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Short communication

Harnessing microbial potential: Exploiting heavy oil-laden soil microbiota for sustainable production of high-yield rhamnolipids from waste cooking oil

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ABSTRACT

Oil polluted soil microbiota plays an important role in the production of biosurfactants. In comparison to synthetic surfactants, biosurfactants offer unique advantages, such as lower toxicity, biodegradability, selectivity, and effectiveness under unpleasant conditions. Despite these benefits, the widespread use of biosurfactants is limited by enormous production costs. To address this challenge, this study aimed to explore the adoption of waste cooking for rhamnolipids production. Two prominent bacterial strains: *Kosakonia cowanni* and *Acinetobacter colcoaceticus,* were obtained from heavy oil-laden soil samples, and further demonstrated their capability for rhamnolipids production from waste cooking oil (*Acinetobacter colcoaceticus*: 0.51 g/L, *Kosakonia cowanii*: 0.39 g/L). The biosurfactants obtained were characterized through TLC, FTIR, and H NMR to confirm their rhamnolipid identities as mono-rhamnolipids. The findings in our study emphasizes the potential of cost-effective production of rhamnolipids that possess interesting biotechnological features through the synergy of oil-polluted environments and waste cooking oil. This study contributes significantly to the development of sustainable rhamnolipid production using non-pathogenic strains. By harnessing these microorganisms, we advance towards addressing critical environmental challenges, such as heavy metal contamination in water. This research aligns with broader sustainability goals, including clean water and sanitation.

1. Introduction

The imperative to address environmental challenges and simultaneously seek alternative sources for bio-based compounds has spurred interest in the utilization of marginal and contaminated environments. The heavy oil-laden soil, characterized by its hostile conditions and limited nutrient availability, poses a challenging ecological liability. However, it presents as a promising environment for microbial communities, as it harbours a hidden wealth of microbial diversity (mostly extremophiles) that have evolved unique metabolic capabilities to thrive in such harsh conditions (presence of hydrocarbons and heavy metals). These microorganisms are potential candidates for converting environmental liabilities into valuable products. Given the growing demand for sustainable alternatives, there is an urgent need to shift from synthetic surfactants to biosurfactants which are derived from microorganisms, plants and

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animals (Marchant and Banat, 2012). Their amphiphilic nature makes them ideal components in cleaning and personal care products, such as detergents, soaps, and cosmetics. In addition, surfactants have varied applications in food, remediation, textile, pesticide, and biomedical sectors. Thus, they are used widely. However, their widespread use has negative impact on the environment. Especially since they are used in high volumes by the aforementioned sectors and they take a long time to be completely degraded, hence, they have high environmental persistence (Naughton et al., 2019). Several advantages that render them preferable are their nontoxic nature, begnin interaction with the environment and higher biodegradability. As an addition, they essentially possess a lower CMC which makes it more valid for various applications. Biosurfactants are classified as Low molecular weight (glycolipids and lipopeptides) and High molecular weight (polymeric compounds). Rhamnolipids are among the most studied biosurfactants due to their outstanding properties. Rhamnolipids, known for their biodegradability, low toxicity and versatile surfactant properties, have gained considerable attention in various industries, including bioremediation, petrochemical, pharmaceuticals, and agriculture. This phenomenon is substantiated by the estimated US$ 6.40 billion the biosurfactant global market is expected to reach by 2027, driven by a 5.5% compound annual growth rate since 2022 (Market Research Engine (2024)). However, the cost-effective production of these compounds remains a challenge. The high cost of raw materials and the input of the process lead to the high cost of production of rhamnolipids, which limits the large-scale promotion and application of biosurfactants (Dias and Nitschke, 2023). Currently, surfactants mainly derived from petroleum products as a result of the low cost of production are not biodegradable and may lead to environmental problems. The chronic and sublethal toxicities of some of these chemical surfactants to aquatic animals occur even at a concentration of 0.1 mg/L (Lewis, 1991). The quest for sustainability and nontoxic practices is gaining momentum, and harnessing rhamnolipids produced from waste materials provides a safe and cost-effective alternative to replace toxic surfactants. To this end, exploring alternatives such as the use of waste cooking oil as substrates is advantageous in significantly reducing about 60% of overall production costs while advancing the transition towards a circular economy. Worldwide, approximately 41–67 million tons of waste cooking oil is generated annually (Kim et al., 2021). Waste cooking oil, a ubiquitous by-product of culinary practices, poses its own set of environmental challenges. Its improper disposal leads to pollution and resource wastage, emphasizing the need for innovative strategies to convert this waste into valuable products (Foo et al., 2022). Plant-based surfactants, often derived from palm or coconut oils, require chemical processes like ethoxylation. This can lead to the formation of byproducts such as 1,4-dioxane and raises concerns about the environmental impact of palm oil production, including deforestation and increased carbon emissions (Jamaludin et al., 2019). To address these issues, there's a growing interest in microbially produced biosurfactants. In line with this, countries like Estonia are prioritizing resource efficiency and reduced greenhouse gas emissions as part of their economic transition. Oil-based carbon sources are often considered optimal for achieving high rhamnolipid yields due to their high fatty acid content, a key precursor in rhamnolipid biosynthesis. The oil in waste hinders the natural composting of food waste, decreasing its overall biodegradation efficacy, leading to soil and water pollution. Microorganisms are pivotal in rhamnolipid production, with strain selection directly impacting yield and overall efficiency. While Pseudomonas aeruginosa has been extensively studied, exploring a diverse microbial pool is essential to identify strains that optimize rhamnolipid production while minimizing environmental impact and production costs (Dabaghi et al., 2023, 2023; Zhao et al., 2021a; 2023). Diverse microorganisms may possess unique metabolic pathways and non-pathogenicity, making them valuable for modified and safe environmental application (Rafeeq et al., 2023). Moreover, several regulatory issues associated with genetically modified organisms have limited the applicability of biosurfactants produced by genetically engineered organisms. And That is not the only issue pertain to Rhamnolipids production but many underlying factors still hinder their large scale production (Miao et al., 2024). Microbial surfactants offer a sustainable alternative to synthetic surfactants, as they are produced by microorganisms like bacteria, yeast, or fungi. Their production occurs through fermentation under controlled environments, minimizing their contamination. Furthermore, the versatility of microbial surfactants, influenced by microbial strain, substrate, and production conditions (Adu and Hunter, 2021), allows for tailored production to meet specific application requirements. To date, there is limited research on rhamnolipid production through a circular economy approach that involves bio transforming waste cooking oil using microorganisms indigenous to heavy oil-contaminated soil. Further, this aligns with the principles of circular economy and waste valorisation. Additionally, the sustainable production of biosurfactants contributes to the United Nations' Sustainable Development Goals (SDGs), specifically goals 8 and 12, which emphasize economic growth, decent work, and responsible consumption and production (UN, 2015). Therefore, this study aimed to use waste cooking oil in conjunction with culturable microbiota sourced from various heavy oil-laden samples to produce high-quality rhamnolipids. Furthermore, we aimed to elucidate the interesting structural and spectral characteristics of the produced rhamnolipids using TLC, FTIR and HNMR.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and reagents were supplied by Sigma-Aldrich, Glass world, biolab and Saarchem were used without further purification, as they were all of analytical grade. The methylene blue was obtained from Promark Chemicals (Canada), Magnesium Sulphate from Saarchem (India), Tryptic Soy Broth, LB Broth Miller and Cetrimonium Bromide (CTAB) were from Sigma Aldrich (USA), Sulfuric acid 98 % from Glass world (South Africa), Bacteriological Agar from Biolab, Ferrous Sulphate from Saarchem (India), Calcium Chloride Di-Hydrate from Saarchem (India), Methanol from VWR Chemicals and HCl 32 % from Glass world (South Africa), Anthrone from M chemicals (USA) and Chloroform from Merck (South Africa).

