hydrolysis	Spores
B1	Gram +ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
B2	Gram +ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
B3	Gram +ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
B4	Gram +ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
B5	Gram +ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve

Anuraj et al[12] revealed that *Bacillus sp* has these morphological and cellular characteristics. The same features of *Bacillus sp*. were observed in the finding of Prashanthi et al[24]. Ndlovu et al[26] utilized *Bacillus amyloliquifaciens* for biosurfactant production. The biochemical results were outlined (Table1). These identical characteristics and recognition clues were discussed as *Bacillus sp* in the paper of Santos, E. C. L et al [27].

Screening of Bacteria for biosurfactant production

All the bacterial strains were screened for identifying the bacteria which is more capable to produce biosurfactants. The strains which showed superior results were selected for further studies. The *Bacillus sp* showed extremely positive results for various screening tests as mentioned by Deblina et al.[22] Saravanan & Vijayakuamar[28] reported that more screening procedures should incorporate in screening techniques to recognize the capable biosurfactant producers. John Paul et al[9]detailed that *Bacillus sp*. isolated from oil contaminated soil are proficient to produce biosurfactant.

Hemolytic activity

The results of hemolytic activity of three samples were positive (Fig.3). They showed a transparent zone formation around the colonies. The sample B1 and B2 exhibited the topmost hemolytic activity as 2cm. zone. Two samples B4 & B5 were negative, as they didn't show any zone formation. The positive activity of hemolysis by various *Bacillus* strains were described by Ambrin et al[15]. The hemolytic study of *Bacillus* by Rajesh et al[29]. revealed the identical results. NDIBE et al[23]. suggested that biosurfactant producing organisms can be screened by hemolytic activity. The present result of hemolytic activity was authenticated with the result of M. Garg et al[30]. The organisms which produce biosurfactants will lyse the erythrocytes and produce a transparent zone on all sides of colony. Ewida& Mohammed[13]. reported that the transparent zone of hemolysis is equivalent to the biosurfactant concentration. Based on the literature of Dhouha et al[31].2012, Lipopeptide and Rhamnolipid biosurfactants can be screened by hemolytic assay.

Oil spreading assay

Oil spreading assay is more precise and less time-consuming method to disclose the biosurfactants. There is a straight relationship

between the diameter of zone and the presence of biosurfactants[10]. The existence of biosurfactants persuade clear zone formation by oil displacement. The supernatants of all bacterial strains were added to the plates containing oil. All the strains except sample 5 were positive as they expelled the oil to form a clear zone (Fig.4). Based upon the article of Chikodili et al[19],the oil spreading positive results indicate that the isolates can produce biosurfactants efficiently. Parthipan et al[32]. detailed that the efficiency of oil spreading assay is used to evaluate the production of biosurfactant. The greatest oil displacing activity indicated by sample B1. The sample B5 specified a negative activity result. Similar results were illustrated by Thavasi et al[33]. Saritha et al[10] detailed that biosurfactant produced by *Bacillus sp* are potential to produce oil displacement clear zone.

Emulsification ability assay

It is an indirect technique employed to screen the biosurfactant production. If the cell free supernatant consists of biosurfactants, it will form emulsion with the hydrocarbons which carry in the test solution. When a liquid phase is diffused in a continuous liquid phase in the form of droplets, there will be a formation of amalgam. Emulsification ability assayed with cell free supernatant and two organic solvents such as petrol and coconut oil. The emulsification index E is measured as the correlation of height of the emulsion with the liquid's total height. Bacterial strain (B2) showed more emulsification index in petrol (Fig.5). The outcome acquired from this procedure are thoroughly supported by the description given by M. Garg et al[30]. The present result of emulsification assay was verified with the literature of Magalhaeset al[34]. The potential of biosurfactants can be determined by emulsification ability. Shoeb et al[35] narrated emulsifying ability can decide the yield of bio-emulsifier. Saritha et al[10] justified that biosurfactant has high emulsification ability

Bacterial adherence to hydrocarbons activity

The positive cell hydrophobicity described as the sign of biosurfactant production. Cell adherence with hydrocarbon such as petrol is observed as an indirect method to screen biosurfactant producing bacteria. Biosurfactant producing cells can affix themselves with hydrocarbons. The ability of bacterial cells to adhere to hydrocarbons were tested using BATH assay. The results were noted down (Table 2). The sample B1 showed highest cell adherence. BATH assay revealed positive results which are close

