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ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIA FROM HYDROCARBON CONTAMINATED SOIL

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Abstract

Biosurfactants are the sustainable options for ecologically friendly remediation of hydrocarbon contaminations. The study aimed at isolation and characterization of biosurfactant producing isolates from hydrocarbon contaminated soil from Satara district in Maharashtra, India. Drop collapsing and oil spreading tests and CTAB tests of fermented media were primary screening tests for screening putative isolates. Blood agar lysis, lipase, surface tension reduction and emulsification measurements were used as quantitative tests. Three potential bacterial isolates with significant biosurfactant activities were found. The biochemical characterization and 16S rRNA gene classified the isolates as *Achromobacter xylosoxidans, Bacillus subtilis Pseudomonas aeruginosa* and were registered in GenBank with the accession number OP060997.1, OP002276.1 and OP846851.1 respectively. The biosurfactants in media were purified with column chromatography and the biosurfactant fraction were resolved further using TLC. The spot with highest biosurfactant activity was analysed using LC-MS and FTIR. The FTIR scan of biosurfactant fraction found homology with rhamnolipids. The LC-MS analysis found Hexadecanoic acid methyl ester and Methyl stearate as putative biosurfactants in purified fractions of P11 and NG3W. 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine is a plausible biosurfactant compound whose biosurfactant activity is not previously reported. The identified compounds are naturally synthesized and need to be further studied for its industrial application.

Keywords : Biosurfactant, LC-MS, FTIR, surface tension, column chromatography, oil dispersion.

Introduction

Crude petroleum oil and its derivatives are regarded as one of the most widespread environmental contaminants (Sotoudeh *et al.*, 2023). The use of crude oil is quite profuse and disposal generally results in carbon emission (Dai *et al.*, 2023). The crude oil contaminations traverse right from the drilling site to washing off into water bodies from urban regions (Marquez *et al.*, 2023). Most of the

oil contaminations are spillage, leakage based or accidental (Kardena & Helmy, 2023). But this might turn to be a carrier for many toxic chemicals including polycyclic aliphatic hydrocarbons or sulfur, nitrogen or heavy metal carrying compounds (Prior, 2023).

Crude motor oil is a significant hydrocarbon contaminant in urban regions. It can withstand temperatures over 315 °C and are highly saturated. These engine oils already have detergents and dispersants added to reduce the formation of oil sludge (Khuong *et al.*, 2016). Polycyclic aromatic hydrocarbons (PAH) are formed in the engine oil, which are highly stable to bioconversion and resistance to extreme conditions (Abdel-Shafy & Mansour, 2016). The toxicity of PAH or other compounds from the oil have different magnitudes of toxicity relative to the exposure time (Abdel-Shafy & Mansour, 2016).

The disposal of crude oil is associated with novel technologies for remediation, economical applicability with sustainability. Compared to all the strategies applied in the remediation of crude of oil, bioremediation using microbes is the recognized as most sustainable and economical means (Adetunji *et al.*, 2021). But microbial degradation of crude oil has long been implemented for ecorestoration or generation of degrading agents (Xu *et al.*, 2018). The biodegradation of crude oil in niche using microbes has several limiting factors like absence of organic compounds or nutrients, recalcitrancy of the substrate, stability of the oil degrading compounds or growth rate of the microbes in given conditions (Atlas, 1991; Sun *et al.*, 2021). Hence, discovering the novel microbes with putative traits of biosurfactant production and oil degradation is essential.

The biosurfactants are amphipathic molecules that can form bond with aliphatic molecules as well as water (Bhadra *et al.*, 2022). These molecules can be glycolipids, fatty acids, lipopeptides, lipoproteins, phospholipids, or neutral lipids (Aslam *et al.*, 2023). Glycolipids are diverse, stable amphipathic molecules with varied range of applicability, and are readily degradable (Khan & Butt, 2016). These biosurfactants reduce the surface tension in the water oil interface by creating a surfactant oriented monolayer around the hydrocarbon with its aliphatic portion, and pointing the hydrophilic portion outside. This causes the increase in the surface area of hydrocarbon substrate and eases the emulsification. The emulsification increases the availability of hydrocarbon for degradation (Ferreira *et al.*, 2023). This eases the mitigation of hydrocarbon from the environment and is sustainable as compared to the synthetic counterpart.

The biosurfactants secreted by the microbes increases the bioavailability of hydrocarbon through emulsification, which is a limiting factor in effective hydrocarbon remediation. At times, the biosurfactants are not flexible enough to reduce the surface tension or emulsification, but the cosurfactant play a role in microemulsion stabilization. These co-surfactants penetrate between the tails of surfactants and improve its performance. These surfactants can be produced *in-situ* and are crucial for survival in environment where crude oil is the major or only carbon source. Pseudomonas, Bacillus, Achromobacter, Acenetobacter, Alcaligenes, Rhodococcus, Corynebacterium etc. are known to be producing biosurfactants and used in bioremediation of hydrocarbon contaminated regions.

