ISOLATION AND CHARACTERIZATION OF INDIGENOUS BIOSURFACTANT PRODUCING *BACILLUS* AND *STAPHYLOCOCCUS* SPP. DURING MOTOR OIL DEGRADATION

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Abstract. The present study dealt with the isolation and characterization of some biosurfactant-producing bacterial strains obtained from motor oil contaminated soil samples collected from the local auto-workshops of Lahore. Thereby, four bacterial species were selected based on CTAB/methylene blue-agar, emulsification index, and drop-collapse assays, and then, biochemically identified as *Staphylococcus hominis*, *Staphylococcus* sp., *Bacillus flexus*, and *Bacillus oceanisediminis*, and confirmed through 16S rRNA gene sequencing. Fourier transform infrared spectroscopic analysis was done to track the surface chemistry of the isolated bacterial surfaces. The isolated bacterial strains were then employed at different concentrations (1-3%, w/v) of motor oil, and various temperatures, pH values, incubation intervals, and in the absence or presence of certain inhibitors like sodium dodecyl sulfate and the Cr(VI) ions. There observed a maximum of 81.8% (w/w) oil degradation at 1% (w/v) oil concentration with *Staphylococcus* sp. at 37ºC, pH 7, and 96 h of incubation, whereas in the presence of Cr(VI) ions under the same physiological conditions, the oil degradation was suppressed to 6.0% (w/w). The results demonstrate that the identified bacterial strains could effectively be used to bioremediate the motor oil-contaminated soil at drilling sites as well as in the aquifers.
Keywords: *biodegradation, bioremediation, crude oil, emulsion, surfactant*

Introduction

Surfactants, whether derived from chemical or biological origins, possess both hydrophilic and hydrophobic moieties which not only support emulsification but also decrease the surface and interfacial tensions (Raza et al., 2009; Sakamoto et al., 2017; Pinazo et al., 2019), and those of biological origin are called biosurfactants (Singh et al., 2020). They can modify the surface characteristics of certain substrates like surface adsorption, surface energy, and wettability. They can also enhance the bioavailability of hydrophobic substrates for their prompt degradation and biotransformation under certain fermentation conditions (Raza et al., 2007; Henkel and Hausmann, 2019). The biosurfactants represent an assorted class of surface-active molecules and are synthesized by diverse microbes under suitable culture conditions. They are valuable biological molecules with widespread attributes like biocompatibility, biodegradability, specific activity, activity in extreme environments, and so on. This makes them versatile green chemicals for a wide range of industrial and environmental applications (Khubaib et al.,

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2021). The biosurfactants can effectively be employed in medical and industrial applications for emulsification, de-emulsification, encapsulation, enhanced recovery, and ore floatation processes (Gregorich et al., 2015; Naik et al., 2018). Thereby, glycolipids, lipopeptides or lipoproteins, phospholipids, biopolymeric surfactants, and fatty acids are the major varieties of structurally diverse biological surfactants produced by certain bacteria, yeast, and fungi (da Silva et al., 2020).

The rapid and careless use of synthetic chemical reagents has resulted in serious environmental concerns (Karlapudi et al., 2018). It has been well recognized that petroleum-based hydrocarbons and their derivatives like diesel, motor oil, mineral oil, engine oil, and their residues deteriorate the environment. Motor oil being a mixture of certain aromatics, cyclic alkanes, additives, and anticorrosive compounds, is difficult to degrade under ambient conditions (Sharma et al., 2019). Moreover, the used motor oil carries in it a rather enhanced proportion of heavy metals, carcinogenic compounds, toxic metals, and polycyclic aromatic hydrocarbons that cause serious threats not only to human beings but also to vegetation (Bhattacharya et al., 2019).

Different chemo-physical and biological techniques had been used for the degradation of hydrocarbons containing water and soil systems. One way is the emulsify the hydrocarbon oils using synthetic surfactants, but being toxic thus may also cause secondary environmental issues. Thereby, researchers are trying to explore alternative surfactants that may not only be nontoxic and biodegradable but also effective under both mild and severe conditions (Paniagua-Michel and Rosales, 2015). The biosurfactants, employed during bioremediation, would emulsify the hydrophobic substances (like motor oil) in the aqueous systems making them more available as the carbon source for the oil-degrading microbes (Vijayakumar and Saravanan, 2015; Duan et al., 2015; Trellu et al., 2016). The microbes detoxify the hydrocarbon pollutants through different modes including polymerization, transformation, or mineralization. Although sole bacterial strain may be enough to remediate the hydrocarbons yet at times bacterial consortia are used for more effective bioremediation. Mostly, Pseudomonads had been reported as exceptional biosurfactant producing species, nevertheless, they exhibit opportunistic human pathogenicity (Khubaib et al., 2021). In that way, it is inevitable to introduce some non-Pseudomonad especially, non-pathogenic bacterial species as a source for biosurfactant-producing strains to remediate the crude oil contaminated systems. For instance, *Bacillus* species had been reported as lipopeptide producers (Rita de Cássia et al., 2021) whereas *Staphylococcus* species being a lipopeptide producer with double bonds in its fatty acid chains (Varadavenkatesan and Murty, 2013).

The present study is aimed to isolate and identify some novel non-Pseudomonad – non-pathogenic biosurfactant producing bacterial species from the local oil-contaminated soil specimens and use them for the biodegradation of motor oil in the aqueous media under certain physiological and environmental conditions.

Material and methods

Materials

Luria Bertani (LB) agar and agar were purchased from Hi-Media (Mumbai, India), cetyltrimethylammonium bromide (CTAB), and methylene from Merck (NJ, USA), ammonium nitrate (NH_4NO_3), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), ferric chloride ($FeCl_3$), sodium dodecyl sulfate (SDS), potassium chromate (K_2CrO_4), 2,6

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dichlorophenol indophenol (DCPIP) and heavy metal salts of lead sulfate ($PbSO_4$), zinc sulfate ($ZnSO_4$), cobalt chloride ($CoCl_2$), manganese sulfate ($MnSO_4$) and nickel sulfate ($NiSO_4$) from Sigma (Missouri, USA) and petrol (Premium Unleaded) from the local market. The antibiotic discs of Ampicillin, Streptomycin, Tetracycline, Erythromycin, and Kanamycine were purchased from Biolab Pharma, Islamabad, Pakistan.

Specimens' collection

Motor oil-contaminated soil specimens (as 10-100 g) were collected from the three different points of three local auto workshops of Lahore, Pakistan. The specimens were noted for temperature and pH values and transferred to the laboratory under sterile conditions for working.

Isolation of test bacterial strains

A desirable amount of soil sample was dispersed in sterilized normal saline serial diluted up to 10^{-6} spread on LB agar plate prepared in triplicate and placed in an incubator at 37℃ overnight. The next day, 20 different bacterial strains were indicated, four out of which were shortlisted based on different screening tests (CTAB/methylene blue-agar, drop collapse, and emulsification assays) as described in the following text.

CTAB/methylene blue-agar assay

The blue agar plates were prepared as (g/l): 15, 0.2, and 0.005 for agar, CTAB, and methylene blue, in distilled water. The plates were engraved with tiny, cavities and filled with the separate cell cultures of the selected strains and incubated at 37℃ for 24 h to identify any production of extracellular glycolipid. The presence of blue halos around the bacterial colonies indicated the production of biosurfactants (Siegmund and Wagner, 1991).

Drop collapse assay

This technique was employed to screen the biosurfactant-producing strains out of the bacterial strains in hand (Batista et al., 2006). The test bacterial culture was inoculated into LB broth media and further incubated at 37℃ overnight. Later the centrifugation was performed at 4℃ and 14,000 rpm for 5 min to get the supernatant. A 10 µl of supernatant was put on a Petri plate and a drop of motor oil was placed over it followed by a few minutes of equilibration. If the drop is collapsed, it indicates that the bacterial strain has secreted some surface-active molecules in the culture media. The procedure was repeated with distilled water as a control.

Emulsification index

In this test, an aliquot of 2 ml of petrol and an equal amount of supernatant was added in screw-capped test tubes and vortexed vigorously for 2 min. The emulsion hence produced was allowed to settle at 25℃ for 24 h. Then the height of the emulsification column was measured and put into *Eq. (1)* to find out the emulsification index (E_{24}) (Cooper and Goldenberg, 1987).

$$E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of liquids column}} \times 100 \qquad \text{(Eq.1)}$$

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Surface tension measurement

The surface tension changes of the cell-free culture broth were measured using a digital tensiometer (Krüss K10T) calibrated against distilled water (~72 mN/m).

Identification of the bacterial strains

Once selected, the bacterial strains were undergone biochemical assays using QTS 24 kit following the standard protocols. The resultant biosurfactant-producing bacterial strains (coded as F1, F9, SJ20, and SJ32) were then analyzed for 16S rRNA gene sequencing.

FTIR analysis

Fourier transform infrared (FTIR) spectroscopy is widely employed for rapid identification of surface chemistry of bacterial strains and their metabolites containing any lipids, proteins, glycopeptides, polyphosphates, polysaccharides, teichoic acid contents, and so on (Nandiyanto et al., 2019). In the present study, we used the FTIR-attenuated total reflection (ATR) technique for the surface characterization of bacterial cell biomass and a representative isolated biosurfactant.

