A comparative study on chemical characterization and properties of surface active compounds from Gram-positive *Bacillus* and Gram-negative *Ochrobactrum* strains utilizing pure hydrocarbons and waste mineral lubricating oils

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Abstract

Mineral lubricating oils are widely used in various industrial sectors for their applications in maintenance and functioning of machineries. However, indiscriminate dumping of these used oils have resulted in polluting the natural reservoirs which subsequently destroys ecological balance. Bacteria can emulsify or lower surface tension between phases of immiscible substrates and can acquire them as their carbon and energy sources. Such a phenomenon is mediated by production of extracellular polymers which can function as eminent surface active compounds based on their surfactant or emulsifying nature. The comparison between bacterial strains (Gram-positive *Bacillus stratosphericus* A15 and Gram-negative *Ochrobactrum pseudintermedium* C1) on utilization of pure straight chain hydrocarbons, waste mineral lubricating oils as sole carbon source and chemical characterization of the synthesized surface active compounds were studied. Characterization analysis by Ultraviolet Visible spectrophotometry, Fourier transform infrared spectroscopy, Nuclear Magnetic Resonance spectroscopy, Carbon–Hydrogen–Nitrogen analysis has given detailed structural elucidation of surface active compounds. The contrasting nature of bacterial strains in utilization of different hydrocarbons of waste mineral lubricating oils was observed in Gas Chromatography-Mass Spectroscopy analysis. The variation between both strains in utilization of hydrocarbons can be manifested in chemical structural differences and properties of the produced surface active compounds. Scanning Electron Microscopy has given detailed insight into the microstructural difference of the compounds. The utilization of lubricating oils can address waste disposal problem and offer an economical feasible approach for bacterial production of surface active compounds. Our results suggest that these surface active compounds can maneuver applications in environmental bioremediation and agriculture, pharmaceuticals and food as functional biomaterials.
Graphical abstract

Keywords  Surface active compounds · Emulsification index · Environment friendly molecules · Industrial waste mineral lubricating oil · Bioremediation · Biomaterials
Introduction

The detrimental effect on environment due to hazardous waste disposal has been a matter of concern since many decades. Mineral lubricating oils are often required in automobiles, textiles, metallurgical industrial sectors for maintenance of machineries. However, during servicing and repair works, such oils are generally dumped onto the natural reservoirs which can cause a serious threat to living organisms (US EPA 2004; US EPA 2018; Bhattacharya and Biswas 2014; Meiners 2020; Guerin 2008). Mainly consisting of straight chain and branched hydrocarbons, PAH (Poly Aromatic Hydrocarbons), PCB (Poly Chloro Biphenyls) and heavy metals, these oils contribute up to 76% of world’s waste oil disposal. Irrational disposal of such waste oils in natural reservoirs can cause bioaccumulation of non-biodegradable, carcinogenic chemical components in animals and plants which ultimately cause serious implications in human health (Bhattacharya et al. 2015; Kajdas 2014). Therefore, researchers are seeking newer environment friendly remediation methods involving hydrocarbon utilizing bacterial species in minimizing pollution caused by these hazardous pollutants present in waste mineral lubricating oils. Extensive studies involving degradation of these components by bacterial metabolic genes and enzymes have been a topic of great interest since 1980s (Panday and Arora 2020; Bonilla et al. 2005; Vasconcellos et al. 2011; Martínez-Checa et al. 2002; Zhou et al. 2016). The hydrocarbons present in the waste mineral oils provides necessary carbon source for production of bacterial extracellular polymers (Misra and Pandey 2005; US EPA 2004; Das and Chandran 2011). The hydrophilic and lipophilic functional groups of extracellular polymers mediate easier uptake of such hydrocarbons by acting as surface active compound (SAC) (Vasconcellos et al. 2011). Surface active compounds like synthetic emulsifiers or surfactants can emulsify or reduce surface tension or interfacial tension between two immiscible liquids (Uzoigwe et al. 2015). These extracellularly produced surface active compounds by using cost effective carbon sources have gained immense importance due to their biodegradable and less toxic nature. Therefore, these bacterial surface active compounds can be a better alternative with potential applications in environmental bioremediation and agriculture, as additives in daily household products and pharmaceuticals and as preservatives in food while simultaneously reducing pollution caused by chemically synthesized surfactants and emulsifiers (Saha et al. 2020; Banat et al. 2014).

Different myriad species of bacteria like Acinetobacter, Bacillus, Variovorax, Azotobacter, Pseudomonas, Ochrobactrum, Geobacillus, Paenibacillus, Exiguobacterium, Halomonas, Alcaligenes, Aeribacillus are known to be producer organisms of various surface active compounds (Bhattacharya et al. 2014; Sana et al. 2017a; Cai et al. 2017; Zhou et al. 2016; Nerurkar et al. 2009; Toledo et al. 2008). Few studies involving gram-negative, aerobic, rod shaped bacilli Ochrobactrum sp. of family Brucellaceae has been investigated for production of extracellular polymers having surfactant and emulsifier properties (Bezza et al. 2015; Zarinviasgh et al. 2017; Calvo et al. 2008). Ochrobactrum pseudintermedium has shown ability to degrade pure hydrocarbons and waste oils with simultaneous production of surface active compounds (Bhattacharya et al. 2014, 2015). Similarly, Bacillus sp. in presence of varied short and long chain hydrocarbons has produced extracellular polymeric emulsifiers and surfactants in many studies (Gurjar et al. 1995; Toledo et al. 2008; Jemil et al. 2016; Dadasnina and Ismail 2015; Ayed et al. 2014). But studies involving production of extracellular polymeric substances from gram-positive, rod shaped Bacillus stratosphericus belonging to the family Bacillaceae are few (Hentati et al. 2016). Our earlier work has reported about production of SACs by utilization of fish fat as the only substrate (Sana et al. 2017b). Here in this work, Bacillus stratosphericus A15 has been explored for production of SACs from different petroleum hydrocarbon substrates and compared alongside with that of O. pseudintermedium C1.