Potential biosurfactant producing microbes that function in the given niche is the key to commercial application of the biosurfactants in given region. The rate of biosurfactant production, its efficiency, stability in varied environmental variations, nature of compound, the degradability, etc. are factors that can help

in shortlisting such microbes. There are reports of many novel biosurfactants recorded frequently and yet there is more research needed to find competitive molecules with better stability and efficiency. Hence, the research was aimed towards isolation and characterization of the putative isolates and potential biosurfactants from hydrocarbon contaminated soil samples.

Materials and methods

Crude Oil, Soil Samples, and Chemicals

Crude, used engine oil was obtained from Kadam (17.69° N, 74.00° E) and Swaraj 17.70° N, 73.98° E) petrol pump, Satara district, India and had been used throughout the study. Soil samples were collected from an oil logged area at a petroleum refilling station for bacterial isolation. Himedia, Merck, and Sigma purity grade media and chemicals were used throughout the study.

Enrichment and isolation of biosurfactant producing isolates

1 gm of hydrocarbon-contaminated soil samples was added in mineral salt medium (MSM) that had 2% (v/v) crude oil as sole carbon source. Mineral salt media (K_2HPO_4 , 1.73 g; KH_2PO_4, 0.68 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.03 g; NH_4NO_3 , 1 g; $CaCl_2 \cdot 2H_2O$, 0.02 g per 1000 mL distilled water at pH 7) was prepared in conical flasks as enrichment media (Patowary *et al.*, 2015). It was then incubated at 37 °C at 150 rpm for 30 days on rotary shaker. After incubation, 1 ml of the media was serially diluted to 10^{-6}, spread on Nutrient agar plate and incubated at 37 °C for 24 h. The resultant distinct colonies were picked and streaked on sterile NA plates to get pure bacterial isolates.

Screening of biosurfactant producing isolates

The putative isolates were inoculated in 200 ml NB to get an OD_{600} of 1. The flasks were then centrifuged to get a cell free supernatant (CFS), which was used for screening biosurfactant producing isolates. Following tests were used to screen the biosurfactant producing isolates.

Drop collapsing test

The drop collapsing test was carried out with coconut oil using standard method (Jain *et al.*, 1991). A grease free slide was evenly spread with coconut oil using adsorbent cotton. Single drop of 48 h grown bacterial cell free suspension was put on this slide using a micropipette. Unfermented NB served as control. The slides were observed if the drop of cell free suspension collapsed and spread without any surface tension (Jain *et al.*, 1991). The cultures that showed highest spreading were desirable isolates.

Oil spread assay

A glass plate containing 20 mL distilled water was added with 20 µL of crude engine oil. 10 µL of filtered supernatant was dropped onto the center of the oil layer. The diameter of the clear zone on the surface of the oil layer was measured using a scale in centimeters and compared to the positive and negative controls (Morikawa *et al.*, 2000). Triton X-100 was used as positive control. Unfermented NB was used as negative control.

CTAB plate assay

Sterile CTAB agar plates (0.15 g of cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich, Oakville, ON, Canada), 0.005 g Methylene blue (Sigma-Aldrich), and 12 g of agar per liter of distilled water at pH 7) were prepared (Siegmund & Wagner, 1991). The plates were then spot inoculated with bacterial isolates. Plates were incubated at 37 °C for 48 h. blue halo around the colonies indicated that that the isolates had biosurfactant activity.

Blood agar lysis

Each strain was spot inoculated and streaked onto blood agar plates and incubated for 48 h at 37 °C to observe β-haemolysis. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production. The diameter of the clear zones was index of the concentration of the biosurfactant (Płaza *et al.*, 2006). The zones of clearing were scored as follows: ' - ', no hemolysis; ' + ', incomplete hemolysis; '+ + ', complete hemolysis with a diameter of lysis < 1 cm; '+++ ', complete hemolysis with a diameter of lysis >1cm but < 3cm; and '++++ ', complete hemolysis with a diameter of lysis >3 cm. Two plates for each strain were inoculated and clear zones in several different areas of each plate were analyzed.

Lipase activity by Tributeryn Agar method

Tributyrin, and triolein, were emulsified mechanically in MSM media containing 1 % agar and poured into Petri dish aseptically. The isolates were screened for lipase activity using 1 % tributyrin (w/v). The pH of the medium was adjusted to 7.3 ± 0.1 using 0.1 M of HCl and incubated at 35 °C for 3 days. The plates were examined for zones of clearance around the colonies (Nwaguma *et al.*, 2016).