Fermentation setups

The biosurfactant producing strains were separately inoculated in Bushnell-Hass media containing the components (g L^{-1}) as reported elsewhere (Vandenbergh and Gonzalez, 1984): KH_2PO_4 (1.0), K_2HPO_4 (1.0), $CaCl_2$ (0.02), NH_4NO_3 (1.0), $MgSO_4$ (0.2), and $FeCl_3$ (0.05). The pH of the minimal media was set (at 6, 7, or 8) and autoclaved, accordingly. Then the desirable amount of autoclaved motor oil (as 1, 1.5, 2, 2.5, or 3%, w/v) was added as the sole carbon source into the minimal media and incubated on an orbital shaker at a desirable temperature (of 32, 37, or 42℃) and 200 rpm for a desirable period (as 24, 48, 72 or 96 h) in the absence or presence of certain inhibitors including SDS and Cr(VI) ions. All the experiments and analyses were performed in triplicates and their respective analyses were done thrice for concordant data sets. The results are reported as averages on all respective values with standard deviation (SD).

Motor oil biodegradation assay

The motor oil degradation behavior of the test biosurfactant-producing strains was investigated by adding 2,6 dichlorophenolindophenol (DCPIP: as 1% w/v, 10 µl) as a redox indicator in the respective culture media on the orbital shaker at 200 rpm, 37℃ for 120 h and after the incubation period, the culture was centrifuged at 1,000 rpm and 4℃ for 15 min. Any changes in the color of DCPIP were observed by measuring its optical density on a UV-visible spectrophotometer at 750 nm. The oil degradation was indicated by a change in color of the cell-free culture broth.

Heavy metal resistance profile

A modified version of the method reported elsewhere was used for determining the heavy metal resistance profile of the test bacterial strains (Miranda and Castillo, 1998). The bacterial tolerances to 5 heavy metals, i.e., lead (Pb^{2+} as $PbSO_4$), zinc (Zn^{2+} as $ZnSO_4$), cobalt (Co^{2+} as $CoCl_2$), manganese (Mn^{2+} as $MnSO_4$), and nickel (Ni^{2+} as $NiSO_4$) were measured on the LB agar plates provided with heavy metal ions (100 to

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40,000 μg mL^{-1}). Then, overnight bacterial cultures were streaked onto the prepared LB agar plates and incubated for 24 h at 37℃. The bacteria exhibiting resistance above 500 μg mL^{-1} were tested against higher concentrations to achieve maximum resistance against the above-mentioned metals.

Antibiotic resistance profile

The test bacterial strains were investigated for antibiotic resistance against some model drugs (at 20 μg ml^{-1}) including Ampicillin, Streptomycin, Tetracycline, Erythromycin, and Kanamycine on the LB agar media. Fresh bacterial strains were spread on LB agar plates and the above-mentioned antibiotic discs were placed on media with the help of sterilized forceps. The above-mentioned antibiotics discs were placed on the LB agar plates and incubated at 37℃ for 24 h. Then the zone of inhibition was observed for determining the antibiotic resistance of selected bacterial strains. The width of the clear zone was determined using the *Eq. (2)*:

$$\text{Width of clear zone, mm} = \left[\frac{\text{Dia. of test specimen plus clear zone, mm}}{\text{Dia. of the specimen, mm}}\right] \div 2 \quad \text{(Eq.2)}$$

Statistical analysis

Analysis of variance (ANOVA) was used for statistical analysis of the experimental data. The *p*-value usually indicates if a factor were either significant (<0.05) or insignificant (>0.05). The F-value indicates if the ratio of variances of two data sets were significantly different.

Extraction and quantification of biosurfactants

The biosurfactants were extracted from the CFCB of the culture media using the solvent extraction method. Briefly, an aliquot of 200 ml of CFCB mixed with twice equal volumes of ethyl acetate in a separating funnel. The aqueous layer was discarded whereas the organic layer was removed on a rotary evaporator till a brownish residue was obtained which was redissolved in methanol and filtered. The filtrate was recondensed on the rotary evaporator to collect the product (Carrillo et al., 1996).

Results and discussion

The contaminated soil in the vicinity of workshops contains a high proportion of used or spilled off motor oil where the oil-degrading bacteria naturally reach. The oil and water phases being immiscible need a dispersant for making them miscible at the oil-water interface. Herein, it might be some bacterial secretions behaving as surface-active agents (biosurfactants) which disperse the oily phase into the aqueous media thus making it accessible for bacterial uptake and degradation (Zhao et al., 2015; Gao, 2018). The biosurfactants find extensive applications in various fields of science and technology due to their surface-active and emulsification properties even under extreme conditions (Dhote et al., 2018).

Isolation and characterization of strains

There observed 20 distinct bacterial strains from the oil-contaminated soil samples (*Table S1*). The isolates were undergone for emulsification indices and morphological

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and biochemical analyses. The CTAB/methylene blue agar assay was done, being a semi-quantitative assay for the identification of anionic surfactants and any extracellular glycolipids in the vicinity of secreting microbes. The appearance of dark blue halos indicated the secretion of glycolipid biosurfactants (*Fig. 1a*). The separate cell-free supernatants of the selected strains were undergone a drop collapse assay on a glass surface (*Fig. 1b*). The supernatants containing any surface-active inclusions collapsed the droplet of motor oil whereas the motor oil droplet remained intact in beaded shapes when it was placed over the control sample or a supernatant not containing any surface-active secretions. Based on the above results, four distinct bacterial strains (designated as F1, F9, SJ20, and SJ32) were indicated as biosurfactants producers which were used for the ongoing study.

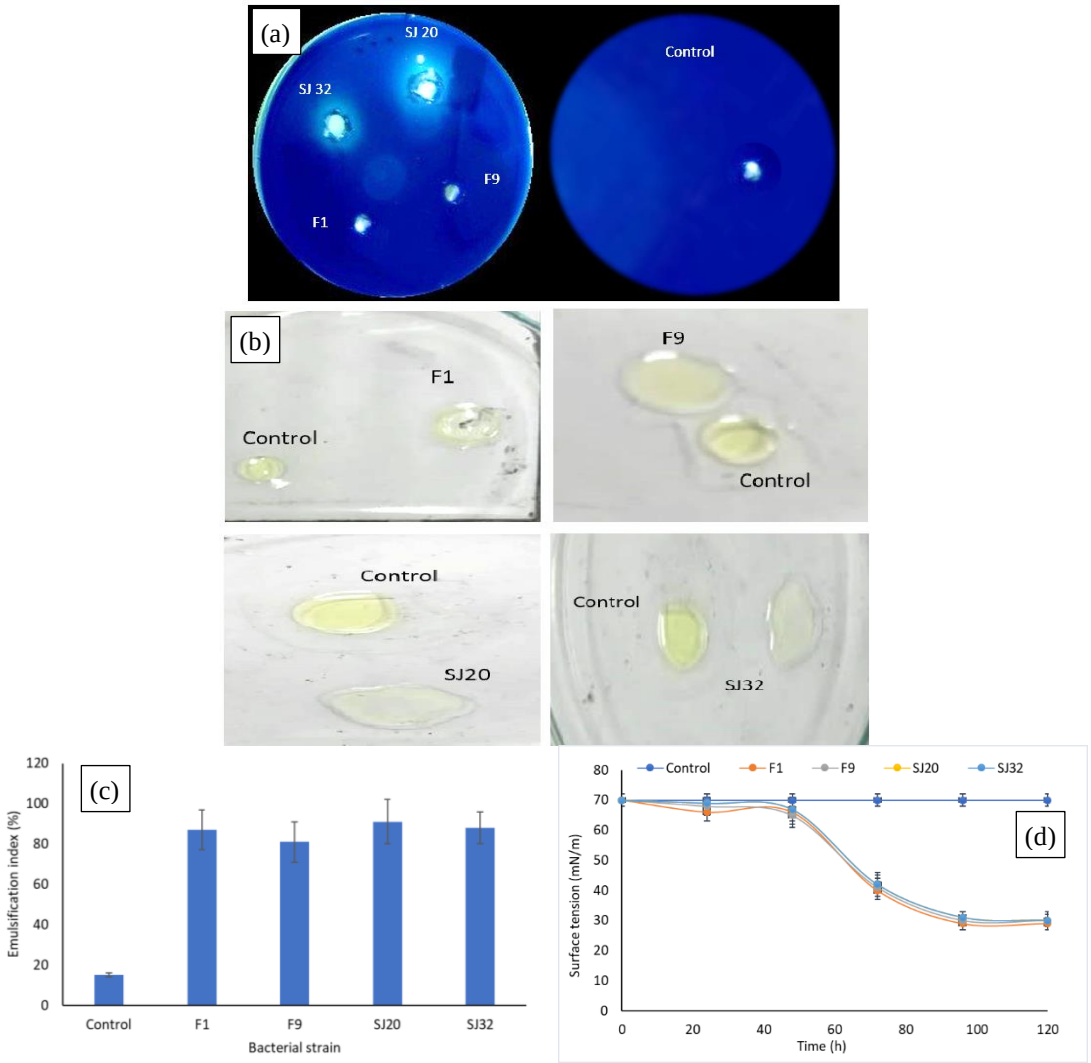

Figure 1. *(a) CTAB/methylene blue-agar assay of selected bacterial strains and the control, (b) drop collapse test results selected of bacterial strains and the control, (c) emulsification index of cell-free culture broth of selected bacterial strains and the control against petrol, and (d) surface tension profiles of respective cell-free culture broths; bar = SD*

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The selected bacterial strains of F1, F9, SJ20, and SJ32 exhibited the emulsification indices of 87±10, 81±10, 91±11, and 88±8%, respectively against petrol (*Fig. 1c*). The results indicated that the selected bacterial strains exhibited the ability to extracellularly secrete some surface-active metabolites (may be biosurfactants) into the culture media. The results were further confirmed by measuring surface tension changes of the cell-free culture broths of the respective bacterial strains. The biosurfactants due to extracellular secretions reduced the surface tension of minimal media from ~70±2 mN/m to below 30±1 mN/m (*Fig. 1d*).