2.2. Preliminary screening

Rhamnolipid-producing bacteria were screened from three samples: home waste cooking oil, restaurant waste cooking oil and heavy oil laden soil (Fig. 1). The estimated characteristics of the oil samples have been corroborated by Awogbemi et al. (2019) in

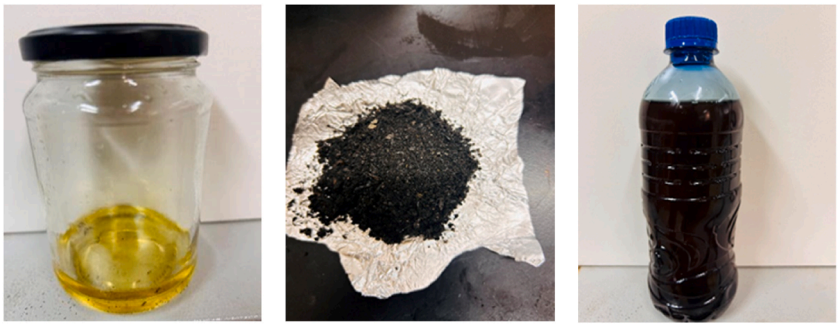

Fig. 1. Sample used for the isolation of bacteria. The waste cooking oil was obtained from a fast-food restaurant (Chicken Centre, Hatfield (PTA) and the other sample is a home waste cooking oil and oil-laden soil was collected from a car servicing yard (0–5 cm depth).

Table 1. The oil samples were filtered using 75 μm mesh before use. The samples (5% v/v oil and 5% w/v soil) were further enriched in a sterile broth consisting of (g/L): $NaNO_3$ (2), KH_2PO_4 (0.2), K_2HPO_4 (0.6), $MgSO_4.7H_2O$ (0.5), $CaCl_2.2H_2O$ (0.01), $FeSO_4.7H_2O$ (0.01), NaCl (0.5), per 95 mL of distilled water in 250 mL Erlenmeyer flasks, then incubated in a shaker at 170 rpm, 30 °C for 54 h to ensure complete mixing of the samples. In a parallel setup, a CTAB-Methylene blue agar medium was added to the aforementioned selection medium (400 mL) for the agar base and sterilized at 121 °C for 15 min. The composition of CTAB-methylene blue (g/L): CTAB (0.4), Methylene blue (0.004) and Bacterial agar (6 g). Sixfold decimal serial dilution was performed on the enriched samples and homogenized 100 μL aliquots were spread on CTAB-methylene blue agar plates. The Petri dishes were incubated at 25 °C under aerobic conditions for 48 h. After incubation, the dark blue halo colonies were identified as presumptive rhamnolipid-secreting isolates. Their pure cultures were then screened again on the CTAB-methylene blue agar plate to measure their area, which was used as a criterion for further evaluation.