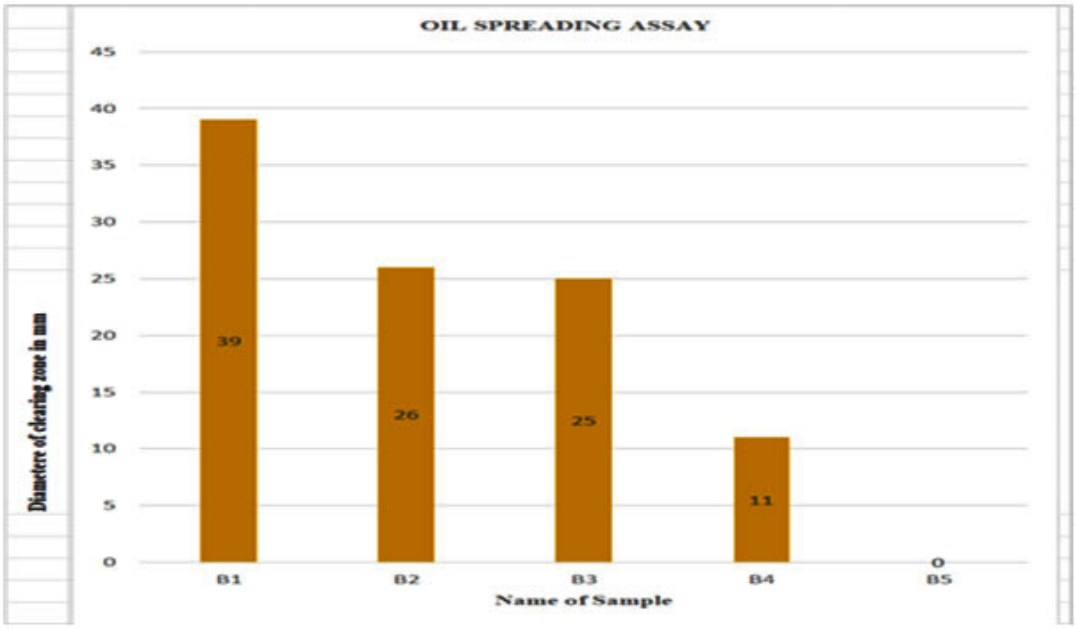

Fig. 4. Oil Spreading Assay

to the studies detailed by Arif et al[36]. Strains of *Bacillus sp* has cell adherence to crude oil which is corroborated with the findings of Ambrin et al[15]. Thavasi et al[33] validated that the positive results of cell hydrophobicity are the sign of biosurfactant production. Anuraj et al[12] authenticated that *Bacillus sp* has the highest cell adherence compared to other bacteria such as *E coli* and *Staphylococcus.*

Drop collapse assay

Drop collapse assay is a liable test and it is not a complicate test to perform. A little amount of cell free broth is needed for this test. The bacterial strains which are potential to generate extracellular biosurfactants indicate the positive result. The results acquired by drops collapse assay was accurate and authentic when compared to the result recorded by Ndibe et al[23]. The drop collapse assay is based on the disruption of liquid drop by biosurfactant containing cell free bacterial extract. The positive collapse test assures the surface activity against oil. If the supernatant contains surfactants, the drops of liquid will collapse as interfacial tension between liquid and hydrophobic surface was decreased. All the bacterial strains except sample 5 showed positive result (Table 3). Collapsed drop approved the presence of biosurfactant in the sample as suggested by Rani et al[16]. Biosurfactant produced by *Pseudomonas* also showed positive result for drop collapse assay correlate the findings of Sumathi et al[1]. Arif et al[36] has done research in biosurfactant production by *Gordonia sp* which work also disclosed that biosurfactant can collapse the drops as interfacial tension decreases. *Bacillus* drop can collapse within 58 seconds which is explained by Anuraj et al[12]., that is verifying current study. The triumphant