The selected extracellular surface-active metabolites producing strains then undergone biochemical characterization (*Table S1*). The strains F1 and F9 showed positive results against catalase, mannitol salt agar, methyl red, and motility tests while negative results against other mentioned biochemical tests. However, the strains SJ20 and SJ32 exhibited positive results against catalase, oxidase, and mobility tests. Eventually, the results demonstrated that the bacterial strains of F1and F9 were Gram-positive cocci whereas the SJ20 and SJ32 strains as the Gram +ve bacilli.

The phylogenetic analysis of four selected bacterial strains was carried out and the resultant 16S rRNA sequences were sent to the GenBank for assigning the accession numbers as MT107124, MT107125, MT103051, and MT103052 for the selected isolates of F1, F9, SJ20, and SJ32, respectively (*Fig. S1*). The BLAST analysis indicated that F1 and F9 isolates expressed 99% homology to the genera *Staphylococcus,* whereas SJ20 and SJ32 isolates showed homology with the genera *Bacillus* (*Fig. S2*). Hence, the selected isolates of F1, F9, SJ20, and SJ32 were identified as *Staphylococcus hominis*, *Staphylococcus* sp., *Bacillus flexus,* and *Bacillus oceanisediminus*, respectively, being of non-Pseudomonad origin. The results reported in this study have been supported by the literature (Kumar et al., 2018).

FTIR analysis of bacterial surfaces

The FTIR analysis of cell surface of *Staphylococcus hominis* F1 expressed a sharp peak at 3301 cm^{-1} for C-H and H-N stretching vibrations, the latter indicated the presence of amino group (*Fig. 2a*). The peaks at 2919 and 2874 cm^{-1} indicated the presence of -CH_2-and -CH_3 moieties, respectively. There observed a peak at 1637 cm^{-1} designated for the CO-N stretching. The peaks at 1457, 1244, and 1111 cm^{-1} expressed C-H, C-O-C (ester), and C-O stretching vibrations, respectively. The FTIR spectrum of the cell surface of *Staphylococcus sp* F9 (*Fig. 2b*) indicated a peak at 3243 cm^{-1} for N-H bond, and two more peaks at 2966 and 2863 cm^{-1} for asymmetric stretching of -CH_3 and -CH_2- moieties, respectively. Two peaks at 1747 and 1686 cm^{-1} indicated C=O stretching vibrations. The peaks at 1652, 1557, and 1264 cm^{-1} corresponded to the presence of peptide bond, N-H deformation, and C-N stretching, respectively. The FTIR spectrum of the cell surface of *Bacillus flexus* (SJ20) (*Fig. 2c*) expressed a sharp peak at 3413 cm^{-1} corresponded to the N-H stretching vibration of the amino group. A peak at 1650 cm^{-1} indicated -CO-N stretching vibration which might be due to some lipopeptide secretions on the cell surface (Kanmani et al., 2017). The peaks at 2924 and 2880 cm^{-1} indicated symmetric and asymmetric stretching of C-H, respectively. A peak at 1101 cm^{-1} could be due to the C-O-C stretching vibration belonging to the ester moieties. The FTIR spectrum of *Bacillus oceanisediminis* (SJ32) (*Fig. 2d*) expressed peaks at 2860 and 2960 cm^{-1} indicating the presence of -CH_2- and -CH_3 bonds while that in the range 2800-3400 cm^{-1} indicated the presence of OH stretching. The sharp peak at 1735 cm^{-1} showed the presence of ester linkage. The peaks at 1456 cm^{-1} and 1348 cm^{-1} indicated bending vibrations of -CH_2- and

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-CH_3 moieties while that at 3381 cm^{-1} indicated the presence of N-H/C-H bonds. A peak at 1559 cm^{-1} indicated the presence of N-H bending vibration.

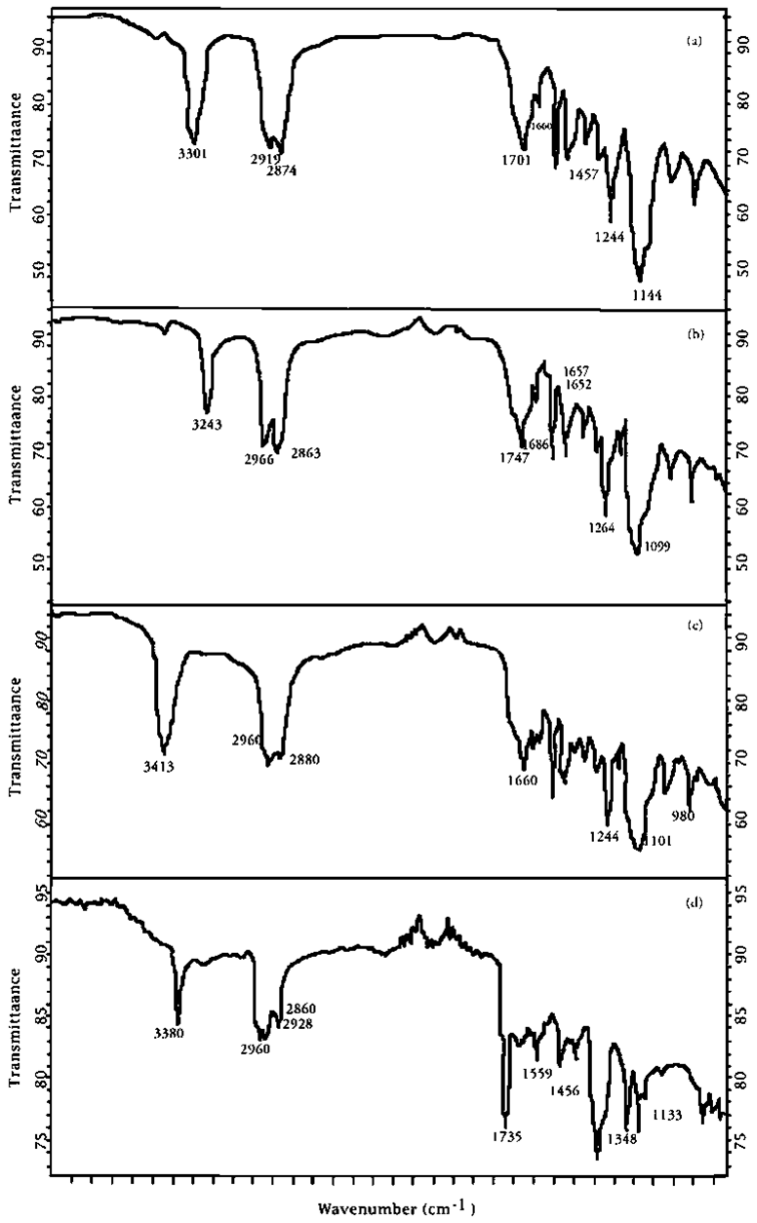

Figure 2. *FTIR spectra of cell surfaces of (a) Staphylococcus hominis F1, (b) Staphylococcus sp. F9, (c) Bacillus flexus SJ20, and (d) Bacillus oceanisediminis SJ32. (The transmittance has been reported in percent)*

Antibiotic resistance profile

The biosurfactant producing bacterial isolates were also tested for resistance against different commonly used antibiotics (as 20 μg mL^{-1}) as shown in *Fig. 3* and the respective data is expressed in *Table S2*. The selected bacterial isolates were found to be resistant against Ampicillin, Streptomycin, Tetracycline, Erythromycin, and Kanamycine, while one isolate i.e., *Bacillus oceanisediminis* expressed sensitivity towards Kanamycin.

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Metal resistance test

The results demonstrated that most of the selected biosurfactant-producing strains showed resistance against the heavy metals of lead (Pb) and manganese (Mn). The least resistance was observed against Zn^{+2} followed by Co^{+2}, and Ni^{+2} ions with all bacterial isolates (*Table S2*). In general, the heavy metal resistance profile of all the biosurfactant-producing strains was as follows: $Pb^{+2}>Mn^{+2}>Ni^{+2}>Co^{+2}>Zn^{+2}$. Heavy metals are regarded as one of the influential parameters that play a significant role not only in bacterial growth but also in their degradation behavior. For determining the potential of bacterial strains to resist heavy metals, the cross-metal resistance of bacterial strains was determined. This is helpful especially concerning heavy metals bioremediation because bacteria can behave more effectively if they can grow in the presence of toxic heavy metals.