2.3. Rhamnolipid production from waste cooking oil

Choice isolates were twice washed and re-suspended in physiological saline (0.85% w/v NaCl), where they were standardized to $OD_{600\ nm}$ of 0.8 after 18 h of incubation. They (5% v/v) were further used to ferment waste cooking-supplemented media. The waste oil (15% v/v) was added as sole carbon source to the fermentation media comprising (in g/L): $NaNO_3$ (2), KH_2PO_4 (0.4), K_2HPO_4 (0.6), NaCl (0.5), $MgSO_4.7H_2O$ (0.5), and the composition of trace elements in g/L: $CaCl_2$ (0.1), $FeSO_4.7H_2O$ (0.1), $ZnSO_4.7H_2O$ (0.76), $MnCl_2.4H_2O$ (0.15), EDTA (0.001), $NiCl_2.6H_2O$ (0.08) in 300 mL of deionized water and autoclaved at 121 °C for 15 min. The broths which were in 500 mL Erlenmeyer flasks were subject to orbital incubation at 30 °C at 180 rpm, where samples were aseptically withdrawn every 18 h for cell growth and rhmanolipid concentration.

2.4. Growth evaluation and rhamnolipid estimation

Fermented samples (1 mL) were withdrawn at intervals (0 h, 18 h, 36 h, 54 h, 72 h and 96 h) and centrifuged (mini-Spin) at 8000 rpm for 10 min. The supernatant was separated for rhamnolipid estimation, while the cell pellet was washed twice in 1.5 mL deionized water and re-suspended in the centrifuge. The cells were homogenized using a vortex mixer which was then followed by absorbance readings ($OD_{600\ nm}$) using a spectrophotometer (Ultrospec 3000, UV–visible). Rhamnolipid estimation was done using the anthrone-sulfuric acid method. A volume of 10 mL of chromogenic reagent (freshly prepared sulfuric acid solution) containing 0.2% anthrone (v/v) was slowly added to 1.5 mL of the supernatant solution, followed by cooling in an ice bath. After sufficient mixing, the sample solution was placed in boiling water to develop coloration (10 min). The sample solution was quickly cooled to room temperature (25 °C) and its absorbance was measured at 620 nm. The standard curve was $y = 0.0015x - 0.1942$, where x and y were rhamno-

Table 1
Basic physical properties of waste cooking oil (Awogbemi et al., 2019).

	Neat oil	Waste cooking oil
Density @ 20 °C (kg/m^3)	919.21	920.4
Viscosity @ 40 °C (mm^2/s)	28.224	31.381
pH	7.38	5.34
Congealing temperature (°C)	−8.65	−5.15
Molecular weight (g/mol)	670.82	51.94
Acid value		2.29
Iodine value (cg/g)		111.1
Palmitic acid	32.21	0.36
Linoleic acid	21.98	0.10
Caprylic acid	0.22	0.20
Saturated fatty acid (%)	71.48	32
Polyunsaturated fatty acid (%)	28.52	6
Monounsaturated fatty acid (%)	_	62

lipids (mg/L) and the absorbance of the sample solution, respectively. The linear correlation coefficient was 0.886. Results are expressed as the mean of three independent tests ± standard deviation

2.5. Extraction and purification

Preparation of the rhamnolipid sample, 25 ml of fermentation broth was spun in a centrifuge at 8000 rpm and 4 °C for 10 min. The pH of the supernatant was first adjusted to 2.0 using hydrochloric acid solution (6 M). The crude biosurfactant was treated with chloroform: methanol (2:1), approximately 13 mL of chloroform, 6.6 mL of methanol until the organic and aqueous phase appeared. The extraction process was carried out 3 times in the organic phase containing Rhamnolipids. The pure biosurfactant was obtained by releasing the aqueous mixture by rotary evaporation.

2.6. Molecular identification of isolates

Genomic DNA was extracted from choice isolates using the ZR Fungal/Bacterial DNA kit™ (Zymo Research). Their 16S target region was amplified using Dream Taq DNA polymerase (Thermo Scientific™) and the universal primers, 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1429R: 5′-CGGTTACCTTGTTACGATT-3′. Amplicons obtained from PCR run were gel extracted (Zymo Research, Zymoclean™ Gel DNA Rocover Kit), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyzer. Purified sequencing products (Zymo Research, ZR-96 DNA sequencing clean-up kit™) were analyzed using CLC Main Workbench 7 followed by a BLAST search (NCBI database) for most probable strain identification.

2.7. Physicochemical characterisation

2.7.1. Oil spreading test

The oil spread test was performed for the two biosurfactant samples. About 20 μL crude oil was placed on to the surface of 40 mL distilled water in a 90 cm Petri dish to develop a thin membrane of oil. Five (5) μL of the crude biosurfactant was carefully dropped on to the centre of the oil membrane The diameters of the clear halos produced were measured, all tests were performed in triplicates at room temperature and mean values were recorded.