Our work focuses on utilization of pure straight chain hydrocarbons and industrial waste mineral lubricating oils for production of extracellular surface active compounds by gram-negative Ochrobactrum pseudintermedium C1 and gram-positive Bacillus stratosphericus A15. The study further compares their chemical composition, surface activity, morphological, microstructural and thermal stability properties. Waste mineral lubricating oils provide an economical carbon source for production of biodegradable SACs and additionally provide a better alternative in minimizing pollution caused by oil disposal.

Materials and methods

Chemicals and media required for the study

Waste mineral lubricating oils like spindle oil, compressor oil, hydraulic oil were collected from an oil factory named Asianol Pvt Ltd., Kolkata, India. Pure alkane hydrocarbons like nonane, tetradecane, hexadecane, eicosane, octacosane and chemical reagents of analytical grade were purchased from Sigma Aldrich Co. USA. Bacteriological media (Nutrient agar and Bushnell-Haas broth) were purchased from Hi-Media Laboratories, India.
Bacterial strains used in the study

*Bacillus stratosphericus* A15 (Genebank accession no. KU644139) and *Ochrobactrum pseudintermedium* C1 (Genebank accession no. KJO94035) isolated in the laboratory of Department of Chemical Technology, University of Calcutta were subjected to 16S rRNA sequencing and their respective accession numbers were obtained (Bhattacharya and Biswas 2014; Sana et al. 2017b). The bacterial strains were subcultured in sterilized nutrient agar slants and grown at 37 °C followed by aseptic preservation at 4 °C.

Culture conditions, maintenance and production of surface active compounds (SACs)

The growth of the bacterial strains and subsequent production of SACs was done using Bushnell Haas broth (BH) [in g/L: 1.0 KH₂PO₄, 1.0 K₂HPO₄, 1.0 NH₄NO₃, 0.2 MgSO₄·7H₂O, 0.02 CaCl₂·2H₂O, 0.05 FeCl₃·6H₂O]. Erlenmeyer flasks containing 50 ml BH broth were sterilized prior to the experiment. Pure hydrocarbons and waste mineral lubricating oils as sole carbon sources were added to the BH broth containing flasks. Fresh overnight grown cultures were inoculated in sterilized BH broth (OD₆₀₀ = 1.0) and aseptically added to the flasks.

Parameters maintained for synthesis of bacterial SACs in culture broth

Determination of optimal culture conditions by considering parameters like temperature, pH, incubation period, percentage of carbon source and aeration was done. The conditions maintained for growth of bacterial strains and production of SACs were 25–40 °C, pH 2–10, 1–15 days and 2–10% of waste mineral lubricating oils (v/v). The flasks were then kept in orbital shaker incubator (ORBITEK-LJE, Scigenics Biotech Pvt. Ltd, India) under aerobic conditions. Control flasks (non-inoculated flasks) were incubated in the same conditions to verify that no abiotic loss take place in aerobic conditions.

Extraction and drying of SACs

The methods described by Bhattacharya et al. 2014 and Sengupta et al. 2019 were followed for extraction, purification and drying of surface active compounds. After centrifugation (Remi C24 Plus centrifuge, India) and discarding of bacterial cell pellet, n-hexane was added to culture supernatant to collect and separate residual oil for GC–MS analysis (Adebusoye et al. 2007). Precipitated surface active compounds were collected aseptically in microcentrifuge tubes and were kept in vacuum desiccators filled with fused anhydrous CaCl₂ as desiccant. The surface active compounds were again dried in Aberhalden’s drying pistol apparatus for further characterization analysis.

Influence of SACs on cell surface hydrophobicity

The adhesion of bacterial cells to hydrocarbons was estimated by modifying the technique of Rosenberg et al (1980). Waste oils were added to test-tubes containing 5 ml of bacterial cell suspension (OD₆₀₀ = 1.0) and vortexed for 2 min. After keeping them undisturbed for 1 h, the separated aqueous phase was carefully collected by micropipette and optical density was measured at 600 nm. Bacterial adhesion to hydrocarbons (BATH) is expressed as-

\[
\text{BATH} = \left\{ 1 - \frac{A_{600 \text{of the aqueous phase}}}{A_{600 \text{of the initial cell suspension}}} \right\} \times 100
\]

Assessment of surfactant and emulsifying property of SACs

The surfactant property of surface active compounds was estimated at room temperature by following du Nuoy ring method (Lunkenheimer and Wantke 1981) using a tensiometer (Data physics DCAT 11, Germany). Emulsification Index (EI) was estimated by following method of Cooper and Goldenberg (1987) against hydrophobic substrates tetradecane, hexadecane, motor oil, diesel, kerosene, vacuum gas oil, engine oil and crude oil. The emulsification index was measured after 24 h according to the following equation (Cooper and Goldenberg 1987).

\[
\text{Emulsification Index(%) } = \frac{h_{\text{emulsion}}}{h_{\text{total}}} \times 100
\]

where, \(h_{\text{emulsion}}\) is the height of emulsion layer formed in between immiscible phases by emulsifying property of SAC and \(h_{\text{total}}\) is the total height of the liquid mixture.

Structural elucidation of the SACs produced by bacterial strains utilizing waste mineral lubricating oil as substrates.

Chemical analysis of the purified SACs

The carbohydrate content was estimated by following phenol-sulphuric acid method for total carbohydrates according to Dubois et al. (1956) and the protein content was estimated according to Lowry’s method for proteins.
(Lowry et al. 1951) by using Ultraviolet-Visible spectrophotometer (Shimadzu UV-1800, Japan). The Folch et al. (1957) method was followed for lipid estimation.