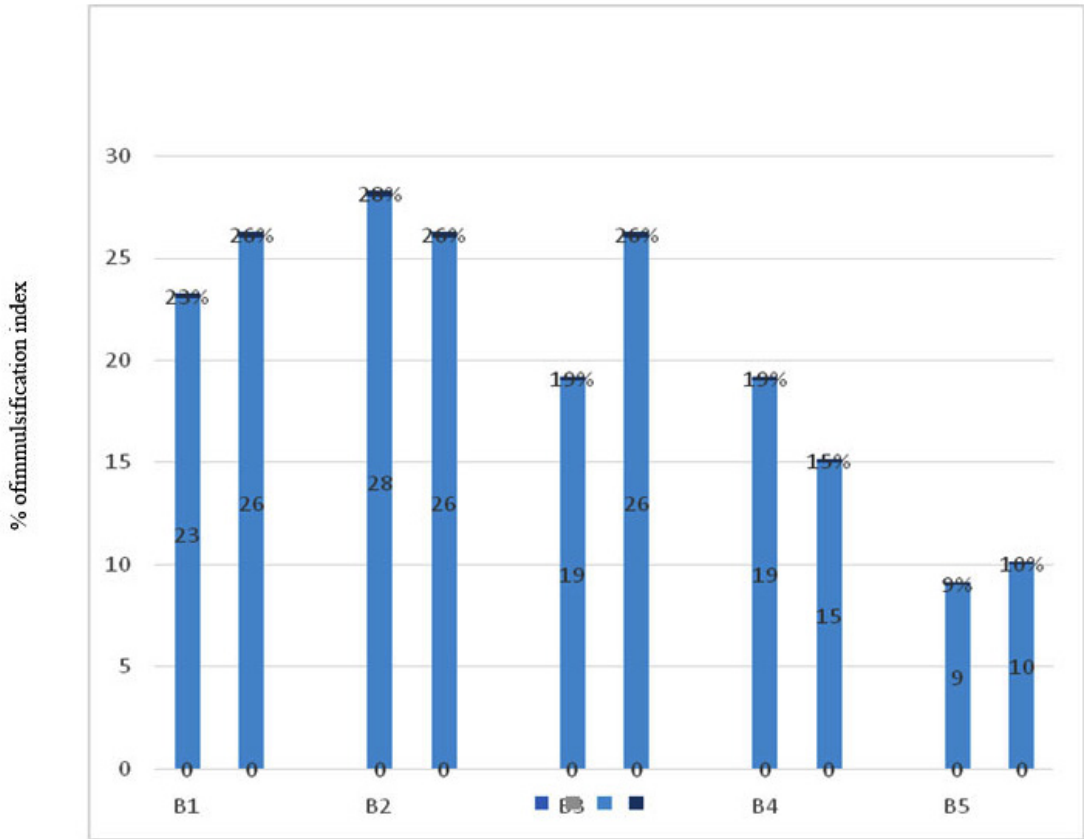

Fig. 5. Emulsification ability Assay

collapse test guarantees the extracellular production of biosurfactant and destabilization cum capability as mentioned by Ewida& Mohammed[13].

Biomass estimation

The dry mass estimation normally gives a much more reliable result than the wet weight. The cell biomass was estimated by taking the difference of empty tube's weight and the Eppendorf tube with pellet. Day 4 showed more cell biomass for all 3strains (Fig. 6). The biomass was determined using dry mass method which was adopted from Araujo et al[3]. The growth of bacterial cells can be tracked by computing the cell biomass[37]. Dry weight of biosurfactant almost close to the result which was narrated by Anuraj et al[12].

Table 2. BATH assay

Sample	A	Ao	H %
B1	0.340	0.993	65
B2	0.372	0.935	60
B3	0.387	0.938	58
B4	0.261	0.402	35
B5	0.189	0.278C	32

Identification of bacteria using molecular techniques

After screening and preliminary identification, DNA of selected bacterial strains were extracted (Fig.7) and 16S rRNA was amplified using Polymerase Chain Reaction (PCR). 1.5 % of Agarose gel with 0.5 µg /mL. Ethidium Bromide was used to verify the PCR products which showed the fragment of 760 base pairs (Fig.8). The amplified 16S rRNA of B2 was purified and performed 16S rRNA gene sequence. Based on BLAST analysis, B2 isolate showed maximum identity 99%. The phylogenetic tree was constructed using MEGA