Surface tension reduction measurement

The surface tension of all samples was tested using the du Noüy ring tensiometer (SEO DST 30 Surface tension metre, Korea) (Du Noüy, 1919). 50 ml of sample in 100 ml beaker was used for each test. The distance between the immersed ring and the liquid surface was set at 4.5 mm to ensure a clean break of the meniscus on the immersed platinum iridium ring. The manufacturer manual specified the ring circumference (R) and the ring dimensions ratio (R/R0) as 0.5960 cm and 53.3906, respectively. The input parameters like ring dimension, density of sample and temperature were set at standard room temperature of 25 °C. The calculation of surface tensions were pre-programmed in the software in the tensiometer. The surface tension value was computed using correction factor proposed by Zuidema and Waters. The surface tension of the sample was recorded from the display.

Emulsification activity

A volume of 1 mL of the cell-free supernatant was added to 5 mL of 50 mM Tris buffer (pH 8.0) in a 30 mL screw-capped test tube. Crude oil (5 mg) was added and vortexed for 1 min and then the emulsion mixture was allowed to settle for 20 min. A negative control consisted of only buffer solution and crude oil with Triton X-100 was used as the positive control (Bento *et al.*, 2005). The emulsification index was calculated using the equation mentioned below.

$$\text{Emulsification index (\%)} = \left(\frac{\text{Height of emulsion}}{\text{Height of total solution}}\right) X100$$

Identification of biosurfactant producing bacteria using biochemical tests

The identification of bacterial isolates was done using IMViC test and enzyme production tests (catalase and oxidase activity). Phenyl alanine and arginine utilization along with ureolytic and H_2S production tests were performed. Gram staining was also performed to discriminate the Gram positive and negative bacteria. Carbohydrate utilization tests were used to discriminate specific bacteria. The pigmentation of the isolates were also recorded.

Identification of Biosurfactant producing bacteria using 16S rRNA

Using 16S ribosomal DNA gene specific universal primers 27F 50 -AGA GTT TGA TCC TGG CTC AG-30 and

1492R 50 -GGT TAC CTT GTT ACG ACT T-30 (Sigma), the 16S rRNA gene was amplified with 50 µL reaction mixture containing 1X reaction buffer (10 mM Tris [pH 8.3], 50 mM KCl and 1.5 mM MgCl2), 20 µM dNTPs, 0.05 U Taq DNA polymerase enzyme (Sigma, USA), 0.5 mM of each primer and 1 ng template DNA. The thermal cycling conditions were: 5 min at 94 °C for initial denaturation; 31 cycles of 30 s at 95 °C, 1 min at 54 °C, 2 min at 72 °C, and a final extension for 5 min at 72 °C. The amplification reaction was performed with a thermal cycler (MyCycler, Bio-Rad, USA) and the PCR amplicons (approximately 1500 bp) were resolved by electrophoresis in 1% (w/v) agarose gel to confirm the expected size of the product. The partial sequences of the 16S rDNA were deposited to the Genbank database to obtain their accession numbers. OP060997.1, OP002276.1, OP838932.1, OP838968.1, OP846821, OP846851, OP846822 were the accession numbers registered in GenBank. These were coded as P3B, P5A, P5B, P5C, P10A, NG3W and P11 respectively in this research.

Phylogenetic analysis

The 16S rRNA gene sequence obtained in this study was aligned with the sequences registered in NCBI. The sequences were aligned with the highly homologous sequences and phylogenetic tree was constructed using MEGA X. The phylogenetic tree for relationship among strains was constructed by the Neighbor Joining Method with a bootstrap of 500 using Kimura-2 parameter.

Extraction of biosurfactant

The solution of MSM broth with 2 percent glucose, was incubated for 72 hours on an orbital shaker set at 120rpm. After fermentation, the broth was centrifuged at 10000 rpm for 10 minutes at 4 °C to produce a cell free media. This was later filtered through 0.2 µm Whatman filter paper. This filtrate was acidified with 6N HCl to pH 2. Chloroform: methanol mixture (2:1 ratio) was added in equal volume and agitated. The mixture was then placed in a separating funnel and the organic phase was collected. This phase was later evaporated at 45 °C and the remaining solutes were redissolved in chloroform: methanol solution (Twigg *et al.*, 2021).. The stock of this crude biosurfactant was maintained at a concentration of 1mg/ml. This was used for all the analysis.

Purification of fractions with biosurfactant activity

Column chromatography was done with chloroform: methanol: water (70:26:4) (Yang, 1969) as the developing solvent system and 60 g activated silica gel slurry in 30 cm X 40 cm diameter column. 1 g of the crude biosurfactant was micro filtered (pore size 3 µm) and dissolved in 5 mL chloroform. This was loaded on the silica gel column with the help of Pasteur pipette. The elution was done at a flow rate of 1 mL/min. Biosurfactant fraction was collected and tested using oil spread assay. Rest of the fractions were discarded.