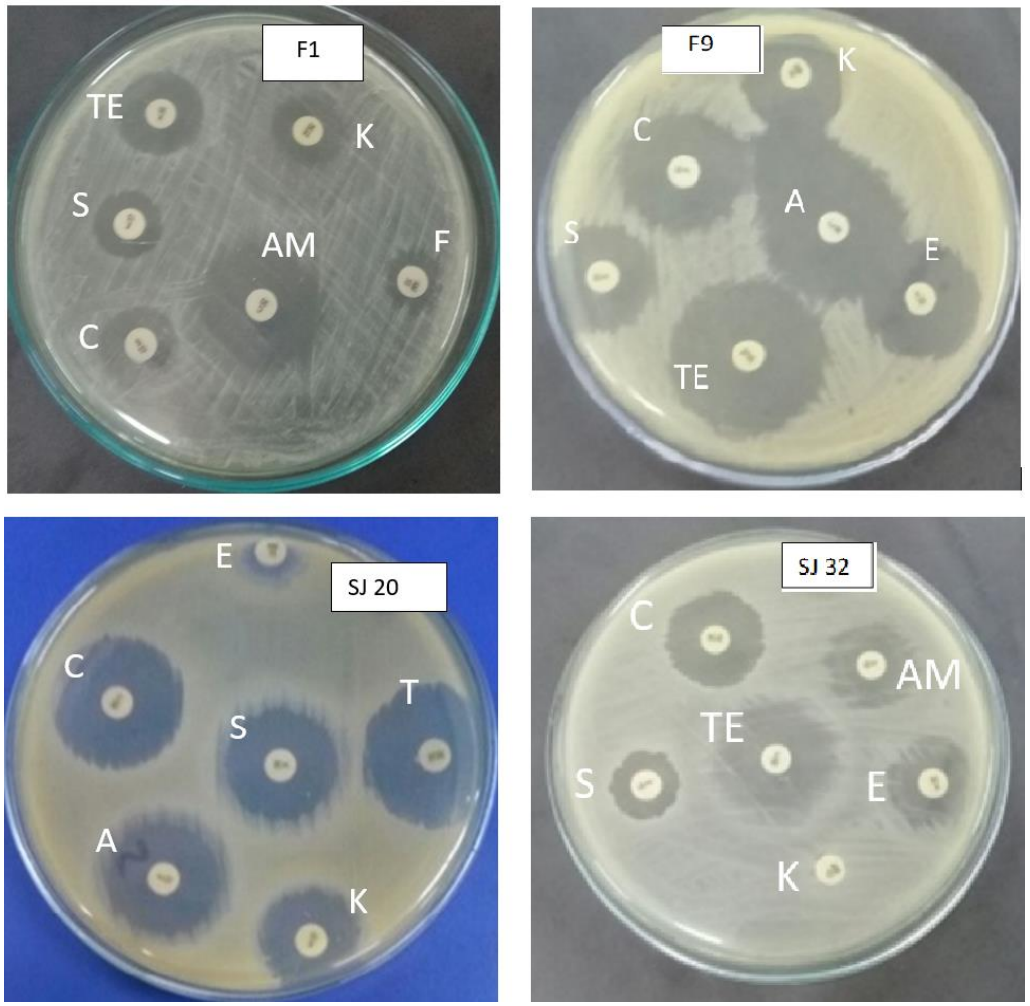

Figure 3. *Antibiotic resistance profile of selected bacterial strains (as per labeled F1(Staphylococcus hominis), F9 (Staphylococcus sp.), SJ20 (Bacillus flexus), and SJ32 (Bacillus oceanisediminis) against the drugs (as 20 µg/mL) of streptomycin (S), ampicillin (AM), tetracycline (T), kanamycin (K), erythromycin (E), and chloramphenicol (C)*

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Motor oil biodegradation study

Effect of initial motor oil concentration

The effect of different concentrations of motor oil (1-3%, w/v) on the degradation behavior in the minimal media has been investigated using the DCPIP assay. The bacterial degradation of the motor oil was observed as the reduced color intensity of 2,6 DCPIP in comparison to the control. The DCPIP, being an electron acceptor, ensures the capability of the test bacterial strain in utilizing the hydrocarbon substrate; as on biodegradation, the change of DCPIP color from blue to colorless shows the change from oxidized state to the reduced state. Thereby, all the selected bacterial strains possessed the capability to use motor oil as the sole carbon source, though to different extents, under certain fermentative conditions (*Fig. 4*). The trend of percent removal of motor oil decreased on increasing the respective initial concentration of motor oil. On increasing the carbon source of the minimal media, a decrease in percent carbon source utilization had also been reported in the literature (El-Sayed et al., 1995; Bayat et al., 2015). This might be due to excessive carbon contents in the minimal media which might imbalance the C/N ratio, in that case. The highest percent removal of motor oil could be observed with the *Staphylococcus* sp. at 1% (w/w) motor oil at 37℃, pH 7, 200 rpm after 96 h of incubation. In general, the lowest percent motor oil removal was observed at 3% (w/w) initial oil concentration. This might be due to the excess availability of carbon contents in the culture media which might form a miscible layer on the aqueous minimal media, particularly in the early days of incubation. However, after a certain time interval, enough biosurfactant contents could be secreted into the culture media thus enabling both phases to miscible into each other which facilitates the motor oil degradation. In an earlier study, the maximum hydrocarbon utilization of up to 73.97% (w/w) was achieved by the bacterial cultures with 1% (v/v) *n*-hexadecane as the sole carbon source. On increasing the *n*-hexadecane concentration to 2 and then to 3% (v/v), the oil degradation was limited to 61.90 and 47.33% (w/w), respectively (Cameotra and Singh, 2009). The carbon source contents above a certain limit also hider the bacterial growth hence the oil degradation too (Abid et al., 2016).

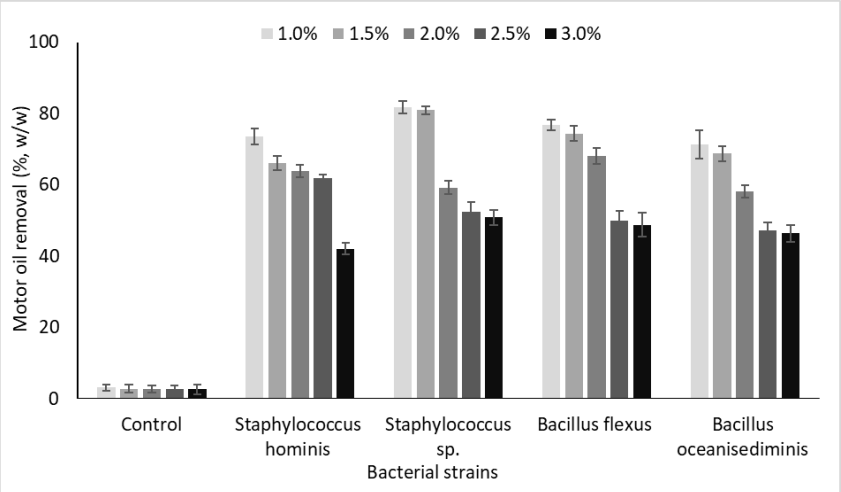

Figure 4. *Effect of motor oil concentration on biodegradation behavior with the selected bacterial strains at 37℃, pH at 7, 200 rpm after 96 h of incubation; control = in media without any bacterial strain, bar = SD*

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The combined effect of pH and motor oil concentration

The pH value of the culture media is also an important factor that affects the oil degradation behavior of bacterial strains. Since the extracellular metabolites might fluctuate the pH of the media so the fermentation was conducted in the phosphate buffer saline at the desirable pH values (6-8) with the desirable initial motor oil concentrations (being 1-3%, w/v) (*Fig. 5*). In general, at either motor oil consideration or with any selected bacterial strain, the highest percent oil removal was observed at pH 7 of the culture media. The highest percent oil removal was observed at 81.8 % (w/w) with 1% (w/v) motor oil at pH 7 by the bacterial strain of S*taphylococcus* sp. after 96 h of incubation at 37℃ and 200 rpm (*Fig. 5a*). The acidic pH, in general, was least favorable for bacterial growth which was reflected by the lowest respective motor oil degradation results. The lowest oil degradation being 9-5% (w/w) was observed at 3% (w/w) initial oil concentration in the presence of *S. hominies* (*Fig. 5e*). These results are consistent with the findings of other researchers who found that different bacterial strains showed maximum oil biodegradation at pH 7 (Palanisamy et al., 2014; Sivagamasundari, 2017; Behera et al., 2021).