Table 3. Drop collapse assay

Sample	Drop Collapse Assay
B1	+ + +
B2	+ + +
B3	+ + +
B4	+
B5	-

"+ + + "- Excellent, "+ + "- Good, "+ " - Fair, " – " - Absent

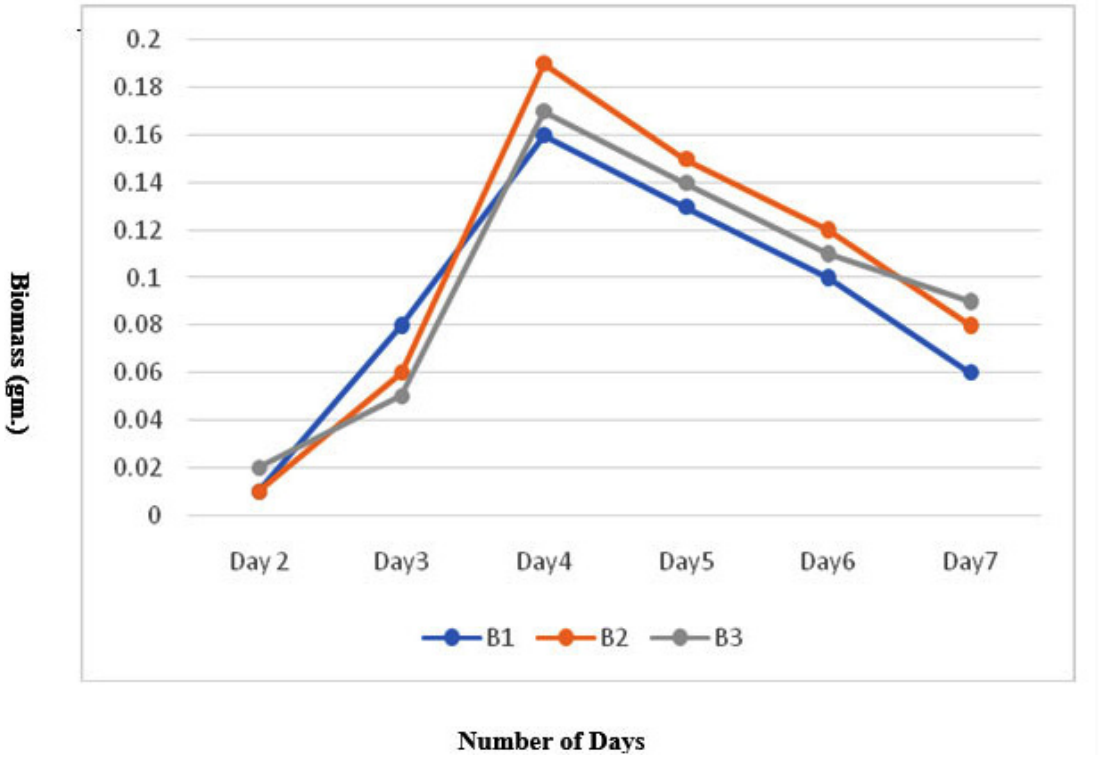

Fig. 6. Biomass Estimation

10 software (Maximum likelihood tree) with 0.1 distance. The Phylogenetic tree for bacterial isolate is shown (Fig.9) Santos E.C.L. et al[27]. has outlined that biosurfactant producing bacteria as *Bacillus sp* by molecular characterization and phylogenetic tree as in the present study. The molecular analysis done by Nurul et al[38]. showed that 16S rRNA gene sequence and phylogenetic studies of genus *Bacillus*, which is more or less similar to the current research work. In hydrocarbon contaminated soil, the presence of *Bacillus sp* and their molecular studies were discussed in the literature of Aabed et al[39]. which corresponds current study. In previous research, molecular study of bacteria from soil by John et al[9] revealed that bacteria as *Bacillus sp*. The gene sequences were submitted to National Center for Biotechnology Information (NCBI) for accession number. Accession numbers were

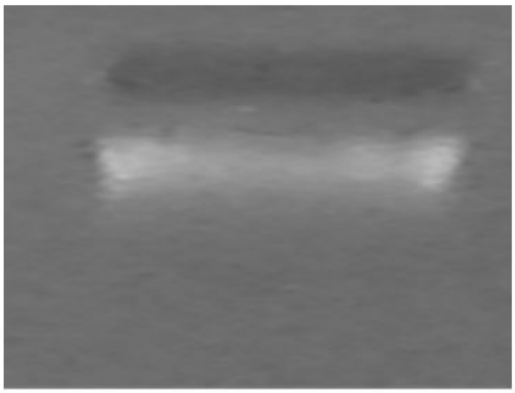

Fig. 7. DNA of *Bacillus sp*
Lane 1: DNA ladder (2kb)
Lane 2: *Bacillus sp* (760 bp)

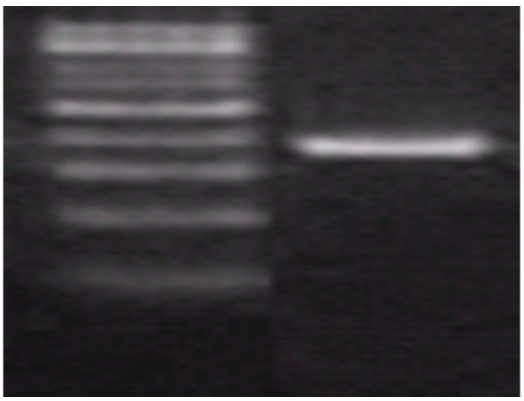

Fig. 8. PCR Product

Fig. 9. Phylogenetic Analysis of *Bacillus sp.*

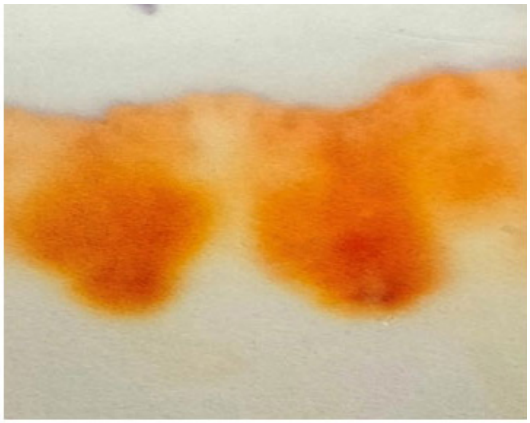

Fig. 10. TLC Analysis of Biosurfactant with surfactin

granted from NCBI as M N 243657 – *Bacillus sp*.