TLC of the biosurfactant fraction

The biosurfactant was characterized using TLC (Thin Layer Chromatography) with a Silica gel 60 F254 plate obtained from Merck, Darmstadt, Germany. The mobile phase employed was a mixture of chloroform (65%), methanol (25%), and water (4%) (Vigneshwaran *et al.*, 2021). This mobile phase aided in separating the components of the biosurfactant on the TLC plate. The solvent front on reaching three fourth of the plate, was removed and air dried. Ninhydrin was sprayed, air dried and visualized under UV light to detect the fractions. The spots were scraped and checked for surfactant activity by oil displacement method. The most active fraction was used for LCMS analysis.

LCMS of the samples

Biosurfactant portion of the TLC fractions were further identified by using

LC-MS (ACQUITY Premier System LC, Waters, USA and Xevo G2-XS QTofMS, Waters, USA). The TLC purified biosurfactant sample was dissolved in methanol and 2μl aliquot was injected into ZORBAX C18 column (2.1 × 50 mm2). The flow rate of the LC was maintained at 0.20 mL/min. Mobile phase of acetonitrile/water gradient (10–90%) with 0.01% formic acid was used in the column. Empower Software was operated in positive ion mode and MassLynx software was used for analysis. Through scanning the range of m/z 150–2000, full scan data were obtained where fragmentor voltage used was 135.0 V. A total run time was 26 min.

FTIR analysis of samples

The FT-IR spectra was recorded in a Jasco-Japan FTIR-4100 FT-IR system, in the spectral region of 4000-400 cm-1 using potassium bromide (KBr) solid cells (Khopade *et al.*, 2012). The air dried biosurfactant sample was ground with a purified potassium bromide salt to remove scattering effects from large crystals. This powdered mixture was then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can be passed. The spectrum was recorded using the standard manual provided by the manufacturer. The peaks were checked to find the functional groups in the compounds and analyse the probable compound (Nandiyanto *et al.*, 2019).

Statistical analysis

The means, standard error of the mean, analysis of variance and post hoc analysis by Tukeys HSD was carried out using IBM SPSS ver 24.

Results and discussion

Primary screening of biosurfactant producing bacteria

From the hydrocarbon-contaminated soil sample, 22 bacterial colonies of various morphologies were extracted, and tested to see their biosurfactant production potential. After adding culture broth, of P3B, P5A, P5B, P5C, P10A, NG3W and P11, the drop of crude oil spread instantly or within a minute. Other bacterial cultures showed a lower magnitude of biosurfactant production and were deselected from the study. The comparative performance of all the isolates is shown in Figure 1. P5C, NG3W and P11, were found to spread spontaneously when dropped on the oil surface, thereby showing a high reduction in the surface tension. The difference was conspicuously visible when compared to control. Therefore, it was affirmed that the selected isolates were biosurfactant producers.

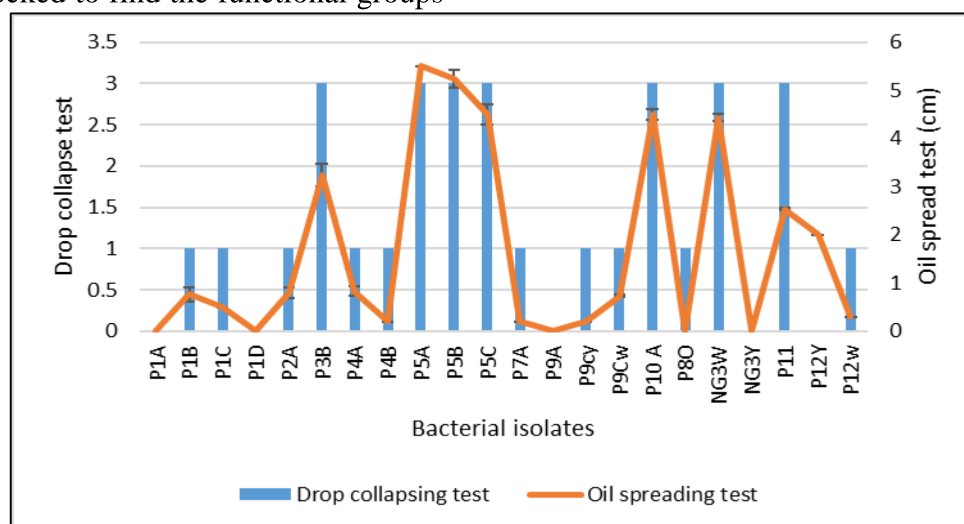

Figure 1 Comparison of biosurfactant activity of bacterial isolates using drop collapsing and oil spread test

Screening of best biosurfactant producing isolate

The CTAB test showed higher biosurfactant activity (blue halo around the isolates) for the isolate P5C, followed by P11. NG3W did not show a positive test for CTAB. There was no significant difference in the hemolysis zone of P5C and NG3W, but a larger halo was shown by P11. The lipase activity of all the shortlisted isolates was significantly different, with P5C showing highest activity. The surface tension reduction was highest in P11. There was no statistical difference in the surface tension reduction of P5C and NG3W. The emulsification activity was highest in P5C. The emulsification activity was significantly different in all the isolates. The details of screening of the top three biosurfactant activities of the isolates are shown in the Table ***1***.