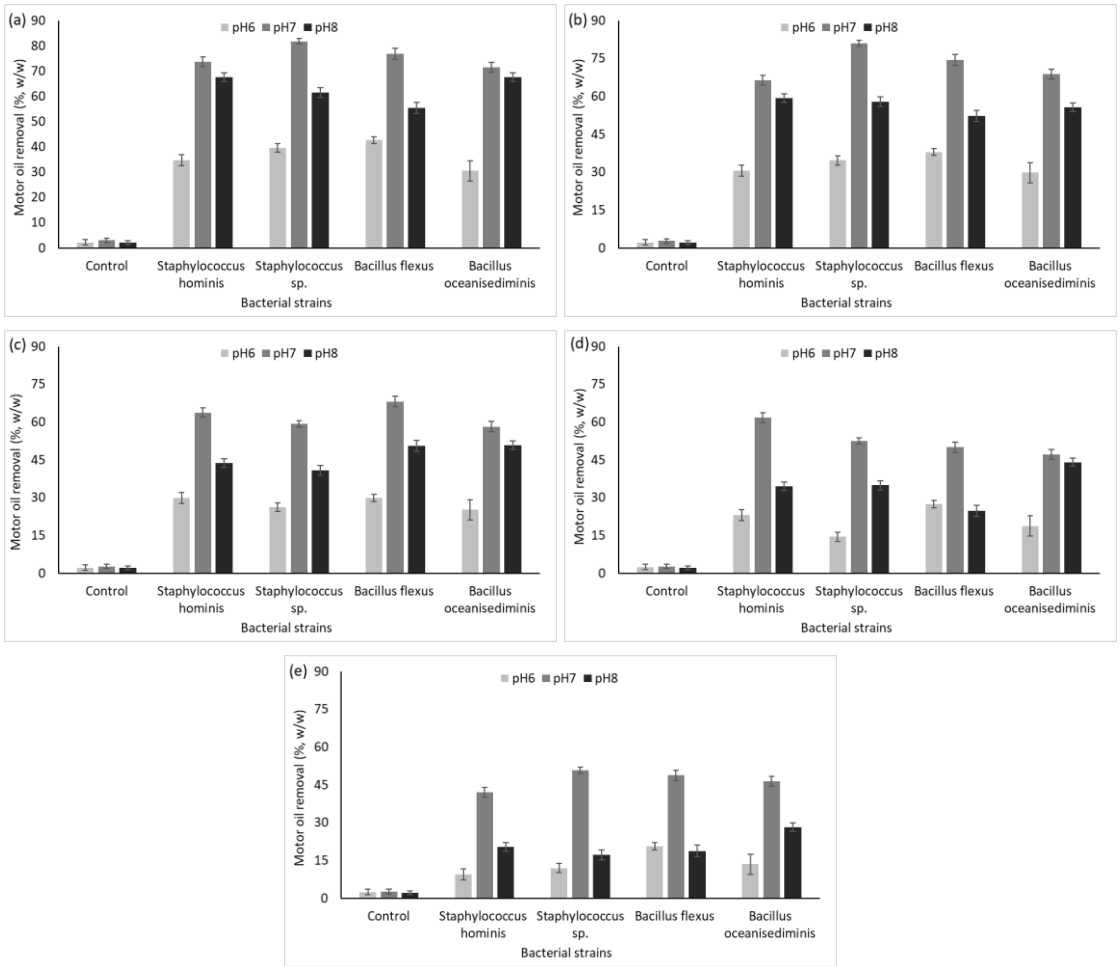

Figure 5. *Effect of bacterial strains at different levels of pH (6,7 and 8) and motor oil concentrations (1.0 (a), 1.5 (b), 2.0 (c), 2.5 (d) and 3 % (w/v) (e)) on degradation profiles at 37℃ and 200 rpm after 96 h of incubation (2, 6 Dichlorophenol indophenol assay at 750 nm); control = in media without any bacterial strain, bar = SD*

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The combined effect of incubation temperature and motor oil concentration

Once the pH of the media was optimized to be 7, the culture media were investigated at various incubation temperatures for all the selected bacterial strains at various initial oil contents (*Fig. 6*). Any changes in temperature from the optimum value may affect the physicochemical and biogenic attributes of the motor oil as the sole carbon source. The experiments were conducted at 32, 37, or 42℃. In general, the optimum incubation temperature was observed to be 37℃ at either initial oil contents with either selected bacterial strain. The highest percent oil removal (81.8%, w/w) was observed at 37℃ and 1% (w/w) initial oil concentration with the bacterial strain of *Staphylococcus* sp. The incubation temperatures of 32 and 42℃ were observed to be unfavorable for bacterial growth at either oil concentration. The bacterial strain of *Staphylococcus* sp. was observed to remove just 1.6% (w/w) oil contents at 42℃ after 96 h of incubation. These findings coincide with the results of Kao et al. (2005) who reported that the biodegradation rate was the highest at the optimum growth temperature of the mesophilic bacteria that ranged between 25-30℃ (Bossert and Bartha, 1984; Rahman et al., 2002; Ren et al., 2021).

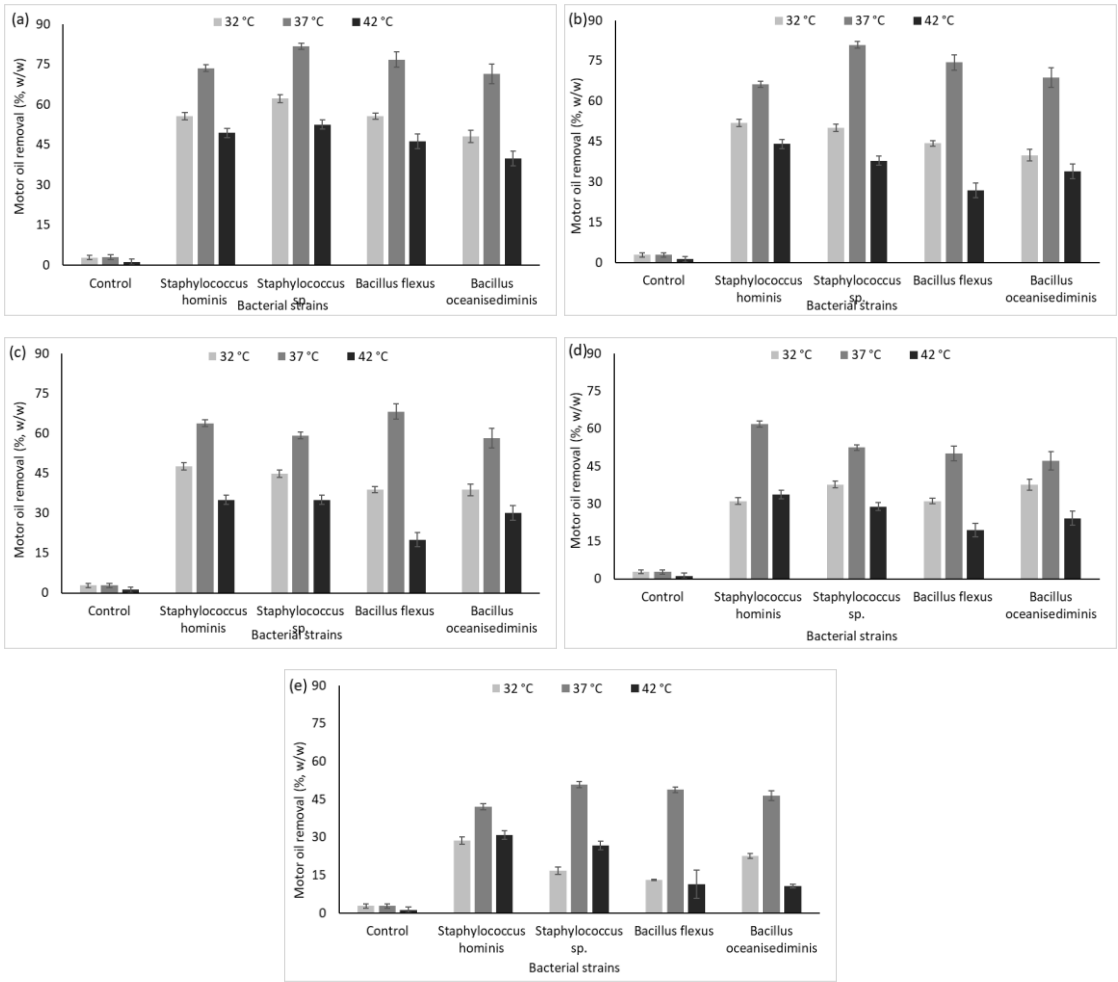

Figure 6. *Effect of bacterial strains at different temperatures (32, 37 and 42℃) and motor oil concentrations (1.0 (a), 1.5 (b), 2.0 (c), 2.5 (d) and 3 % (w/w) (e)) on degradation profiles at pH 7 and 200 rpm after 96 h of incubation (2, 6 Dichlorophenol indophenol assay at 750 nm); control = in media without any bacterial strain, bar = SD*

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The combined effect of incubation time on motor oil concentration

For determining the impact of incubation time on motor oil biodegradation behavior of selected bacterial strains different incubation times (24, 48, 72, and 96 h) were selected. It can be seen from *Figure 7* that as the incubation time was increased the selected bacterial strains showed higher biodegradation of motor oil. It was found that incubation time is directly proportional to the motor oil biodegradation activity of microorganisms (Javed et al., 2015). At 1% (w/v) motor oil concentration, when the incubation time was increased from 24 to 96 h, the biodegradation rate also increased from 40.0 to 81.8% (w/w). Similar behavior of motor oil biodegradation was observed with other motor oil concentrations and it was found that motor oil biodegradation was increased with an increase in incubation time. Although with an increase of incubation time, the biodegradation activity was increased, however, it was found that biodegradation activity was slowed time with the increase of incubation time. The previous literature also shows that higher incubation time favors oil biodegradation (John et al., 2021).