**Characterization studies**

**Fourier transform infrared (FTIR) spectroscopy**

FTIR spectra were obtained in the range 4000–400 cm⁻¹ after preparation of pellets by mixing finely grounded dried potassium bromide and surface active compounds (Perkin Elmer, USA, Spectrum version 10.5.1).

**H¹ Nuclear Magnetic Resonance (H¹-NMR) spectroscopy**

SACs were dissolved in CDCl₃ (for surface active compounds obtained from bacterial utilization of pure hydrocarbons as sole carbon source) and DMSO-d₆ (for surface active compounds obtained from bacterial utilization of waste spindle oil as sole carbon source) and vortexed thoroughly. The samples were then subjected to 400 MHz NMR (Bruker Avance 400 Spectrometer, Germany) and 500 MHz NMR (Jeol ECZ 500 spectrometer, Japan) for H¹-NMR analysis up to spectral width of 15 ppm. The chemical shifts were recorded in ppm relative to the resonance of tetramethylsilane as the internal standard.

**Elemental (CHN) analysis**

4–5 mg of the Purified SACs were taken for estimation of Carbon, Hydrogen, Nitrogen content (Thermofinnigan Flash 1112 Elemental Analyzer, Italy).

**Microstructural analysis by Scanning Electron Microscopy (SEM)**

Dried SACs were observed after glutaraldehyde fixation, sequential dehydration and sputtering with platinum followed by recording images at 15 kV (Zeiss Evo 18 Special Edition, Germany).

**Thermal stability studies**

Thermal stabilities of 12 mg dried, purified SACs were determined by using Thermogravimetric (TG) and Differential thermal (DT) analysis (Perkin Elmer Diamond, USA). Initial and final temperature were set at 30 °C and 800 °C respectively. The heating rate was maintained at 10 °C/min by gradually increasing the temperature.

**Gas Chromatography-Mass Spectrometry (GC–MS) analysis of waste mineral lubricating oil before and after bacterial inoculation**

The n-hexane extracted residual oils before and after bacterial incubation were evaporated for removal of the solvent (EYELA Rikakikai Rotary Evaporator, Japan). The oil samples were then diluted 10 times with HPLC-grade n-hexane and volume of 1 µl were injected into a gas chromatograph equipped with DB5MS column (Thermo Scientific Trace 1300 series, USA). Helium was used as the carrier gas with flowrate 1.5 ml/min. Chromatograms were analyzed by Chromeleon 7.0 program and a library (NIST 2007) search was performed for the identification of peaks.

**Results**

**Selection of carbon source**

Gram-positive Bacillus stratosphericus A15 and Gram-negative Ochrobactrum pseudintermedium C1 showing growth turbidity and better yield of surface active compounds in hydrocarbon substrates were sub-cultured and preserved in sterilized nutrient agar slants at 4 °C. BH broth was enriched with two types of hydrocarbons i.e., pure straight chain alkanes and waste mineral lubricating oils as carbon sources. Carbon sources influencing maximum bacterial growth and SAC production were selected. Ochrobactrum pseudintermedium C1 and Bacillus stratosphericus A15 showed optimal growth when hexadecane and nonane were carbon sources, respectively. Among mineral lubricating oils, waste spindle oil (specific gravity 0.8488) produced maximum growth and yield of SACs by both bacterial strains.

**Parameters for production of SACs**

**pH of the culture medium**

The study was performed under varying pH conditions (pH 2–10). However, yield of surface active compounds increased at alkaline and neutral to slightly alkaline culture medium for Bacillus stratosphericus A15 and Ochrobactrum pseudintermedium C1, respectively.

**Temperature of the culture medium**

The study was done under varying temperature conditions (30–40 °C). Production of SACs by Ochrobactrum
*Ochrobactrum pseudintermedium* C1 were highest at temperatures between 35 and 37 °C, while *Bacillus stratosphericus* A15 has produced SACs between 32 and 33.5 °C.

**Percentage of carbon source, incubation time on bacterial growth and yield of SAC**

It was observed that 4% of the pure and waste hydrocarbons as sole carbon sources provided optimum growth of bacterial strains and subsequent production of SACs. In this study, bacterial strains were grown in pure straight chain hydrocarbons i.e., starting from hexane (C-6) to octacosane (C-28). It was observed that bacterium *Ochrobactrum pseudintermedium* C1 can degrade long chain hydrocarbons (C-14 to C-18) and produce SACs whereas, on the contrary, *Bacillus stratosphericus* A15 can degrade short chain hydrocarbons (C-8 to C-12) and produce SACs. Identical degradation pattern of alkanes was reported in a study by Zheng et al. 2011. The maximum growth was observed between 10th and 12th day of incubation. The maximum optical densities were 0.21 and 0.5 for *Bacillus stratosphericus* A15 and *Ochrobactrum pseudintermedium* C1 respectively (Fig. 1a). The yield of SACs were highest between 12th and 13th day for *Bacillus stratosphericus* A15 and between 14th and 15th day for *Ochrobactrum pseudintermedium* C1. The SAC 3 produced by strain A15 utilizing spindle oil was ethanol.
precipitated and the total yield was around 150 mg/L, while strain C1 has produced SAC 4 with total yield of 300 mg/L following the same process. Ethanol precipitated SACs after isolation and extraction have shown an amorphous nature. These compounds were then subjected to various characterization and chemical analysis to further evaluate their surface activity and to elucidate their chemical structure and composition (Fig. 1bi, bii). A comparative table on various parameters for bacterial production of SACs in culture medium is given in Table 1A.