The combination of biosurfactant screening tests have been recorded previously (Astuti *et al.*, 2019; Cai *et al.*, 2015). Many findings have shown that single screening test is not sufficient to screen the biosurfactant producing bacteria (Ayoib *et al.*, 2023; Henkel *et al.*, 2017). The CTAB agar plate method would identify the glycolipids, lipopeptides or anionic biosurfactants (Walter *et al.*, 2010). This was found in P5C and P11, but not in NG3W, showing that the active compounds could be glycolipids. Blood agar lysis method is able to identify surfactin and rhamnolipids but can also provide false negative or positive results (Youssef *et al.*, 2004). Albeit, these tests supports preliminary screening of putative isolates. The lipase plays a vital role in emulsification and greater activity is desirable. The lower surface tension designates higher biosurfactant activity. This was also proven from the fact that emulsification was highest in P5C. The current findings showed that NG3W might have shown negative biosurfactant for CTAB test, but it possessed significant biosurfactant activity. Hence, the finding supports that several tests are necessary for identification of biosurfactant traits of the bacteria.

Table 1 Comparison and screening characteristics of the highest biosurfactant producing isolates

Bacterial isolates	CTAB test	Blood agar lysis (cm)	Lipase activity (cm)	Surface tension reduction (mN/m)	Emulsification activity (%)
P5C	+++	$2.4^{a} \pm 0.3$	3.8 ± 0.3	$28.94^{a} \pm 0.63$	58.06 ± 0.76
NG3W	-	$2.4^{a} \pm 0.3$	2.6 ± 0.3	$28.16^{a} \pm 0.63$	42.85 ± 0.72
P11	++	3.5 ± 0.3	3.1 ± 0.3	35.50 ± 0.35	50.11 ± 0.38

Any mean followed by ± is the standard error of the mean. The means with similar superscript alphabets in the column are similar means at 0.01 level of significance.

Identification of the biosurfactant producing isolates

Phenotypic and biochemical characterization placed the isolates P5C, NG3W and P11 is shown in Table 2. P5C was registered to GenBank with accession number OP060997.1 and identified as *Achromobacter xylosoxidans* (Figure 2), NG3W was registered with accession number OP002276.1 and identified as *Bacillus subtilis* (Figure 3) and P11 was registered with accession number OP846851.1 and identified as *Pseudomonas aeruginosa* (Figure 4). The phylogenetic analysis of the 16S rRNA gene of the sequences generated confirmed the identification of the isolates.

Table 2 Biochemical characterization of the potent biosurfactant producing bacterial isolates.

Isolates	Catalase	Oxidase	Liquificat	Phenylalanine	Argnine	Starch	Urea	H2S	Nitrate Reduction	Indole	MR	VP	Citrate	Pigment	Arabinose	Fructose	Dextrose	Lactose	Maltose	Mannitol	Sorbitol	Sucrose
P5C	+	+	+	-	+	-	-	-	+	-	-	-	+	Green	+	+	+	+	+	+	+	+
NG3 W	+	+	+	-	+	+	-	-	+	-	-	-	+	White	-	+	+	-	-	+	-	+
P11	+	+	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	-	+	+	+	-

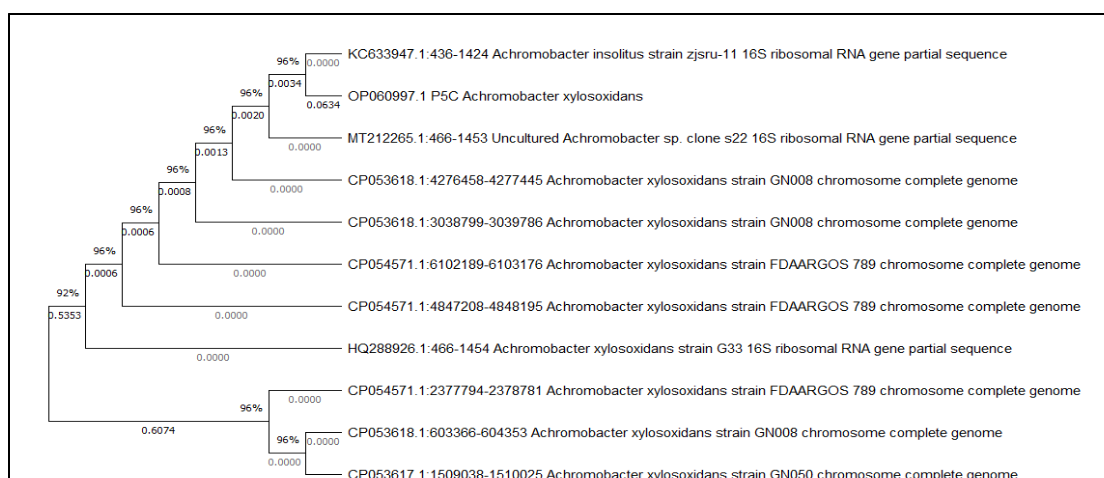

Figure 2 Neighbor-joining phylogenetic tree of isolate P5C (OP060997.1)made by MEGA X (Tamura et al., 2013). Bootstrap values of >50 % (based on 1000 replicates) are given in the nodes of the tree. Nucleotide substitution mode used was Jukes and Cantor. NCBI accession numbers are given in parentheses.