2.7.2. Determination of the emulsification index

The emulsification activity was determined by adding an equal volume of n-hexane to cell-free supernatant. The mixture was homogenized in a vortex for 2 min by vigorous shaking and then kept undisturbed for 24 h at room temperature. E_{24} was obtained by calculating the ratio of the height of the emulsified layer (mm) to the total height of the liquid column (mm) (Jiang et al., 2020):

$$E_{24}\% = \frac{height\ of\ emulsion\ layer}{height\ of\ total\ mixture} \times 100 \quad (1)$$

2.8. Structural characterization

Separation was performed on a TLC Silica gel 60 F254, by the solvent system (chloroform: methanol: 20% aqueous acetic acid) (65:15:2) as a mobile phase, in the presence of a standard of rhamnolipid. Fourier transform infrared (FTIR) spectroscopy of prepared rhamnolipids was collected by the KBr pressed-disk technique. The mass ratio of the sample to KBr was 1: 1500, and each sample was pressed o nto transparent sheets with a uniform thickness of 1 mm. The resolution was 4 cm^{-1} and the scanning range was from 400 to 4000 cm−1, respectively. Protonated (1H) NMR was performed to analyse the chemical structure of the prepared rhamnolipids. The prepared rhamnolipids were dissolved in dimethyl sulfoxide and 1H NMR spectra were recorded using a 600 MHz NMR instrument, Bruker, Ascend 600, at 25 °C. The 1H NMR spectrum was then determined using an NMR spectrometer carried with deuterated chloroform ($CDCl_3$) as a solvent. Chemical shifts were expressed in parts per million (ppm), which were shifted downfield from (internal standard) tetramethyl silane TMS.

3. Results and discussions

3.1. Evidence of biosurfactant-producing bacteria

Microbiota of oil and soil samples were evaluated for rhamnolipid secretion through the CTAB Methylene blue agar plates, where dark blue halos were inferred the rhamnolipid production (Fig. 2). Here we observed that only soil samples consistently produced dark blue colonies after two weeks of consistent strain purification and screening. This mechanism is specially conducted for biosurfactant detection, as it was developed to reveal anionic surfactants having the quality to form dark blue insoluble ion pairs with various cationic solutions like CTAB which is contained in the aqueous solution. Although CTAB might inhibit the growth of some microbes, the successful growth and positive screens of our choice isolate (soil samples) evince their environmental hardiness, which superseded the tolerance of the strains from oil samples with no history of exposure to or proliferation in extreme environments. Moreover, the adoption of CTAB as a medium constituent has also been surmised to facilitate the abundance of hydrolytic strains (Li et al., 2023) Investigations carried out by Chafale et al. (2023) and Das et al. (2024) revealed lighter halos against a darker dye background within 5–7 days. However, in our study, darker halos were observed over 48 h, further strengthening the assumption that the positive strains possessed interesting biotechnological potentials.

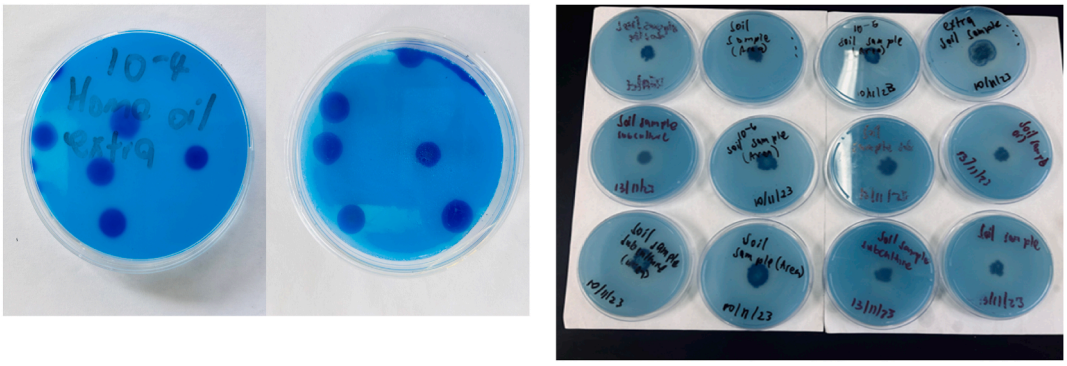

Fig. 2. The CTAB Methylene blue screening for biosurfactant production a.) 4 days after incubation of the preliminary screening b.) two weeks after pure culture was performed.

3.2. Growth and biosurfactant production studies

Five excellent performers of the preliminary screening were selected for the fermentation of a waste cooking oil based medium in time course study, which was monitored for cell growth and rhamnolipid production at 18 h intervals over 96 h. The Biosurfactants produced were presumed to be glycolipids as they showed positive reaction with anthrone sulfuric reagent indicating the presence of sugars. It also reacted separately with both iodine vapours indicating the presence of lipid moiety (Brahma et al., 2024). Based on anthrone sulfuric test that was conducted, positive results were observed to elicit a green colour signifying the presence of Carbohydrates, specifically Rhamnose (Fig. 3).