Analytical methods

Thin Layer Chromatography

The crude biosurfactant was scrutinized on the TLC plate that suggested the presence of biosurfactant with lipopeptide nature. This could hypothesis that the extract has different composition. The Rf value of derived biosurfactant was measured as 0.68 in TLC chromatogram (Fig.10). The similar Rf value of the lipopeptide biosurfactants were reported by Joy et al[40]. This preliminary profile revealed that the extracted biosurfactant has the similarities with the standard surfactin (0.71Rf value). Ramyabharati et al[41] also reported similar results with biosurfactants produced by *Bacillus sp* while contrasting with

Table 4. Functional groups identified by (FTIR)

Vibrational Assessment	Observed Frequency	Functional G r o u p	Intensity
O - H	3340.71	alkanes	strong
C=C	1635.64	alkenes	Weak
C-O-C	1211.3	ester carbonyl	strong
C-Cl	686.66	halogen	strong
C-Cl	601.79	halogen	strong
C-Br	563.21	alkyl halides	strong

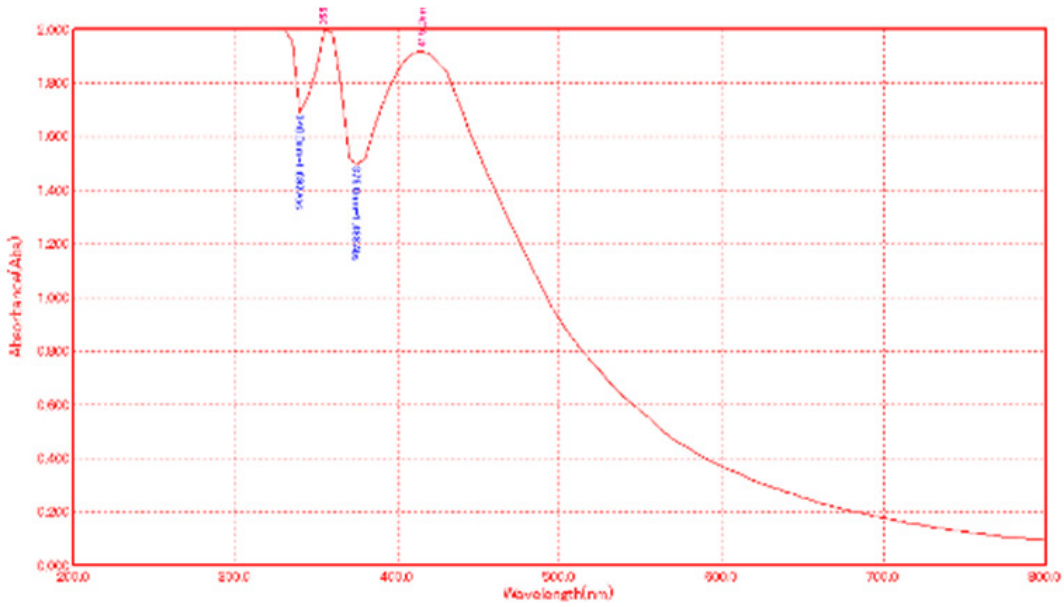

Fig. 11. UV analysis of biosurfactant

standard surfactin. Rf value was calculated by the ratio of distance travelled by solute and distance travelled by solvent. Lipopeptide biosurfactant can be explored using ninhydrin as red spots which was outlined by Ndibe et al[23]. The derived biosurfactant from *Bacillus sp* is closely associated to lipopeptide type according to Rf value data base. The similar findings were observed by the report of MBS Donio et al[42].Sumathi et al[1]reported that biosurfactant such as rhamnolipids produced by *Pseudomonas* shows yellow spots in TLC. Okore etal[43]. authenticated that lipopeptide biosurfactant produced by *Bacillus sp* will develop red spot while spraying Ninhydrin reagent in TLC plate.