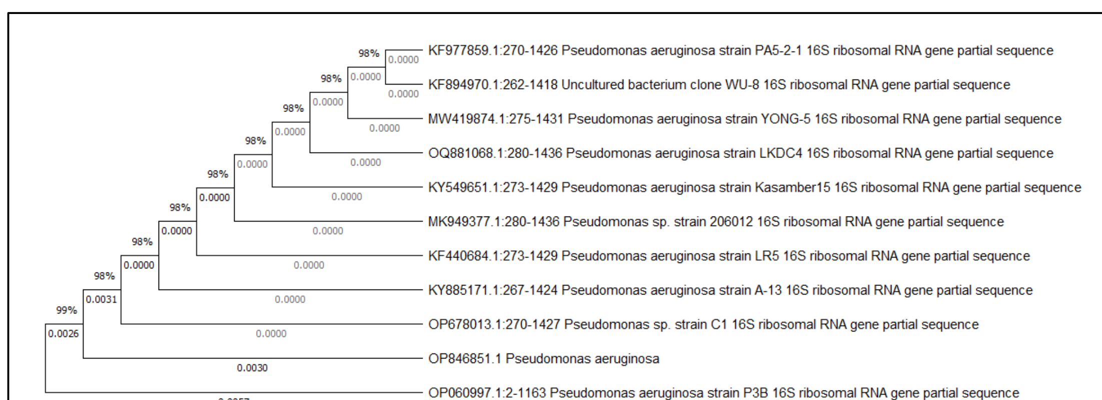

Figure 3 Neighbor-joining phylogenetic tree of isolate P11 (OP846851.1) made by MEGA X (Tamura *et al.*, 2013). Bootstrap values of >50 % (based on 1000 replicates) are given in the nodes of the tree. Nucleotide substitution mode used was Jukes and Cantor. NCBI accession numbers are given in parentheses.

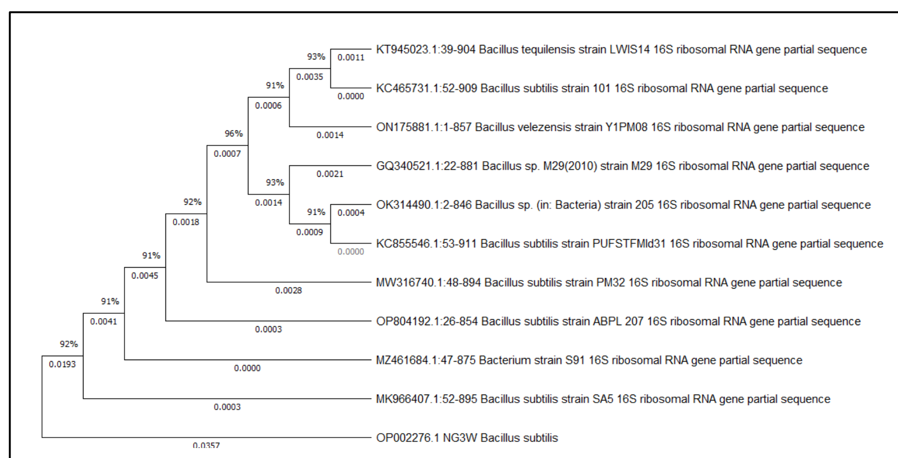

Figure 4 Neighbor-joining phylogenetic tree of isolate NG3W (OP002276.1) made by MEGA X (Tamura et al., 2013). Bootstrap values of >50 % (based on 1000 replicates) are given in the nodes of the tree. Nucleotide substitution mode used was Jukes and Cantor. NCBI accession numbers are given in parentheses.

FTIR analysis

The FTIR spectrum of TLC purified biosurfactant of P5C gave a broad and strong band of hydroxy group at 3334 /cm, typically corresponding to the stretching vibration of the O-H bond (Figure 5). The broad nature of the peak suggests that multiple hydroxyl groups with different hydrogen bonding environments were present in the analyzed sample. The width of the peak can be influenced by factors such as hydrogen bonding strength or intermolecular interactions. The wavenumber 2945/cm and 2832/cm is associated with the asymmetric stretching vibration of C-H bonds in alkanes, aldehydes, or ketones. The wavenumber 1656/cm often corresponds to the stretching vibration of the carbonyl group (C=O) in ketones, aldehydes, carboxylic acids, or esters. This wavenumber 1453/cm is often associated with the bending vibration of the methyl (CH3) group. Finally, the wavenumber 1110/cm and 1018/cm can indicate the presence of C-O stretching vibrations in alcohols, ethers, and esters. These results suggest that the most probable compound can be a derivative of fatty acid with a polar group.