Assessing the emulsifying, surfactant and cell surface hydrophobicity of SACs

Gram-positive Bacillus stratosphericus A15 and Gram-negative Ochrobactrum pseudintermedium C1 have shown 38% and 56% adherence to waste spindle oil with decrease in absorbances of the aqueous phase to 0.62 and 0.57 at 600 nm respectively as estimated by BATH assay. Such phenomenon is further explained by the tendency of the strain Bacillus stratosphericus A15 to produce SAC 1 and SAC 3 after utilization of nonane and waste spindle oil with the capacity of lowering the surface tension from 71 mN m⁻¹ to 53 mN m⁻¹ and 51.7 mN m⁻¹ respectively (Fig. 2ai). On the contrary, SACs produced by Ochrobactrum pseudintermedium C1 have shown no significant surface tension reduction. SAC 2 and SAC 4 produced from strain Ochrobactrum pseudintermedium C1 could reduce surface tension to 63.9 mN m⁻¹ and 61 mN m⁻¹ when hexadecane and waste spindle oil, respectively, were present as carbon sources. Unlike SACs produced by Bacillus stratosphericus A15, Ochrobactrum pseudintermedium C1 has produced surface active compounds with emulsifying properties and exhibited better results when diesel, engine oil and mustard oil were used as hydrophobic substrates in comparison to SACs produced by strain A15. SAC 4 produced by Ochrobactrum pseudintermedium C1 has shown emulsification index of diesel (2 %), vacuum gas oil (2.5 %), hexadecane (10 %), tetradecane (12.5 %), mustard oil (37.5 %), motor oil (35 %), crude oil (50 %) and engine oil (88 %) after 24 h. The emulsions remained stable for up to 48 h (Fig. 2a(ii) (Table 1B). The findings are in correlation with earlier reported studies where petroleum hydrocarbons were present in the growth medium (Toledo et al. 2008; Gudiña et al. 2015).

Chemical composition and structural elucidation of SACs

Chemical analysis, Ultraviolet Visible Spectrophotometry and CHN analysis

The surface active compounds were found to be composed of carbohydrates, protein and lipid in varying percentages. The obtained results were further corroborated by CHN analysis (Table 2). The results obtained can be further interpreted by FTIR and H¹-NMR analysis. In previous studies by Zheng et al. (2011) and Kourmentza et al. (2019), bacterial surface active compounds with similar varied constituents was reported.

Characterization of SACs by FTIR studies

The surface active compounds produced by bacterial strain A15 (SAC 1) and C1 (SAC 2) utilizing pure hydrocarbons nonane and hexadecane respectively were primarily characterized by FTIR followed by detailed structural elucidation by H¹-NMR. It is worth mentioning that when compared to spectra of pure nonane and hexadecane, significant differences were observed in bacterial produced surface active compounds. The bands between 3300 and 3450 cm⁻¹ and 2000–2500 cm⁻¹ can be attributed to the hydroxyl –OH and alkyne stretch respectively. Further bands at 1650 cm⁻¹, 1385 cm⁻¹ and 1050 cm⁻¹ corresponds to alkene C=C stretch or carbonyl stretching of amides, CH₃ bends and C–O stretch of ether respectively in both the structures (Beltrani et al. 2015; Bhattacharya et al. 2014; Amaral et al. 2006). Presence of small bands in the fingerprint region (858–934 cm⁻¹) indicated characteristic presence of carbohydrates (Beltrani et al. 2015) (Table 3A) (Fig. 2bi).

The FTIR spectra of surface active compounds produced by strain A15 (SAC 3) and strain C1 (SAC 4) utilizing waste spindle oil have shown some differences (Table 3B) (Fig. 2bii). Hydroxyl –OH, alkyne stretch and C=C stretch of alkenes or carbonyl stretching of amides (C=O) were present in both SAC 3 and SAC 4 (Amaral et al. 2006). The C=O stretch of amide group was further supported by bands at 1461 cm⁻¹ and 1570 cm⁻¹ which corresponds to C–N stretch and N–H bends respectively in SAC 4 (Amaral et al. 2006; Beltrani et al. 2015). Sharp bands (2925, 2855 cm⁻¹) in SAC 4 were due to CH stretch and N–H bends respectively (Beltrani et al. 2015; Gudina et al. 2015). Additional bands in fingerprint region denoted presence of ether and anomicar carbon of carbohydrates in both the spectra (Bhattacharya et al. 2014; Sengupta et al. 2019; Beltrani et al. 2015).

Characterization of SACs by H¹-NMR studies

H¹-NMR analysis of SAC 1 and SAC 2 produced by bacterial strains A15 and C1 utilizing pure hydrocarbons nonane and hexadecane as sole carbon sources respectively have shown distinct differences in structures when compared to proton NMR spectra of pure hydrocarbons. Aliphatic saturated hydrocarbon chain of lipids, unsaturated hydrocarbons and ether functional groups were present in both the structures. A strong signal at 2.984 ppm in SAC 2 can be assigned
Table 1  Parameters maintained for bacterial production of surface active compounds and their properties

A. Culture medium parameters for production of bacterial surface active compounds

| Variable parameters for production of SACs in culture medium | Bacillus stratosphericus A15 | Ochrobactrum pseudintermedium C1 |
|-------------------------------------------------------------|-----------------------------|---------------------------------|
| Carbon source                                               | Nonane followed by tetradecane and hexadecane influence maximum SAC yield. In waste mineral lubricating oils, spindle oil influenced maximum growth and SAC yield | Hexadecane followed by tetradecane and nonane influence maximum SAC yield. In waste mineral lubricating oils, spindle oil followed by compressor oil influenced maximum growth and SAC yield |
| Percentage of carbon source                                 | 4% of carbon source was effective for maximum bacterial growth and SAC production in culture medium | 2–4% of carbon source was effective for growth of the bacterial strain. 4% of the carbon source influenced maximum SAC production in culture medium |
| pH of culture medium                                         | 8                           | 7.2                             |
| Aeration rate                                               | 90–92 RPM                   | 95–100 RPM                     |
| Temperature                                                 | 32–33.5 °C                  | 35–37 °C                       |