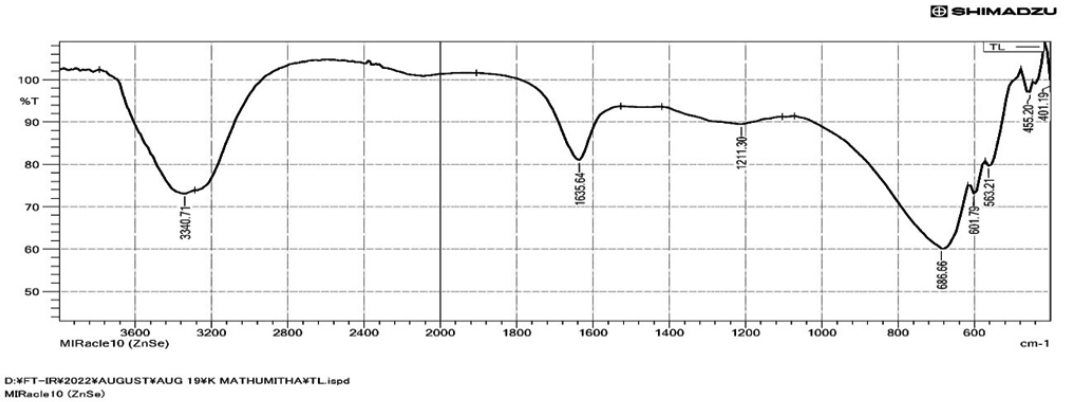

Fig. 12. FTIR analysis of biosurfactant

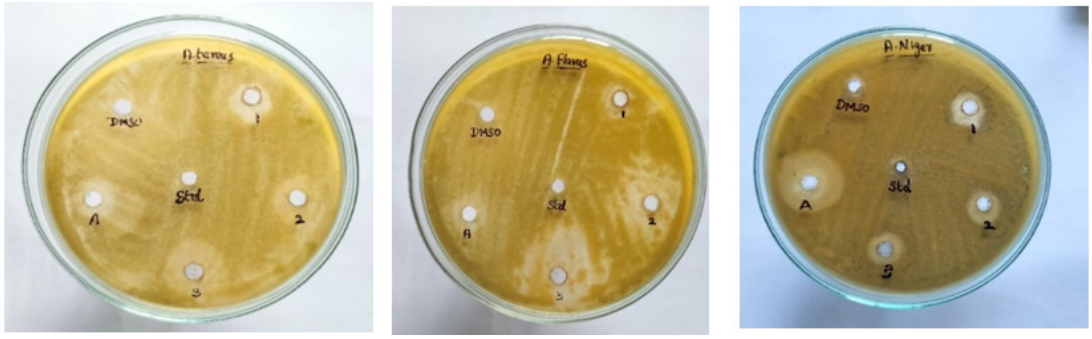

Fig. 13. Antifungal Activity of Biosurfactant with commercial surfactin against human pathogens

Table 5. Major functional compounds recognized by GCMS analysis

Retention time	Functional compounds	Molecular weight	Molecular formula
36.26	Benzeneacetic acid, 4-(2,2,3,3,3-pentafluoro-1-Oxopropoxy)-, methyl ester	166.17	C12H9F5O4
4.31	Silane diol, dimethyl-	92.17	C2H8O2Si
4.62	(S)-(+)-1,2-Propanediol	76.09	C3H8O2
4.74	Silane, ethoxy triethyl-	160.33	C8H20OSi
4.90	1H-Tetrazole	70.05	CH2N4
4.98	Butanoic acid, propyl ester	130	C7H14O2
4.99	4-Pentenoic acid, 2,2-diethyl-3-oxo-5- phenyl-,ethyl ester	274.35	C17H22O3
7.58	D-Alanine, N-(4-ethylbenzoyl)-, decyl ester	361.5	C22H35NO3
9.88	Malic Acid	134.08	C4H6O5
12.75	Succinic acid, 4-cyanophenyl 4- isopropyl phenyl ester	337.36	C20H19NO4

Biosurfactant with Rf value 0.68 was recorded in previous literature of Deepansh et al[44].Surfactin and Iturin by *Bacillus subtilis* were corroborated on TLC by Phulpoto et al[14] with the Rf value of 0.3and 0.75 respectively.

UV-Visible spectroscopy analysis

The production of biosurfactants were confirmed by UV-Visible spectrophotometer. UV-Vis Spectroscopy is a quantitative technique used to measure how much a chemical substance absorbs light. The existence of certain type functional groups can be checked by UV- Vis spectroscopy (Fig.11). The extracted biosurfactant showed maximum peak for *Bacillus sp* at 415nm.The UV absorptions at 340nm revealed the presence of azo chromophore with n- ð* transition. The peak of absorption at 375nm indicates the existence of arene group such as anthracene with n – ð* transition. The results revealed that the biosurfactant is sizeable with complex molecule which consist of many functional groups[18] Dharman et al[45] published that biosurfactant from *Bacillus safensis* displayed 530nm of maximum absorption.

Fourier Transform Infrared Spectroscopy (FTIR)

The extracted biosurfactant of *Bacillus sp* had extreme peak at 3340 cm $^{-1}$ which correlates -OH groups. The existence of this peak denotes that sample carries alcohol group in agreement with M Garg et al[30].Peaks representing C=C alkene group

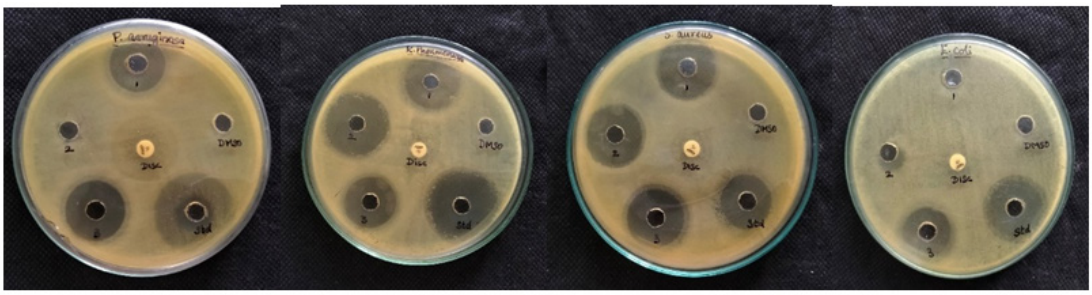

Fig. 14. Antibacterial Activity of Biosurfactant with commercial surfactin against human pathogens