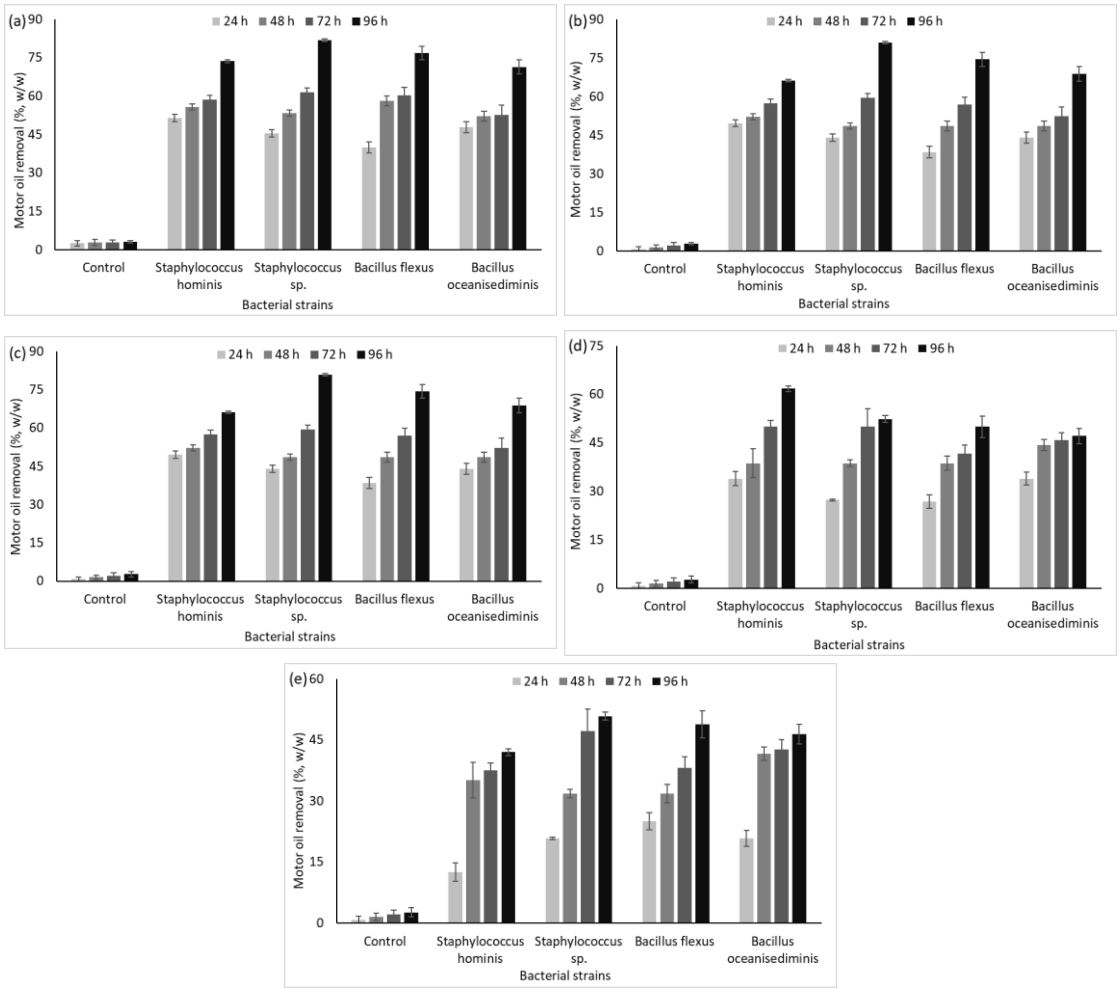

Figure 7. *Effect of bacterial strains at different incubation time (24, 48, 72 and 96 h) and motor oil concentrations (1.0 (a), 1.5 (b), 2.0 (c), 2.5 (d) and 3 % (w/v) (e)) on degradation profiles at 37 ℃, pH at 7 and 200 rpm after 96 h of incubation (2, 6 Dichlorophenol indophenol assay at 750 nm); control = in media without any bacterial strain, bar = SD*

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Effect of inhibitors

The SDS was tested if it could inhibit the growth of biosurfactant-producing bacteria. Two concentrations of SDS (as 1 and 2%, w/v) were tested at different concentrations of motor oil (*Fig. 8a,b*). At SDS 1% (w/v), maximum inhibition of 3 to 11% was observed with 1 and 2% (w/v) motor oil concentrations, respectively. On increasing the SDS content to 2% (w/v), the inhibition of 0.8% was observed at 3% (w/v) oil concentration, while other test concentrations of motor oil did not exhibit significant inhibition. Heavy metals are also present in the environment in a small proportion; however, their proportion is increasing due to the increase in industrial activities. Heavy metals can either inhibit or increase the biodegradation process. In this study, Cr(VI) ions were selected for studying the inhibitory effect of heavy metals on the biodegradation process. When 1,000 μg/ml concentration of Cr(VI) ions was used the maximum inhibition of biodegradation was shown by bacterial strain *Staph. hominis* at 1% (w/v) oil concentration. At a higher concentration of heavy metals, the micro-organisms showed higher inhibition as they instantaneously stop their activities (Javed et al., 2015).

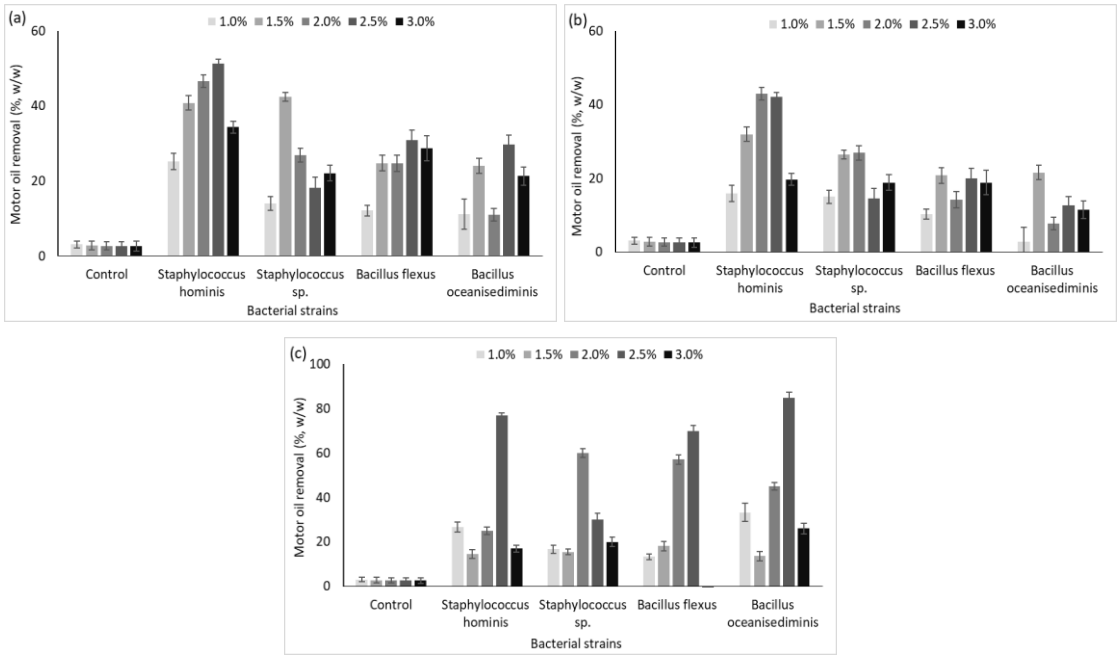

Figure 8. *Effect of inhibitors: SDS at 1% (a) and 2% (w/v) (b) w/v and chromium (1,000 μg/ml) (c) on the motor oil degradation behavior at different concentrations (1.0, 1.5, 2.0, 2.5 and 3 %, w/v) by using selected bacterial at 37℃ and 200 rpm (2, 6 Dichlorophenol indophenol assay at 750 nm); control = in media without any bacterial strain, bar = SD*

The statistical analysis indicated that the initial oil concentration, pH value of the culture media, incubation time, and SDS and Cr(IV) ions concentrations were significant factors at either fermentation setup whereas the bacterial strain type was observed to be an insignificant parameter in either case except on changing the SDS concentration in the culture media (*Table 1*). Since the selected bacterial strains were all capable to degrade the motor oil so the choice of the bacterial strains becomes insignificant in the comparison. However, the prior presence of surfactant (i.e., SDS) in the culture media supports the motor oil degradation in the culture media due to its emulsification behavior.