The FTIR spectrum of TLC purified biosurfactant of NG3W and P11C gave similar spectral readings and revealed similar banding pattern (Figure 6 & Figure 7). Both compounds revealed typically stretching vibration of the O-H bond at 3322-3325 /cm. 2946 and 2948 /cm showed with a higher transmittance correspond to aliphatic C-H bonds. The peak at 2829 /cm with nearly 87 percent transmittance corresponds to presence of methylene (CH2) stretching vibrations. Peak at 1671 /cm was found in NG3W, corresponding to C=O bond in the compound. The 1412 /cm peak was found in both samples corresponding to methyl or methylene vibrations. The final peak at 1020-1021 /cm correspond to C-O stretching vibration, commonly associated with alcohols, ethers and esters.

Overall, with some minor differences, all the graphs showed close resemblance to the spectra of biosurfactants found by (Deepika *et al.*, 2017; Saikia *et al.*, 2012; Samak *et al.*, 2014). (Deepika *et al.*, 2017) mentions the identified compound to be rhamnolipids. The findings were also supported by the fact that the biosurfactant gave blue halo in CTAB agar which is generally given by glycolipids.

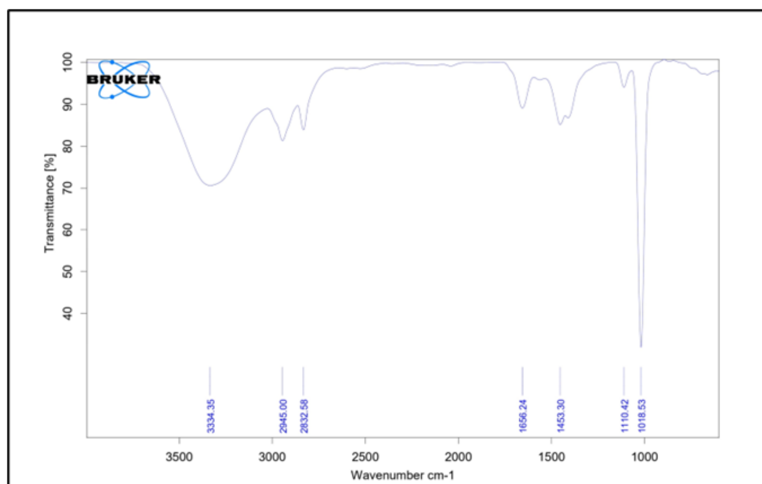

Figure 5 FTIR scan of TLC purified biosurfactant fraction of isolate P5C

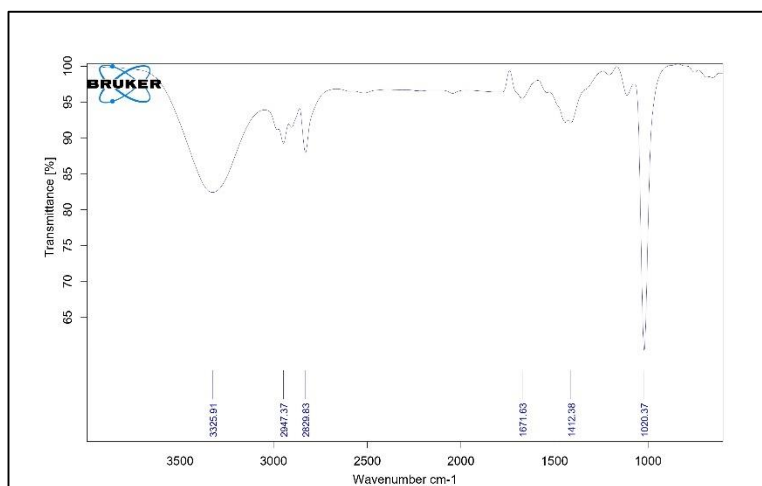

Figure 6 FTIR scan of TLC purified biosurfactant fraction of isolate NG3W

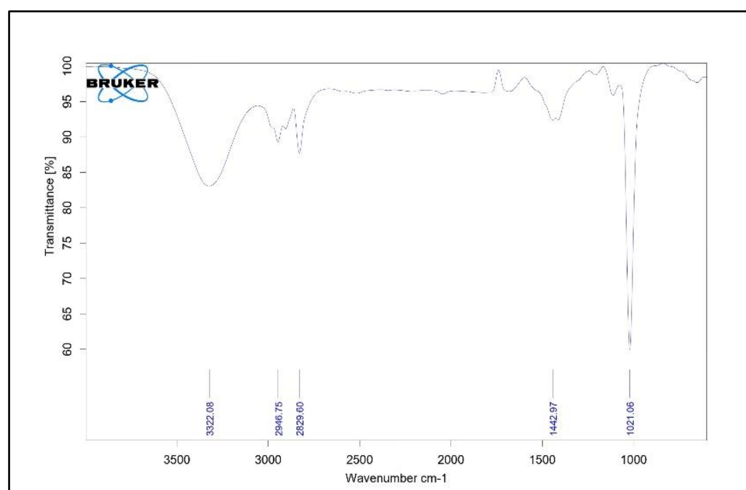

Figure 7 FTIR scan of TLC purified biosurfactant fraction of isolate P11

LC-MS analysis of the samples

The LC-MS analysis of the TLC purified biosurfactant fraction of NG3W (*Bacillus subtitlis*) resulted in Hexadecanoic acid, methyl ester, Bis(2-ethylhexyl) phthalate and Methyl stearate

Table *3*). The most predominant compound detected was Bis(2-ethylhexyl) phthalate, which accounted for 89.52% of the peak area. Bis(2-ethylhexyl) phthalate, also known as DEHP, is a synthetic compound commonly used as a plasticizer. While it is not typically considered a biosurfactant, the possibility that its presence in the biosurfactant fraction due to contamination were very low. *Bacillus subtilis* is reported to produce DEHP (Lotfy *et al.*, 2018) in different media. This research is probably the second report to find DEHP produced by *B. subtilis*. There are no supporting evidences for the surfactant properties in this compound. Hence, the surfactant property may be attributed due to other compounds in the fraction. Moreover, there aren't any evidences to state any biosurfactant activity improvement by introduction of DEHP. Methyl stearate, representing 4.42% of the peak area, is a fatty acid methyl ester derived from stearic acid, which is a saturated fatty acid. Similar to methyl palmitate, methyl stearate is not commonly considered a biosurfactant but can exhibit surfactant properties in specific applications. Methyl stearate is found in biosurfactant fractions in many researches (Andrade *et al.*, 2018; Nataraj *et al.