Waste streams have been increasingly espoused as sustainable feedstock for biosurfactant production (Begum et al., 2023; Femina et al., 2023); the heterogeneity of these wastes might influence the type and subclass of biosurfactant produced, as well as its concentration. However, the concentration and featrues of biosurfactant produced might also depend on the microbial strain adoption for its production. In our study, it was observed that SsB (0.51 g/L; 90 h) and SsE (0.39 g/L; 72 h) gave the highest rhamnolipids yield over 90 h of waste cooking oil fermentation (Fig. 4a–e). In comparison, rhamnolipids concentrations secreted by the other strains could not exceed 0.25 g/L, and also recorded relatively high cellular densities. This phenomenon is not economically prudent for largescale production in the industry, especially if the microbial strains employed possess no value as cellular proteins. Hence, our choice of SsB and SsE was in favour a low cell-rhamnolipids ratio, which was similar in pattern to the observation of Kourmentza et al. (2018). The aforementioned study reported a maximum rhamnolipids production from waste cooking oil at 120 h; however, a phenomenal increase in production was observed at 72 h. In another study, a Pseudomonas strain achieved maximum conversion of waste cooking oil to rhmnolipid at 96 h of fermentation, concomitant with a decrease in cell density (Shi et al., 2022). This kinetic study reveals the applicability of the aforementioned microorganisms for large scale production with added bonus of no pathogenicity.

3.3. Bacterial identifications

The possible identification obtained for the 2 bacterial isolates is provided below (Table 2). The identification of the strain was based on the ±600 bp partial sequence of the 16S rRNA gene of the organisms. The sequences were compared against GenBank of the National Centre for Biotechnology in the United States of America using a basic BLAST search. The isolation and screening of *Kosakonia cowanii* and *Acinetobacter calcoaceticus* from oil laden soil or a hydrocarbonoclastic environment is not unusual, as studies have

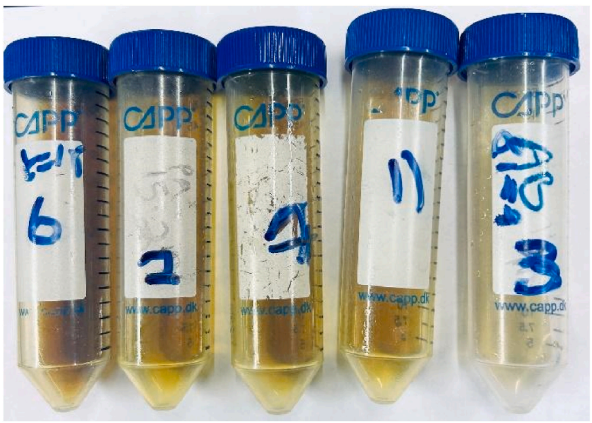

Fig. 3. Results obtained from anthrone sulfuric acid results obtained for the samples.