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Table 1. *Effect of multiple factors on the motor oil degradation (%) under various fermentation setups*

Setup	Constants	Factors	Hypothesis	F-value	P-value	Remarks
I	pH = 7 Incubation time = 96 h Temp. 37℃	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	1.73	0.214	Insignificant
		Int. oil conc.	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	25.25	0.00	Significant
II	Incubation time = 96 h Temp. 37℃	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	0.34	0.80	Insignificant
		Int. oil conc.	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	5.01	0.002	Significant
		pH	H_o: $\mu_{10}=\mu_{11}=\mu_{12}$ H_1: at least two means are unequal	20.4	0.00	Significant
III	pH = 7 Incubation time = 96 h	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	0.63	0.59	Insignificant
		Int. oil conc.	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	7.73	0.00	Significant
		Temperature	H_o: $\mu_{13}=\mu_{14}=\mu_{15}$ H_1: at least two means are unequal	32.57	0.00	Significant
IV	pH = 7 Temp. = 37℃	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	0.16	0.92	Insignificant
		Oil conc.	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	12.60	0.00	Significant
		Incubation time	H_o: $\mu_{16}=\mu_{17}=\mu_{18}=\mu_{19}$ H_1: at least two means are unequal	25.12	0.00	Significant
V	pH = 7 Incubation time = 96 h Temp. 37℃	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	2.84	0.039	Significant
		Int. oil conc.	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	8.45	0.00	Significant
		SDS	H_o: $\mu_{20}=\mu_{21}$ H_1: Two means are unequal	4.40	0043	Significant

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Setup	Constants	Factors	Hypothesis	F-value	P-value	Remarks
VI	pH = 7 Incubation time = 96 h Temp. 37℃ Cr(IV) = 1000 µg/ml	Bacterial strains	H_o: $\mu_1=\mu_2=\mu_3=\mu_4$ H_1: at least two means are unequal	0.66	0.594	Insignificant
		Oil concentration	H_o: $\mu_5=\mu_6=\mu_7=\mu_8=\mu_9$ H_1: at least two means are unequal	8.83	0.001	Significant

Designate: H_o = Null hypothesis (All means are equal), H_1 = Alternate hypothesis (At least two means are unequal), μ_1, μ_2, μ_3 and μ_3= Population mean of bacterial strains, μ_5, μ_6, μ_7, μ_8, μ_9 = Population mean of initial motor oil concentration, $\mu_{10}=\mu_{11}=\mu_{12}$ = Population mean of different pH, $\mu_{13}=\mu_{14}=\mu_{15}$ = Population mean of different temperatures, $\mu_{16}=\mu_{17}=\mu_{18}=\mu_{19}$ = Population mean of different incubation time, $\mu_{20}=\mu_{21}$ = Population mean of SDS concentrations

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Extraction and characterization of biosurfactants

The test bacterial strains were investigated for possible biosurfactant secretions in its culture media up to 96 h of incubation. After solvent extraction, we obtained the crude biosurfactant yields of 0.9, 1.8, 1.6, 1.7g L^{-1}, respectively, with *Staphylococcus hominis*, *Staphylococcus sp.*, *Bacillus flexus*, and *Bacillus oceanisediminus* strains. Previously, a bacterial strain of *Bacillus licheniformis* resulted in 1.28 g L^{-1} of biosurfactant (Kumar et al., 2016). Likewise, *Bacillus subtilus* had been reported to produce 3.24 g L^{-1} of biosurfactant (Nayarisseri et al., 2018). The bacterial strain of *Staphylococcus* sp. had been reported to produce 2.1 g L^{-1} biosurfactant (Eddouaouda et al., 2012). The biosurfactant produced from *Staphylococcus* sp., being the highest motor oil degrader and biosurfactant producer, was investigated using the FTIR analysis. The FTIR spectrum of biosurfactant collected from the *Staphylococcus* sp. is shown in *Fig. 9*. A sharp absorbance peak at 3292 cm^{-1} confirmed the existence of -NH and -OH functional groups in the biosurfactant, which indicated the presence of amino groups in the product. Other peaks at 2956, 2925, and 2854 cm^{-1} confirmed the existence of -C-CH_3 vibrations. The presence of a peak at 1664 cm^{-1} indicated the CO-N stretching vibrations which are similar to the lactone ring present in the lipopeptides. The presence of peaks at 1456 and 1406 cm^{-1} corresponded to -C-CH_2 and -C-CH_3 vibrations of aliphatic chains, respectively. The peak at 1194 cm^{-1} indicated the probable existence of C-O-C vibration of the ester linkage. The results demonstrate that the biosurfactant produced from the bacterial strain of *Staphylococcus* sp. was a lipopeptide.

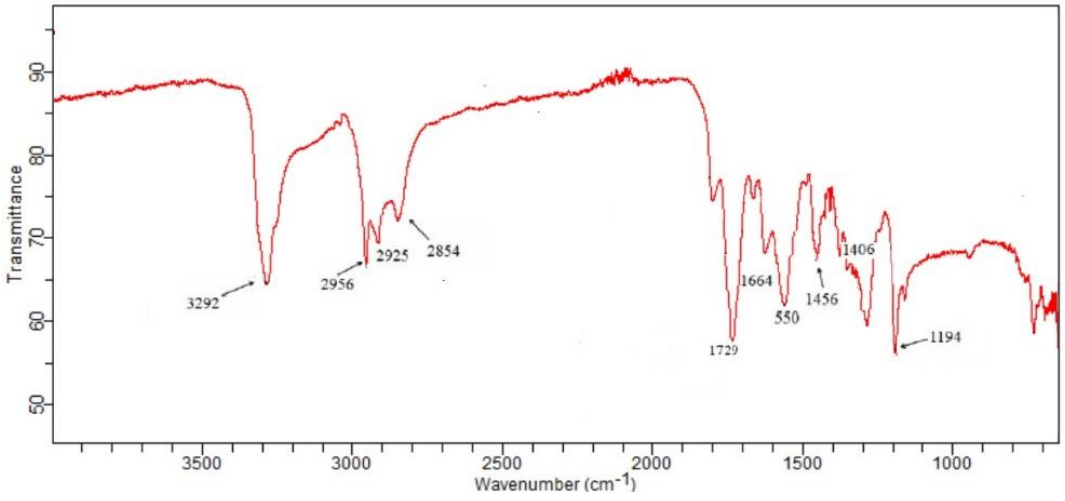

Figure 9. *Representative FTIR spectrum of the biosurfactant isolated from Staphylococcus sp.*

Conclusion

The isolated and identified non-Pseudomonads at different motor oil concentrations, pH, temperatures, incubation time, and parameters showed different levels of biodegradation. The trend of higher biodegradation was observed at low motor oil concentrations while a higher concentration of motor oil declined the bacterial growth. The biosurfactants behaved more effectively in the neutral medium as compared to in the acidic or alkaline media. The SDS and Cr(VI) ions inhibited the process of motor oil biodegradation in the presence of microbial isolates. It was concluded that the identified

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non-Pseudomonads isolated from soil of auto workshops could effectively be employed for successful biodegradation of motor oil and its biotransformation into biosurfactants. The future developments in this area might be to further tune the biosurfactant-producing strains for enhanced yields and to obtain particular congeners using the advanced techniques of synthetic and molecular biology. The bacterial consortia may also be investigated for enhanced remediation of crude oils contaminated soils.

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Conflict of interests. The authors declared that they have no conflict of interests.

Ethical statement. This article does not contain any studies with human participants or animals performed by any of the authors.

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APPENDIX

Table S1. *Emulsification index (E_{24}) vs. petrol at 25 ℃, and the morphological and biochemical characterization of indigenous bacterial isolates*

Isolate	E_{24} (%)	Morphological characterization					Biochemical characterization					
		Gram stain	Spore stain	Cell shape	Catalase test	Oxidase test	Motility test	Methyl red test	Indole test	Mannitol salt agar test	Triple sugar iron test	
											slant	butt
F1	87±10	+	-ve	Cocci	+ve	-ve	+ve	+ve	-ve	+ve	Y	Y
F9	81±10	+	-ve	Cocci	+ve	-ve	+ve	+ve	-ve	+ve	Y	Y
F19	40±6	+	-ve	Cocci	+ve	-ve	+ve	+ve	-ve	+ve	Y	Y
S6	42±5	+	+	Rods	+ve	-ve	-ve	+ve	-ve	-ve	Y	R
SJ1	49±12	+	+	Rods	+ve	-ve	-ve	-ve	-ve	-ve	Y	R
SJ4	41±10	+	+ve	Rods	+ve	-ve	+ve	+ve	-ve	-ve	Y	Y
SJ10	43±8	+	+	Rods	+ve	+ve	-ve	-ve	-ve	-ve	Y	Y
SJ12	31±16	+	-ve	Cocci	-ve	+ve	+ve	-ve	-ve	-ve	Y	R
SJ15	48±14	+	+	Rods	+ve	-ve	-ve	-ve	-ve	-ve	Y	R
SJ17	40±7	+	+	Rods	+ve	-ve	-ve	-ve	-ve	-ve	Y	R
SJ19	46±9	+	+	Rods	+ve	-ve	-ve	-ve	-ve	-ve	Y	R
SJ20	91±11	+	+	Rods	+ve	-ve	+ve	-ve	-ve	-ve	Y	R
SJ32	88±8	+	+	Rods	+ve	-ve	+ve	-ve	-ve	-ve	Y	R
SJ36	42±6	+	+	Rods	+ve	-ve	+ve	-ve	-ve	-ve	Y	R
SJ37	41±8	+	-ve	Rods	+ve	-ve	-ve	+ve	-ve	-ve	Y	Y
SJ39	47±12	+	-ve	Cocci	+ve	-ve	+ve	+ve	-ve	+ve	Y	Y
SJ40	46±13	+	+	Rods	+ve	-ve	+ve	+ve	-ve	-ve	Y	Y
SJ41	38±7	+	+	Rods	+ve	+ve	+ve	-ve	-ve	-ve	Y	R
SU2	40±9	+	-ve	Rods	+ve	+ve	-ve	-ve	-ve	-ve	R	Y
U8	45±9	+	+	Rods	+ve	-ve	-ve	-ve	-ve	-ve	Y	R