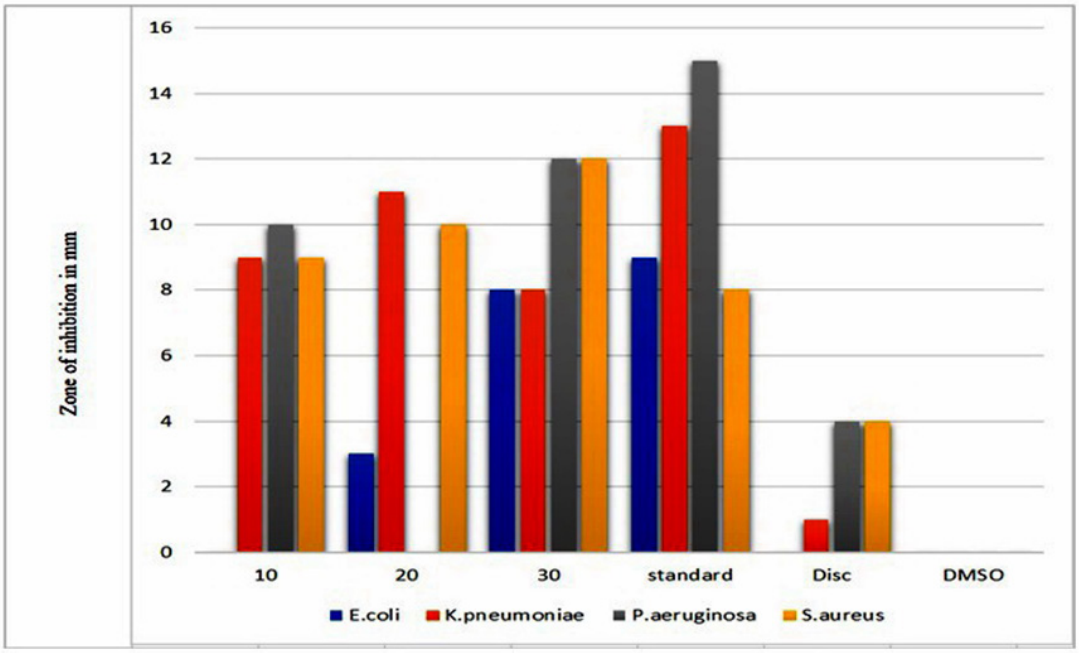

Fig. 15. Antibacterial Activity

was received at wave number 1635 cm^{-1}. This peak indicates the sample comprises peptides. Another vibration at 1211 cm^{-1} approved the presence of glycosidic bond C-O-C ether groups[18]. The absorption frequency at 686 cm^{-1} and 601 cm^{-1} specifies the presence of C-Cl group[37]. The C-Br stretching vibration at 563.21cm^{-1} designated halogen compound. The presence of carbon iodine bond was revealed by the peak at 401 cm^{-1} [42](Table 4). The peaks produced by biosurfactants were compared to the corresponding functional groups. The characteristic vibrations of stretching peaks indicated the presence of lipopeptide biosurfactant[45]. The identical functional groups were disclosed in previous works done by M Garg et al[30]. The characteristics of biosurfactants was assessed by contrasting the IR spectra of sample to commercial surfactin[46]. (Fig.12)

Gas Chromatography - Mass Spectrometry

The GCMS characterization further exposed that biosurfactant produced by *Bacillus sp* was lipopeptide in nature. After performing GC -MS of biosurfactant, the great peaks were monitored at various retention time of 12.75, 10.22, 4.98, 3.87 respectively. The evaluation of biosurfactant fraction exhibited the existence of a prime peak at 12.75 retention time. Fatty acid nature compounds such as methyl ester, carboxylic acid were the vital compounds in the sample which was similar to the result of Parthipan et al[32]. The compounds such as methyl ester, silane, 3-Quinoline carboxylic acid, methyl- dodecyl ester which were present in the sample favour tenso-active property for the biosurfactant. Methyl ester compounds are long chain fatty acids derived from vegetable oil or animal fats which has magnificent biosurfactant properties are easily bio degradable in nature. Dimethyl silane diol is used as a surface modification regent such as hydrophobization of oxidized silicon and oxidized metal surfaces. Silane is used to increase resistance to abrasions as well as increased adhesion thermal stability and cross linking in paints, inks and coatings. Dharman et al[45] disclosed the occurrence of fatty acids