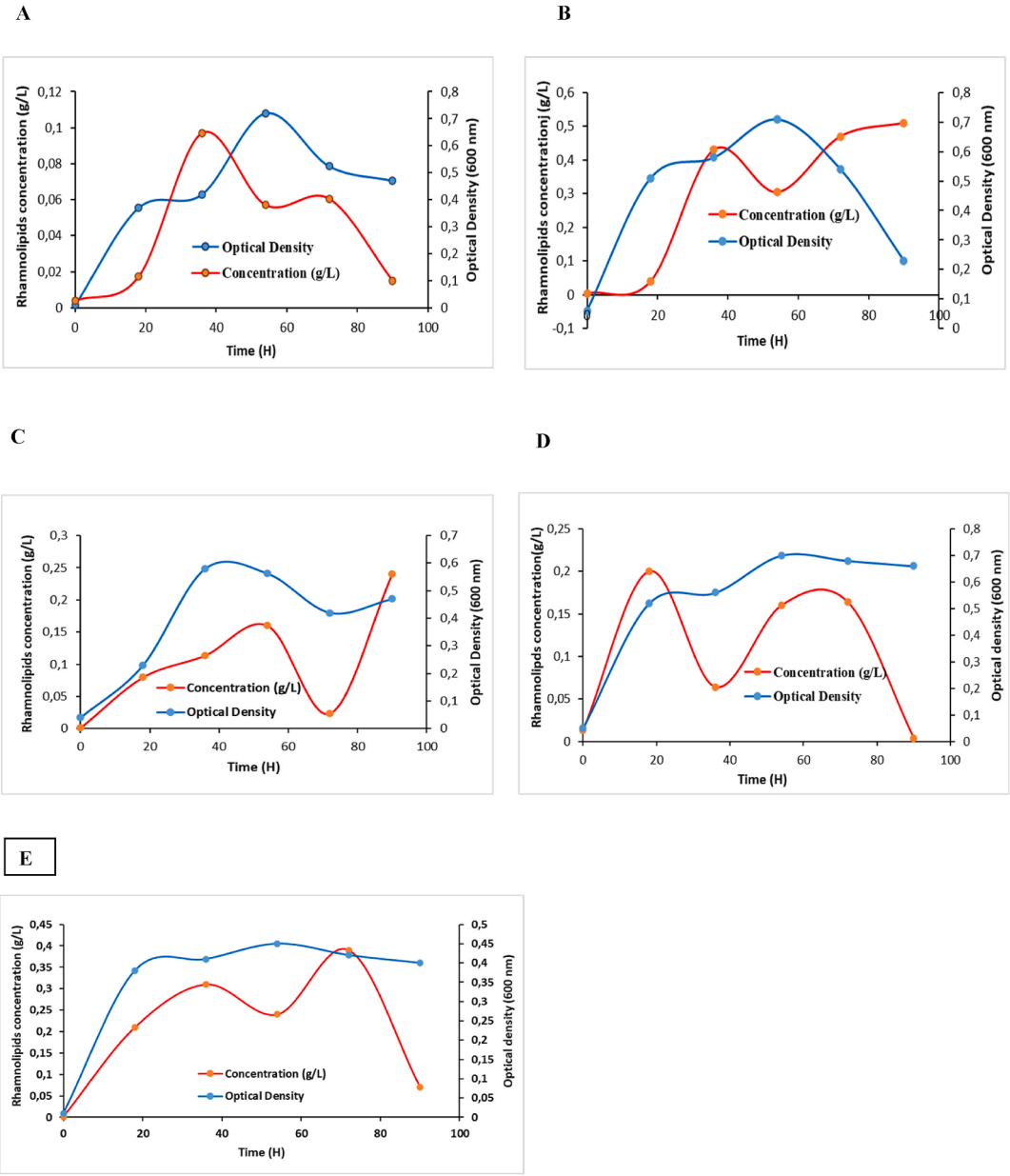

Fig. 4. Representation of optical density versus Rhamnolipids production for various samples in a time course study, a.) SsA b.) SsB c.) SsC d.) SsD e.) SsE.

Table 2
Bacterial strains obtained from the soil sample.

	Isolate	Result	% Sequence Identity
1	SsB	*Kosakonia cowanii*	99.75 %
2	SsE	*Acinetobacter calcoaceticus*	100 %

shown such microbial strains to possess the metabolic machinery for survival as well as environmental remediation (Ren et al., 2020; Moshtagh et al., 2021; Chafale et al., 2023).

Table 3 demonstrates that *Acinetobacter calcoaceticus* outperformed *Kosakonia cowanii* in rhamnolipid production when cultured on waste cooking oil. This study successfully identified strains capable of rhamnolipid biosynthesis from a heavy oil-contaminated environment. In contrast to previous research utilizing *Pseudomonas* species and waste cooking oil (Shi et al., 2022; Haidar et al., 2022), the strains employed here exhibit non-toxic characteristics, making them suitable candidates for environmentally friendly applications. A potential limitation for large-scale production using strains isolated from heavy oil-laden soil is the restricted avail-

Table 3
Rhamnolipid yields from production processes using isolated strains.

Strain	Substrate	Rhamnolipid Yield (g/L)
Kosakonia cowanii	Waste Cooking Oil	0.39
Acinetobacter calcoaceticus	Waste Cooking Oil	0.59

ability of this specific substrate. The inconsistent supply of such soil could disrupt the production process, hindering scalability and commercial viability.

3.4. Characterisation

3.4.1. Emulsifying activity

After incubation for 96 h, E_{24} was calculated to be 56.30 % and 40.47 %, as indicated in Table 4. An E_{24} quotient approximately similar to SsB Rhamnolipid was reported by Pathania and Jana (2020); whereas a range similar to SsE was obtained using hexadecane in another study (Lan et al., 2015). A study using sunflower oil recorded an E_{24} of 55% (Samadi et al., 2023), whereas another study revealed that the hydrophobic organic compounds n-hexane, liquid paraffin and kerosene all had $E_{24} > 40\%$ (Zhou et al., 2019). It has been observed that acidic pH regimes negatively the E_{24} of hydrophobic organic compounds, especially n-hexane, as no appreciable E_{24} was observed against at pH < 3 (Phulpoto et al., 2022). This phenomenon further substantiates the appositeness of our rhamnolipids for direct industrial processes, as the E_{24} were obtained at pH < 5.5. The difference between the E24 values of the two obtained Rhamnolipids could be accounted for by the fact that SsE has a better stabilizing effect SsB, according to Kreischer and Silva (2017), for a biosurfactant to be considered a good emulsifying agent it has to conform to the criterion of reaching an emulsifying index above 50 % for more than 24 h. The form of emulsion that was prepared during this study is O/W as oil was dispersed into water to perform this activity. According to David and Akhondi (2023)hydrophilic surfactants usually foster O/W emulsions. The significance of our results is further corroborated by the pivotal role E_{24} plays in evaluating the readiness of biosurfactant for applications in food, pharmaceutical, nutraceutical, and petroleum industries.

3.4.2. Oil spreading test

Rhamnolipids activity was determined using the Oil spreading test. Five microliters of the CFS resulted in a clear halo with diameters of 5.52 ± 0.09 cm for SsE Rhamnolipid and 4.61 ± 0.08 cm for SsB Rhamnolipid. This reveals that SsE Rhamnolipid has better surface activity than SsB Rhamnolipid. Interestingly, these results are more than fivefold the zone of oil dispersed (9.17 ± 0.29 mm) in another study (El-Housseiny et al., 2020). However, a comparable outcome in literature includes the observation of a recent study by Goveas et al. (2024), where a 3.5 ± 0.88 cm zone was acknowledged. The results of oil spreading experiment further demonstrates the ability and suitability of both rhamnolipids for adoption in oil spill cleanups.