B. Properties of surface active compounds produced from bacterial strains utilizing waste spindle oil as sole carbon source

| Properties of SACs                                         | SAC 3 from Bacillus stratosphericus A15 | SAC 4 from Ochrobactrum pseudintermedium C1 |
|------------------------------------------------------------|----------------------------------------|--------------------------------------------|
| Emulsification index and surfactant property               | Lowers surface tension from 71 mN m⁻¹ to 51.7 mN m⁻¹ when waste spindle oil is the carbon source | Emulsification index values were engine oil (88%), crude oil (50%), mustard oil (37.5%), motor oil (35%) and tetradecane (12.5%), hexadecane (10%) after 24 h |
| Thermal stability of SACs by Thermogravimetric analysis (TGA) | Residual weight of sample remaining- 58%, Total weight loss of sample- 42% (Temperature range is 30–800 °C) | Residual weight of sample remaining- 66%, Total weight loss of sample- 34% (Temperature range is 30–800 °C) |
| Microstructure of SACs by Scanning Electron microscopy (SEM) | Elongated rod structures and few aggregated globular structures were observed in higher magnifications | Porous matrix like formation with aggregated globular and elongated rod like structures were observed in higher magnifications |
Structural differences by H\textsuperscript{1}-NMR analysis of surface-active compounds produced by utilizing spindle oil were obtained (Table 3b) (Fig. 3bi, bii). Multiple signals between 0.7 and 1.4 ppm denote protons in aliphatic saturated chain of lipid moiety i.e., alkyl CH\textsubscript{3}, CH\textsubscript{2} and CH or cholesterol in the structures (Watts 2013). This is followed by unsaturated or carbonyl protons at 1.8–2.4 ppm and protons of ether functional group at 3.6–3.8 ppm. Further downfield, signals between 4.2 and 5 ppm can confirm the presence of anomeric sugars (Duus et al. 2000). Presence of isopropyl groups in SAC 4 was evident by characteristic signals between 1.409 and 1.475 ppm and 4.1–4.2 ppm. However, in SAC 3 isopropyl group was observed in the region between 1.4 and 1.5 ppm. The presence of isopropyl group suggest the branched nature of the structures.

Table 2 Chemical composition and CHN analysis of surface active compounds produced by bacterial strains utilizing waste spindle oil (in percentage)

| Surface active compounds produced by bacterial strains | Lipid (%) | Carbohydrate (%) | Protein (%) | Carbon (%) | Hydrogen (%) | Nitrogen (%) |
|------------------------------------------------------|-----------|------------------|------------|------------|--------------|-------------|
| SAC 3 from Bacillus stratosphericus A15              | 43        | 0.85             | 3.4        | 4.356      | 2.663        | 0.620       |
| SAC 4 from Ochrobactrum pseudintermedium C1          | 80        | 1.85             | 6          | 12.42      | 3.99         | 0.805       |
### Table 3  Structural elucidation of bacterial surface active compounds by FTIR and NMR spectroscopy

**A. Comparative spectroscopy analysis (FTIR and H\textsuperscript{1}-NMR) of SACs produced from bacterial strains by utilizing nonane and hexadecane as carbon source**

| Structural Elucidation | SAC 1 from *Bacillus stratosphericus* A15 | SAC 2 from *Ochrobactrum pseudintermedium* C1 |
|------------------------|--------------------------------------------|-----------------------------------------------|
| **1. FT-IR** (Band range) | Functional group and type of band intensity | Functional group and type of band intensity |
| Single bond (2500–4000 cm\textsuperscript{-1}) | O–H stretch (strong, broad, 3300–3450 cm\textsuperscript{-1}) | O–H stretch (broad, strong, 3300–3450 cm\textsuperscript{-1}) |
| Double bond (1500–2000 cm\textsuperscript{-1}) | C=O or carbonyl stretching of amides (medium, 1650 cm\textsuperscript{-1}) | C=O or carbonyl stretching of amides (medium, 1650 cm\textsuperscript{-1}) |
| Triple bond (2000–2500 cm\textsuperscript{-1}) | Alkyne stretch (weak, 2332 cm\textsuperscript{-1}) | Alkyne stretch (weak, 2332 cm\textsuperscript{-1}) |
| Finger print region (600–1500 cm\textsuperscript{-1}) | –CH\textsubscript{3} bending (1384 cm\textsuperscript{-1}), C–O stretch of ether (strong, 1055 cm\textsuperscript{-1}) and anomeric carbon (877 cm\textsuperscript{-1}) | –CH\textsubscript{3} bending (1384 cm\textsuperscript{-1}), C–O stretch of ether (strong, 1067 cm\textsuperscript{-1}) and anomeric carbon (872 cm\textsuperscript{-1}) |
| **2. NMR** Functional group and chemical shifts (ppm) | | |
| Upfield region | Protons of CH\textsubscript{3} and CH\textsubscript{2} (0.8–1.2 ppm), allylic C–H (1.8–2.2 ppm) and ether CH–OCH\textsubscript{3} group (3.5–3.4 ppm) are present | Protons of CH\textsubscript{3} and CH\textsubscript{2} groups (0.8–1.8 ppm), allylic C–H group (1.8–2.2 ppm), unsaturated groups or carbonyl group (2.984 ppm) and ether CH–OCH\textsubscript{3} group (3.5–3.4 ppm) are present |
| Downfield region | Isopropyl groups present (3.690–3.755 ppm) | Isopropyl groups absent |