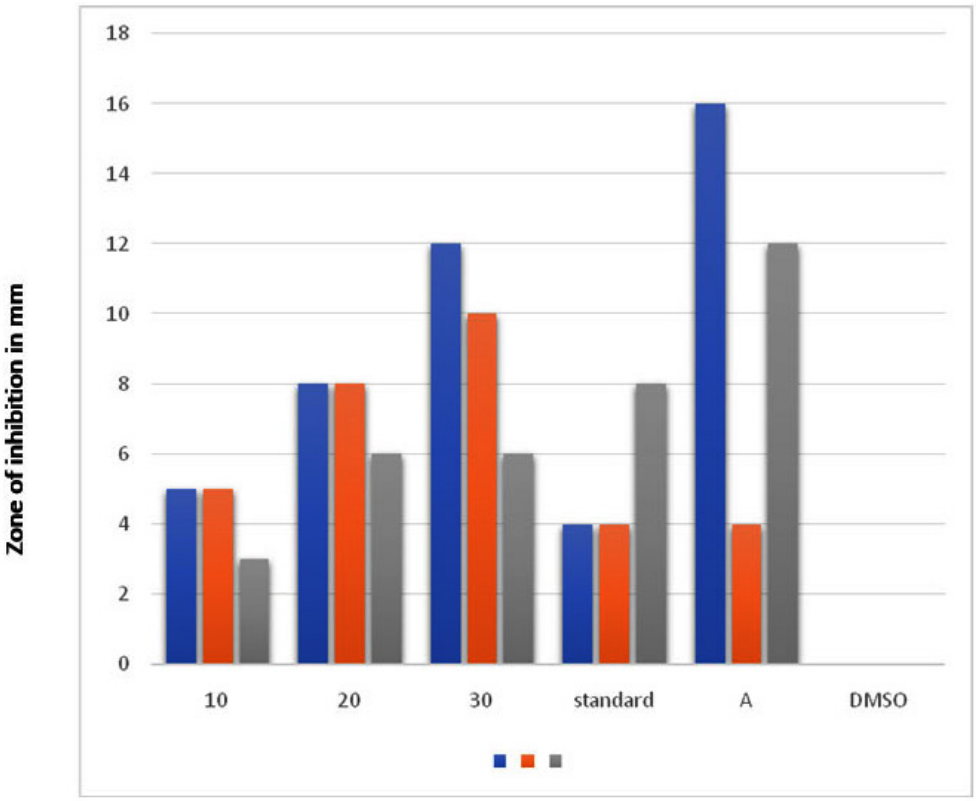

Fig. 16. Antifungal Activity

such as 3- hydroxy decanoic acid in rhamnolipid biosurfactant. GCMS analysis of biosurfactant from soil bacteria which degrade oil was carried out by Santhakumar et al[47].Parthipan et al[32] was disclosed that *Bacillus subtilis* can produce lipopeptide biosurfactant. *Bacillus sp* produce lipopeptide nature biosurfactant was detailed in the study of Deshmukh et al[48]. Retrieval of these constituents such as 2 trimethyl silyl ethanol, 2 methoxy -5-phenol of biosurfactant produced by *Bacillus sp* Lv13 was narrated by Yinghi et al[49]. Halophilic *Bacillus sp* produced biosurfactant consist of glycolipids which was detailed by MBS Donio et al[42].Santhakumar et al[47] summarized that biosurfactant extracted from bacteria hydrocarbon contaminated soil corresponds to fatty acids and fatty acid esters. The major functional compounds recognized by GCMS analysis were inscribed (Table5)

Antimicrobial activity

The antimicrobial activity of biosurfactants against various groups of pathogenic microorganisms has attained more consciousness in the latest years. Antimicrobial assay of biosurfactants was carried out using well diffusion method against bacteria such as *P aeruginosa, E coli, K pneumoniae, S aureus* and fungi *Aspergillus niger, Aspergillus terreus, Aspergillus flavus.* The extracted biosurfactant showed antimicrobial activity in contrast to food borne pathogens. In our recent study, biosurfactants from *Bacillus sp* exhibited inhibitory results against fungal and bacterial human pathogens. The elevated antibacterial activity was noticed in *K pneumoniae* when compared with commercial surfactin. The greatest antifungal inhibition zone observed against *Aspergillus terreus*..The zone of inhibition against clinical pathogens were specified (Fig.13 &14) The biosurfactant from *Bacillus sp* exhibited good inhibitory zone against Gram negative and Gram-positive bacteria (Fig.15 &16). These results are in accordance to pioneer reports by Sharma et al[46]. Prasanthi et al[24] reported that soil bacteria have antibacterial activity opposed to pathogenic bacteria. Lipopeptide biosurfactants from *Bacillus sp* has antibacterial, antifungal and antiviral activity which was revealed by the study of MBS Donio et al[42].M Garg et al[30] noted that refined biosurfactant from *Candida parapsilosis* displayed antimicrobial activity. The study by Araujo et al[3] signified that biosurfactant of which comes under Rhamnolipids has antifungal activity.

CONCLUSION

In the present study, six bacterial strains from hydrocarbon contaminated soil of Kerala and Tamil Nadu, India were screened for potential biosurfactant producer. The capable isolate was identified by 16S rRNA analysis as *Bacillus sp*(MN243657). The nature of biosurfactant was characterized by TLC, UV, FTIR and GC-MS analysis and concluded as Lipopeptide nature. The extracted biosurfactant has promising antimicrobial activity. This bacterial strain can be used for bioremediation of contaminated sites. This can be utilized in pharmaceutical industry as it has antimicrobial activity. Further research on vital applications of this biosurfactant are recommended.

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Conflictof interest

The authors declared that there is no conflict of interest in this research paper.

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