3.4.3. Thin Layer Chromatography (TLC)

The two compounds were found to have similar spots on the TLC plate. They migrated quickly further up with the use of chloroform:methanol solvent system. The long spots witnessed could be accounted for by non-polarity of the rhamnolipids, as they of close proximity to the solvent line, probably due o their affinity for and rapid movement with the solvent. The compounds of interest have Rf values of 0.71 and 0.69 (Fig. 5a), which coincides with the reference (Fig. 5b), further implying that they are most probably monorhamnolipids. Rhamnolipids were first discovered in *Pseudomonas* sp., and they are known to produce both mono- and di-rhamnolipids (Zhou et al., 2019). Notwithstanding, some *Acinetobacter* sp. have also been observed to secrete both forms of rhamnolipids (Dong et al., 2016). Although *Kosakonia* sp. have been reported to participate in hydrocarbon degradation, there no preceding information on the isolation and characterisation of its biosurfactant; only its lipases have been reported so far (Ren et al., 2020; Guergouri et al., 2022). This therefore serves as the earliest report on the characterization of a mono-rhamnolipid secreted by *Kosakonia cowanii* (see Fig. 6).

3.4.4. Fourier Transform Infrared Spectroscopy (FTIR)

Both rhamnolipids were observed to display a seemingly similar FTIR profile. The sharp peak around 3500 cm^{-1} – 3000 cm^{-1} represents an OH group which is present in the rhamnose sugar, as they are known for their sugar moieties. The sharp absorption bands at 2856 cm^{-1} and 2917 cm^{-1} suggest a C-H stretching vibrations from the aliphatic chains, indicating the presence of fatty acid chains in rhamnolipids. The peak around 1745 cm^{-1} represents the presence of -C = O stretching vibrations, which could indicate an ester or carboxylic acid group found in the rhamnolipids structure. Absorption bands around 1463 cm^{-1} to 1600 cm^{-1} show C = C stretching vibrations which could be due to unsaturated fatty acid tails of rhamnolipids. Furthermore, an intense absorption band at 1462 cm^{-1} and 1160 cm^{-1} indicated the presence of the -C = O group and the -C-O-C- group, respectively. The FTIR spectra observed in this study exhibit characteristic features consistent with glycolipids, aligning with similar IR absorption spectra reported for other glycol-

Table 4
Emulsification index of the two biosurfactants obtained.

Rhamnolipid	Emulsified layer (cm)	Height of liquid (cm)	E_{24} %
SsB	1.38 ± 0.01	3.44 ± 0.05	40.47
SsE	1.92 ± 0.04	3.44 ± 0.05	56.30

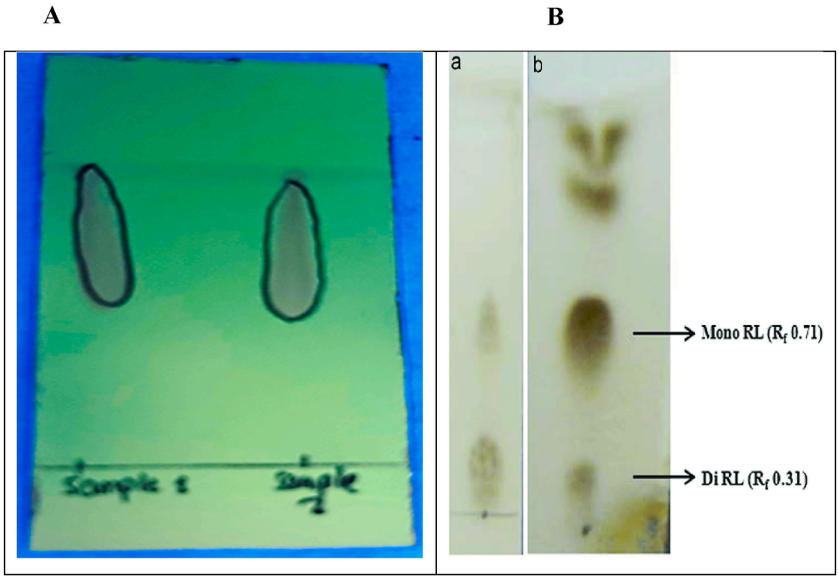

Fig. 5. TLC analysis of the composition of Rhamnolipids composition in the culture broth of *Kosakonia cowanii* and *Acinetobacter calcoaceticus* A.) TLC analysis of the two Rhamnolipids: left blot (*K. cowanii;* Rf 0.71), right blot (*A. calcoaceticus* Rf 0.69) B.) TLC of a reference obtained from Deepika et al. (2022).

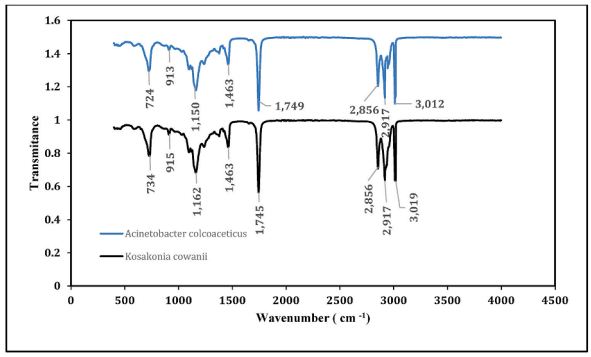

Fig. 6. The FTIR spectrum of the two purified biosurfactants obtained from different bacterial strains.

ipid biosurfactants in literature (Sun et al., 2021; Zhao et al., 2021b; Safari et al., 2023). Altogether, the FTIR profile analysis supports the conclusion that the *Acinetobacter calcoaceticus* and *Kosakonia cowanii* biosurfactants are likely a glycolipid biosurfactants.