**B. Comparative spectroscopy analysis (FTIR and H\textsuperscript{1}-NMR) of SACs produced from bacterial strains by utilizing waste spindle oil as carbon source**

| Structural Elucidation | SAC 3 from *Bacillus stratosphericus* A15 | SAC 4 from *Ochrobactrum pseudintermedium* C1 |
|------------------------|--------------------------------------------|-----------------------------------------------|
| **1. FT-IR** (Band range) | Functional group and type of band intensity | Functional group and type of band intensity |
| Single bond region (2500–4000 cm\textsuperscript{-1}) | O–H stretch (broad, strong, 3431 cm\textsuperscript{-1}) | O–H stretch (broad, strong, 3388 cm\textsuperscript{-1}), CH\textsubscript{2} and CH\textsubscript{3} groups of C–H stretch (sharp, 2925 and 2855 cm\textsuperscript{-1}) |
| Triple bond region (2000–2500 cm\textsuperscript{-1}) | Less distinct alkyne stretch | Distinct alkyne stretch (weak, near 2332 cm\textsuperscript{-1}) |
| Double bond (1500–2000 cm\textsuperscript{-1}) | Alkene –C=O or carbonyl –C=O group (medium, 1642 cm\textsuperscript{-1}), C–H bending of alkane (medium, 1384 cm\textsuperscript{-1}). Less intense bands denoting presence of N–H bend and C–N stretch | Alkene –C=O or carbonyl –C=O group (strong, 1652 cm\textsuperscript{-1}), N–H bend (medium, 1570 cm\textsuperscript{-1}), –C–N stretch (medium, 1461 cm\textsuperscript{-1}), C–H bending of aldehyde (medium, 1384 cm\textsuperscript{-1}) |
| Finger print region (600–1500 cm\textsuperscript{-1}) | C–O stretch of ether (strong, 1065 cm\textsuperscript{-1}), anomeric carbon of carbohydrates | C–O stretch of ether (strong,1066 cm\textsuperscript{-1}), anomeric carbon (950–700 cm\textsuperscript{-1}) |
| **2. NMR** Functional group and chemical shifts (ppm) | | |
| Upfield region | Protons of –CH\textsubscript{3}, –CH\textsubscript{2} and –CH of lipid or cholesterol (0.8–1.2 ppm), allylic C–H (1.8–2.2 ppm), isopropyl (1.3–1.5 ppm), carbonyl (2.2–2.4 ppm) groups | Protons of –CH\textsubscript{3},–CH\textsubscript{2} and -CH of lipid or cholesterol (0.8–1.2 ppm), isopropyl (1.3–1.4 ppm), allylic C–H (1.8–2.2 ppm), carbonyl (2.2–2.4 ppm) groups |
| Towards downfield | Less distinct signal for protons corresponding to ether CH–OCH\textsubscript{3} group (3 ppm and 3.839 ppm) and anomeric sugars (4.2–4.6 ppm) | Sharp signals for protons attached to isopropyl (4.162–4.2 ppm), ether CH–OCH\textsubscript{3} group (3.389 ppm) and less pronounced signals for protons attached to anomeric sugars (4.1–4.2 ppm) and amide –NH (7.7–7.8 ppm) |
Microstructural studies of SACs by scanning electron microscopy

The SACs produced by both strains have shown some contrasting differences. Strain A15 produced surface active compound with few elongated rod structures in low magnifications whereas in higher magnifications presence of spherical structures were more prominent (Fig. 4ai, aii) (Table 1B). Surface active compound produced from strain C1 has shown irregular spherical structures in lower magnifications whereas in higher magnifications few elongated rod structures and globular spherical structures adjacent to web like porous matrix was observed (Fig. 4bi, bii) (Table 1B).

Thermal stability studies of SACs

Thermogravimetric analysis (TGA) of SACs produced by bacterial strains A15 and C1 utilizing waste spindle oil have shown initial weight reduction in between 80 and 150 °C. This phenomenon can be attributed due to loss of moisture and solvents in both cases. Thermogram of samples displayed similar gradual downward curve with increase in temperature thus implying decomposition of organic matter. During the second phase i.e., from 150 to 350 °C rapid degradation in both the samples was observed. Maximum degradation has started from 350 °C and continued till 800 °C. Upon completion of the analysis, residual weights of SAC 3 and SAC 4 were found to be 58% and 66% respectively suggesting more thermal resistant nature of SAC 4 when compared to SAC 3 (Fig. 5a) (Table 1B). Similar thermal degradation of surface active extracellular polymeric substances have been reported in earlier studies (Sengupta et al. 2019). Thermal Analysis (DTA) of both samples validate with their respective TGA graphs. The endothermic peak near 100 °C is characteristic of dehydration reaction. Another significant exothermic peak was observed at 338.06 °C and 380.54 °C.
for SAC 3 and SAC 4 respectively corresponding to organic oxidation of both the compounds (Fig. 5b).

**GC–MS analysis of waste industrial mineral lubricating oils before and after bacterial incubation**

GC-MS chromatogram analysis of waste spindle oil before and after bacterial incubation, have revealed some interesting differences (Fig. 5c). Complete disappearance of few peaks corresponding to cyclohexane, hexadecynol and heptadecyne derivatives in the chromatogram of *Ochrobactrum pseudintermedium* C1 utilized spindle oil fraction was observed whereas there has been decrease in relative abundance to the peaks corresponding to hexadecynal, eicosane intermediates in C1 utilized fraction of spindle oil (Fig. 5 di, diii). Comparing chromatograms before and post incubation by bacterial strains, less degradation capability of hydrocarbons by strain A15 was observed (Fig. 5 di, dii). The peak corresponding to hexadecynal was observed in bacterial strains utilized chromatograms. However, decrease in concentration of hexadecynal in strain C1 utilized fraction when compared to strain A15 utilized fraction of waste spindle oil suggested better utilization of long chain hydrocarbons by strain C1 (Fig. 5 di, dii, diii). Presence of organic acid hexadecadienoate and alcoholic intermediates for instance, dodecadienol, octadecatetraenol in the chromatogram of spindle oil utilized by strain C1 also suggests bacterial metabolism and bioconversion of complex hydrocarbon substrates into intermediate compounds. *Ochrobactrum pseudintermedium* C1 has shown similar characteristics in degrading long chain hydrocarbons of waste oils (Bhattacharya and Biswas 2014; Bhattacharya et al. 2015; Sengupta et al. 2019). Although, *Bacillus stratosphericus* A15 has produced surface active compound by utilizing fish fat as carbon source (Sana et al. 2017b), this study reports production of surface active compounds and GC-MS analysis of residual hydrocarbon post bacterial inoculation.