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Table S2. *Antibiotic (at 20 µg/mL) resistance and heavy metal resistant profiles of the selected strains*

Code	Strain	Antibiotic resistance (Inhibition zones, mm)					Heavy metal resistance (µg/ml)					Order of metal resistance
		S	AM	TE	K	E	Zn^{+2}	Pb^{+2}	Co^{+2}	Mn^{+2}	Ni^{+2}	
F1	*Staphylococcus hominis*	17±1	30±2	27±2	18±2	11±2	1,000	40,000	1,000	10,000	5,000	Pb^{+2}>Mn^{+2}>Ni^{+2}> Co^{+2}, Zn^{+2}
F9	*Staphylococcus sp.*	15±2	25±2	27±4	14±2	19±2	700	20,000	800	8,000	1,000	Pb^{+2}>Mn^{+2}>Ni^{+2}>Co^{+2}>Zn^{+2}
SJ20	*Bacillus flexus*	23±2	27±4	32±3	20±3	7±5	500	15,000	500	5,000	2,000	Pb^{+2}>Mn^{+2}>Ni^{+2}>Co^{+2}, Zn^{+2}
SJ32	*Bacillus Oceanisediminis*	11±3	11±4	24±4	0	15±4	700	30,000	700	9,000	4,000	Pb^{+2}>Mn^{+2}>Ni^{+2}>Co^{+2}, Zn^{+2}

Note: S = streptomycin, AM = ampicillin, T = tetracycline, K = kanamycin, E = erythromycin, and C = chloramphenicol

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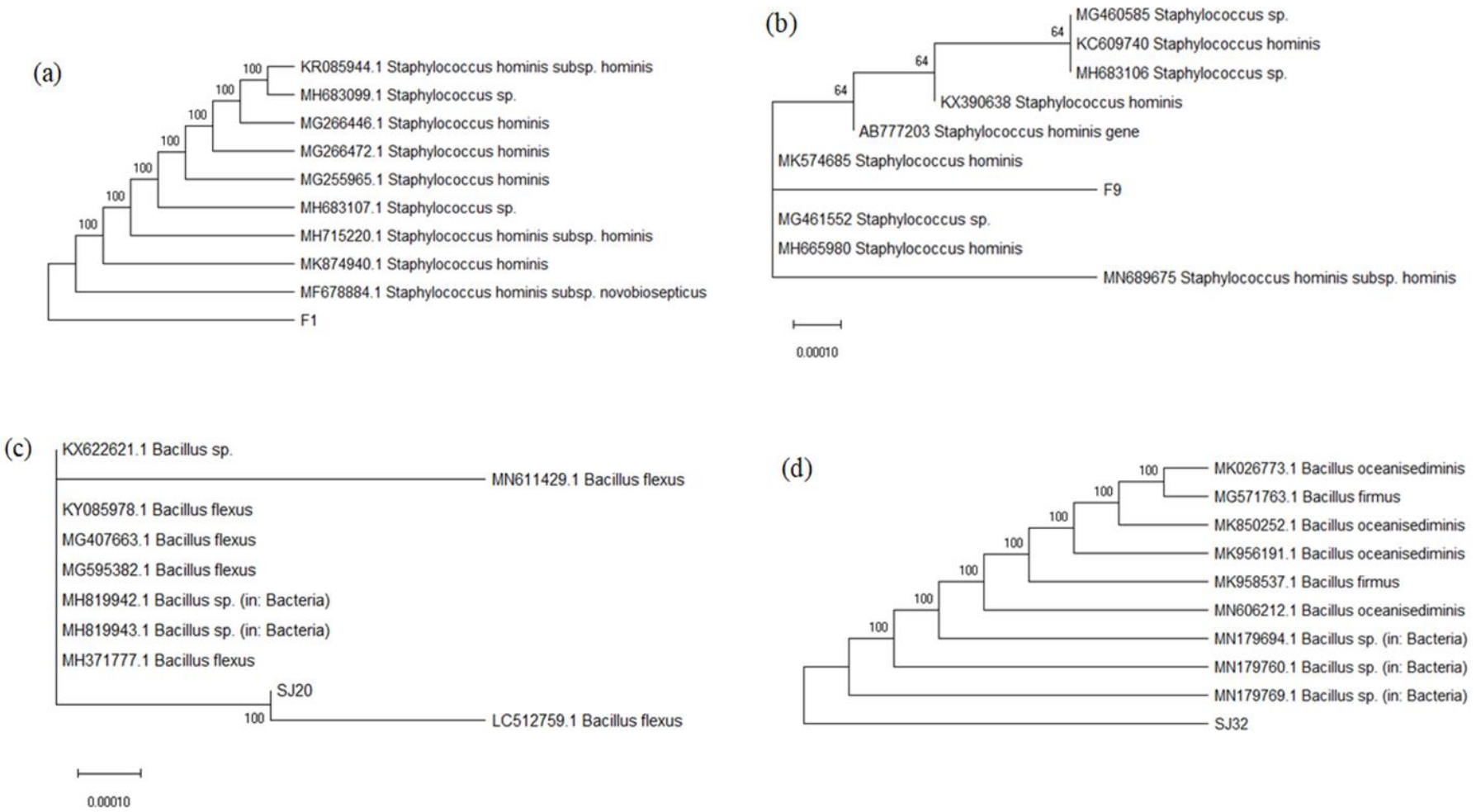

Figure S1. *Phylogenetic trees of selected bacterial strains of (a) F1, (b) F9, (c) SJ20, and (d) SJ32*

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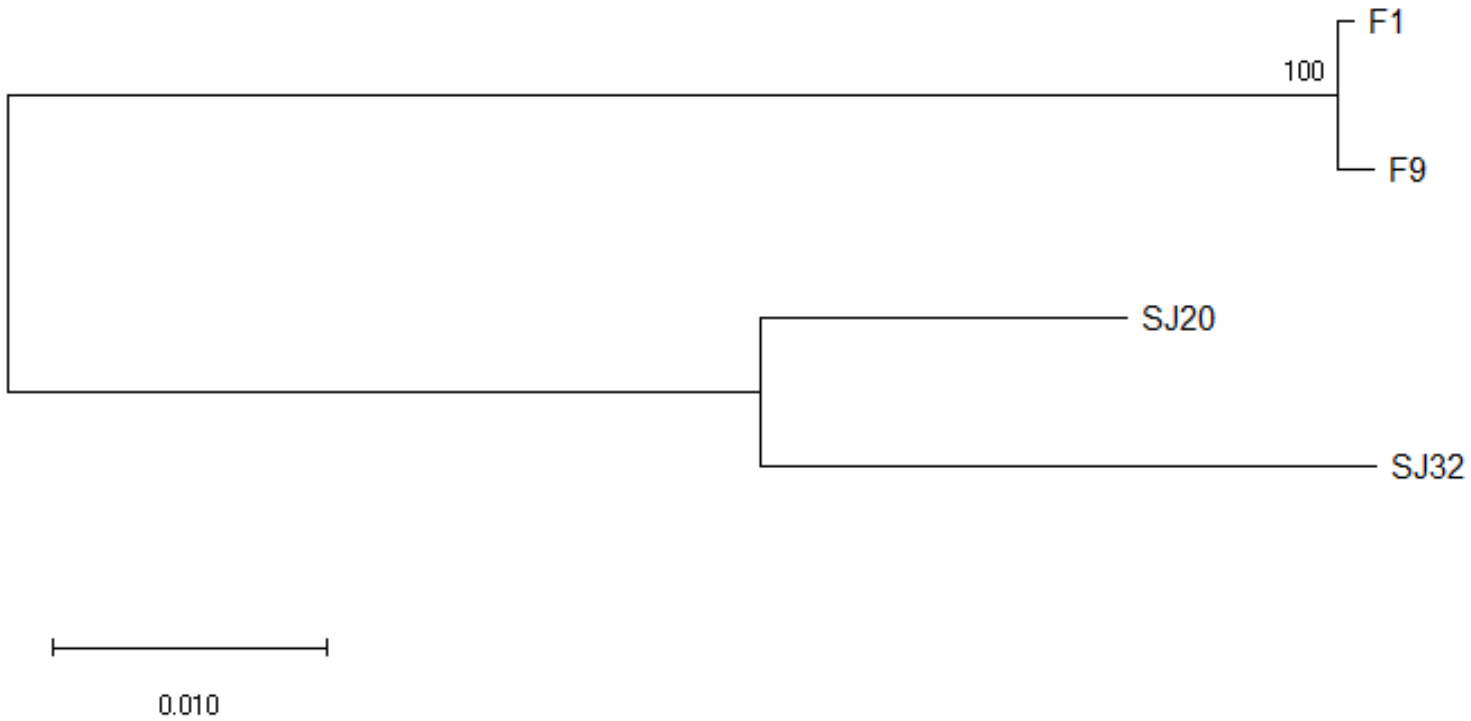

Figure S2. *16 Sr RNA sequencing of the selected strains*

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