*, 2021). Hexadecanoic acid, methyl ester, also known as methyl palmitate, was identified as the second most abundant compound, representing 6.06% of the peak area. The component was found to correlate with the FTIR results where C=O and C-O were found in the compound. Methyl palmitate is a fatty acid methyl ester derived from palmitic acid, a common component of natural fats and oils. While it is not typically classified as a biosurfactant on its own, it can exhibit surfactant properties under certain conditions or in combination with other molecules (Giri *et al.*, 2016). Hexadecanoic acid, methyl ester and Methyl stearate were also found in biosurfactant fraction of P11 with variable concentrations of 71.45 % and 28.55 % respectively.

P5C had a higher concentration of 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine (41.49 %), which is generally found to have antimicrobial activity (Elamin *et al.*, 2021; Li *et al.*, 2012; Ozma *et al.*, 2022). Based on the presence of two pyrrolopyrazine rings and the ethoxy groups, it is conceivable that 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine could have both hydrophilic and hydrophobic regions, potentially allowing it to act as a surfactant. Though, there are no evidence to support its biosurfactant activity. This compound can potentially be a novel biosurfactant compound and need to be researched further. A similar concentration of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- was found as two peaks, probably due to isomeric structure. This has also been reported as antibacterial metabolite (Sahadevan *et al.*, 2014; Shiyamala *et al.*, 2014).

Table 3 LC-MS analysis of NG3W biosurfactant fraction

Sample	Retention time (min)	Name of compound	Molecular formula	Molecular weight (g/mole)	Peak area percent (%)
NG3W	37.010	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	6.06
	37.445	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.55	89.52
	41.973	Methyl stearate	$C_{19}H_{38}O_2$	298.50	4.42
P5C	34.480	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.00	30.76
	37.226	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.00	27.75
	37.886	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	$C_{14}H_{22}N_2O_2$	250.00	41.49
P11	36.993	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.00	71.45
	41.957	Methyl stearate	$C_{19}H_{38}O_2$	298	28.55

Conclusion

The sampling provided 3 potential bacterial isolates with significant biosurfactant activities. A combination of screening tests provided variable results of biosurfactant activities in all the three isolates. Quantitative measurements showed the bacterial isolate P5C to be the best, with higher magnitude of biosurfactant activity. The TLC purified fraction of the biosurfactants and LCMS analysis helped in identification of the compounds. The abundant compounds of the bioactive fractions were found to be Bis(2-ethylhexyl) phthalate, 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, Hexadecanoic acid, methyl ester and Methyl stearate as the active components. DEHP was the only compound which can't probably be a biosurfactant. The identified compounds are naturally synthesized and may require further studied for its industrial application.

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