![Fig. 4 Microstructural scanning electron microscopy (SEM) images of a SAC 3 produced by *Bacillus stratosphericus* A15 and b SAC 4 *Ochrobactrum pseudintermedium* C1 at (i) 5 K (ii) 10 K magnifications](image)
Discussion

Bacterial species generally degrade long chain alkanes more efficiently followed by branched alkane, small aromatics and cyclic alkanes. In a study, several Gram-negative and Gram-positive species of bacteria have shown different degradation rates when exposed to crude oil sludge (Obi et al. 2016; Lăzăroaie 2010). Bacteria do so by adhering to hydrocarbon droplets or by surfactant and emulsifier production which facilitates easier consumption of such immiscible carbon sources (Rojo 2009). Many bacterial species have produced such amphiphilic molecules possessing emulsifying and surfactant properties by degradation of hydrocarbons. However, chemical architecture, nature and properties of such molecules vary and are mainly dictated by the growth parameters and nutrient sources (Rosenberg and Ron 1999; Calvo et al. 2009).

It is necessary to mention that by certain evolutionary strategies, some bacteria can show affinity towards oil or hydrocarbons and consume them as their necessary energy and carbon source. They are called “hydrocarbonoclastic” bacteria for their role in bioremediation of hydrophobic pollutants like petroleum hydrocarbons and discharged oils (Xu et al. 2018; Yakimov et al. 2007). In context of environmental bioremediation, such eco-friendly practices can be done by subjecting bacteria alone or by utilizing bacterial surface active compounds for increasing availability of immiscible carbon sources (Datta et al. 2020; Sengupta et al. 2018). Detailed structural characterization and chemical composition by various spectroscopy and spectrophotometry methods can elucidate chemical structures and hence respective functional attributes of surface active compounds (Saha et al. 2020).

**Bacteria synthesized SACs from pure and waste hydrocarbons as carbon sources**

In this study, Gram-negative *Ochrobactrum pseudintermedium* C1 and Gram-positive *Bacillus stratosphericus* A15 strains capable of metabolizing hydrocarbon and lipid based substrates were chosen for production of surface active compounds (Bhattacharya et al. 2014; Sengupta et al. 2019;
Sana et al. 2017b). Both bacterial strains have shown an initial lag phase during their growth on hydrocarbon carbon sources. The final OD_{600} of strain C1 was found to be greater than strain A15 suggesting its better growth and yield of SACs while utilizing long chain hydrocarbons. Interestingly, production of SACs was observed in another study by Cai et al. 2017 when hexadecane and diesel were used as carbon sources in culture medium. However, bacterial strain A15 has shown better growth and yield of SACs in presence of short chain hydrocarbons. The best yield was observed in presence of nonane and hexadecane for Bacillus stratosphericus A15 and Ochrobactrum pseudintermedium C1 with positive growth turbidity at 600 nm during incubation. Among other mineral lubricating oils, waste spindle oil with increased adherence to the cell surface of bacterial strains and yield of SACs was chosen as carbon source. The yield of SACs by both bacterial strains was found to be maximum during early logarithmic and stationary phase of growth therefore, promoting better uptake of these immiscible hydrocarbon substrates as carbon sources. Utilization of petroleum hydrocarbons like crude oil followed by production of surface active compounds from bacteria or bacterial consortium has been studied in previous works (Cooper and Goldenberg 1987; Bhattacharya et al. 2019).

Other additional parameters like pH, temperature, aeration are necessary for increasing the production of surface active compounds. Extreme conditions of pH, temperature can inhibit bacterial growth and result in inactivation of enzymes required for biosynthesis of these extracellular surface active compounds (Jin and Kirk 2018; Das and Chandran 2011).

**Interpretation of characterization studies for structural elucidation, morphological, tensioactive and thermal stability properties of surface active molecules**

The FTIR and H1-NMR characterization has revealed the presence of some chemical groups (–OH of alcohols, –OCH3 of ethers and –CO of amides) which are not otherwise present in linear chain alkanes. Hence, it can be said that enriching of Bushnell Haas broth with these alkane hydrocarbons lead to simultaneous production of amphiphilic SACs thus increasing cellular uptake of these immiscible hydrocarbons as carbon source (Cai et al. 2017). The FTIR and H1-NMR study has revealed presence of aliphatic groups in the structures along with the hydrophilic groups like hydroxyl, carbonyl and ether thus confirming the results obtained in chemical analysis (Ortega-de la Rosa et al. 2018). Additional presence of –CH2 and –CH3 groups of aldehydes along with –NH and –CN group of amides in FTIR spectrum of SAC 4, is further confirmed by H1-NMR analysis. Further, surface active compounds from Ochrobactrum pseudintermedium C1 have shown emulsification index for wide range of hydrocarbon substrates like pure aliphatic hydrocarbons (hexadecane and tetradecane), petroleum fractions (diesel, engine oil, vacuum gas oil, motor oil and crude oil), and edible oil (mustard oil). The results so obtained suggest that surface active compounds from Ochrobactrum pseudintermedium C1 have shown more efficiency in emulsifying both pure and mixture of hydrocarbons. The ether and hydroxyl functional groups attached to aliphatic chains provide those molecules amphiphilicity thus promoting its emulsifying nature (Gudina et al. 2015; Zheng et al. 2011). The spectroscopy and spectrophotometry results obtained for surface active compounds from strain A15 suggest that percentage variation of protein, lipid and carbohydrate may impart their surface tension lowering properties as demonstrated in work of Dadrasnia and Ismail 2015. The difference in surface active properties can be further understood by adhesion characteristics of individual bacterial strains to waste spindle oil. Both bacterial strains on the basis of their hydrophobicity adheres to either short chain or long chain hydrocarbons. Gram-positive bacteria contains an outer cell wall mainly composed of teichoic acids and peptidoglycan layer while Gram-negative bacteria contains an additional lipopolysaccharide and phospholipid layer surrounding the periphery of the cell wall (Silhavy et al. 2010). The long chain hydrocarbon substrates therefore can adhere to the permeable lipopolysaccharide layer of strain Ochrobactrum pseudintermedium C1, followed by its uptake to the cell’s interior and mediating simultaneous production of SACs with emulsifying properties (Beveridge 1999; Bhattacharya et al. 2014; Sengupta et al. 2019). The presence of teichoic acids and proteins can help in cellular uptake of short chain hydrocarbons by Bacillus stratosphericus A15 leading to simultaneous production of SACs with surface tension lowering properties (Silhavy et al. 2010; Sana et al. 2017b). This surface tension lowering ability can be due to adhesion of SACs to the bacterial outer surface and directing the hydrophilic part of SAC outwards thus decreasing cell surface hydrophobicity or due to alterations in cell surface functional groups during production of these compounds (Kaczorek et al. 2018). The increased and rapid adhesion of hydrocarbons by Gram-negative bacteria has been previously studied by Rosenberg et al. (1980). Chemical characterization studies (FTIR and H1-NMR) has depicted presence of alkene, alkyne stretch of aliphatic hydrocarbon and increased number of isopropyl groups in SAC 4 exhibiting stability at higher temperatures when compared to SAC 3. The elemental analysis has also shown high carbon, hydrogen and nitrogen content in SAC 4 that can further establish its emulsifying character and thermal stability. The scanning electron microscopy images suggest the microstructural differences in SACs produced by Bacillus stratosphericus A15 and Ochrobactrum pseudintermedium C1. The globular structures in both SACs can be attributed to the presence of

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chemical functional groups in proteins and carbohydrates (Bhattacharya et al. 2014). Detailed examination of SEM images have revealed high number of globular structures in SAC 4 when compared to SAC 3. The microstructural differences observed correlate with the structural elucidation of the structures obtained by chemical analysis, FTIR and H^{1}-NMR. The characterization analysis suggest that the surface active compounds are heterogeneous mixture of lipoglycoprotein containing varied percentages of lipids, proteins and polysaccharides rendering them distinct characteristics (surfactant, emulsion forming nature and thermal stability). The structural and chemical composition of the produced extracellular surface active compounds have shown resemblance to the compounds reported in earlier published works of Peele et al. 2016 and Zheng et al. 2011. Emulsan produced by Acinetobacter calcoaceticus RAG-1 has shown similar chemical compositional characteristics with polysaccharides attached to fatty acid groups distributed across the entire hydrophobic molecule. The polysaccharide groups can be replaced by proteins or can be in combination as observed in this present study (Neu 1996).

Interpretation of GC–MS studies of residual waste spindle oil before and after bacterial utilization

GC-MS studies provide a detailed insight into the differential uptake between two bacterial strains in utilization of hydrocarbons present in waste spindle oil. The abundant presence of organic acids, aldehydes and alcoholic intermediate products in the utilized fractions suggest that bacterial strains can undergo β-oxidation after consuming long chain compounds in waste spindle oil which is present in the culture medium as the sole carbon and energy source (Bhattacharya and Biswas 2014). Based on GC-MS findings, presence of hexadecynal and hexadecadienoate and complete degradation of hexadecynol in utilized fraction of waste spindle oil suggests that hexadecynol oxidizes to former two compounds which is corroborated by Adlan et al. 2020 where decreasing abundance of long chain alkanes and conversion to intermediate products proved a better cellular uptake and biodegradation. Unlike Bacillus stratosphericus A15, Ochrobactrum pseudintermedium C1 had shown better utilization of long chain hydrocarbons as evident from the abundance of hexadecynal. Further, absence of peaks corresponding to heptadecane and hexadecane intermediates establishes comparatively efficient metabolism of long chain hydrocarbon by Ochrobactrum pseudintermedium C1. This is further justified from the study by Sierra-Garcia and de Oliveira (2013) that the presence of AlkB gene, which encodes for non-haeme iron monooxygenase participate in oxidation of long or short chain hydrocarbons to respective alcoholic intermediates which might facilitate the production of SACs. Moreover, incorporation of oxygen containing chemical functional groups like hydroxyl, ether and carbonyl groups in the surface active compounds as evident from FTIR, H^{1}-NMR, and chemical analysis can further confirm the involved hydrocarbon biodegradation mechanism for production of these metabolites.

Conclusion

Comparative characterization analysis of bacterial surface active compounds by utilization of pure and waste mineral lubricating oils was done. However, the compounds produced by Bacillus stratosphericus A15 and Ochrobactrum pseudintermedium C1 have exhibited difference in surface active properties and thermal stabilities. Production of such bacterial surface active compounds by using conventional nutrient growth medium can be an expensive process. Waste mineral lubricating oils represent an economic carbon source for production of bacterial surface active compounds. The presence of different chemical groups in these compounds can render them future promising applications.

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Author contribution The study was conceptualized by SD. The methodology of the study was developed by SD, IS and DS. Formal analysis and investigation of the study was done by IS. Original draft of the manuscript was prepared by IS. Revision and editing of the manuscript were done by IS, SD, DB, DS. The study was done under the supervision of SD and DB. The funding acquisition and infrastructural support for conducting the study was arranged by SD and DB.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.
Ethical approval

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

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