3.4.5. Proton Nuclear Magnetic Resonance (H-NMR)

The HNMR spectra of the purified biosurfactant is shown in Fig. 7 a and b above. In figure a) the structure shows different peaks and patterns which solely correspond to different types of hydrogens in the structure of rhamnolipids. As indicated, the peaks between 0.5 and 2 ppm relay information about the type of hydrogens attached to saturated carbons, possibly from fatty acid chains. The peaks from 3 to 5 ppm could correspond to hydrogens in the sugar moiety, which rhamnolipids are also known to be composed of a sugar component (rhamnose). For graph b.) The peak appearing at 5.2 ppm could be an anomeric proton of a rhamnose, this is typically a singlet due to the unique environment of an anomeric carbon. The cluster peaks between 0.8 and 1 ppm represent the methyl group (CH_3) at the end of the fatty acid chains. The multiple peaks between 1.2 and 1.6 ppm show characteristics of methylene protons (CH_2) in the fatty acid chain. The peak at around 2.3 ppm could elucidate protons adjacent to the carbonyl group in the fatty acid chain. The 5–6 ppm region not having any peaks suggests a rhamnolipid that is highly saturated. The region 3–4 ppm shows multiple peaks that could be of protons on the rhamnose sugar. According to the analysis of purified rhamnolipids done by Dabaghi et al. (2023) and Gaur et al. (2023), our spectra peaks relay that the biosurfactant produced in our study are indeed Mono Rhamnolipid, further corroborating the FTIR and TLC results discussed *supra*.

4. Conclusion

This study successfully harnessed the potential of microorganisms from a challenging environment to produce valuable rhamnolipids from waste cooking oil. The isolated *Acinetobacter calcoaceticus* demonstrated superior rhamnolipid production compared to *Kosakonia cowanii*. While promising results were achieved, challenges related to fermentation optimization remain. Future research

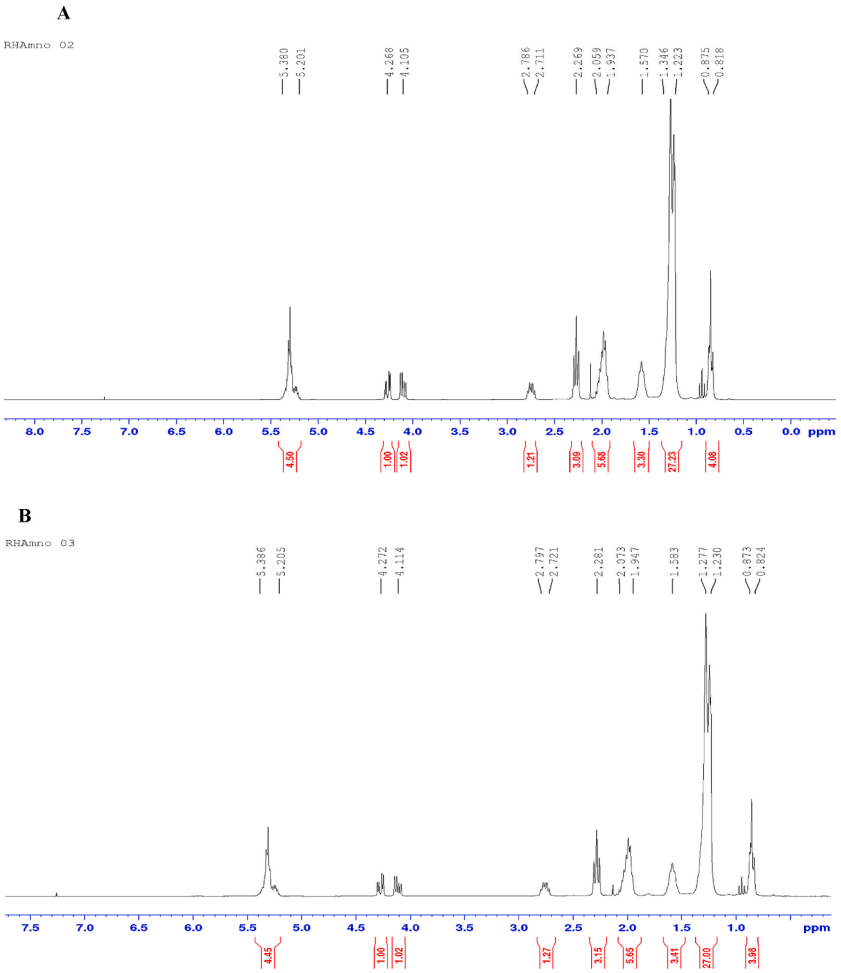

Fig. 7. The HNMR of the two purified biosurfactants produced from a.) *Kosakonia cowanii* and b.) *Acinetobacter calcoaceticus* using deuterated chloroform ($CDCl_3$) as a solvent.

should focus on enhancing production efficiency through process parameter fine-tuning and a deeper understanding of microbial physiology. This investigation contributes to the development of sustainable bio-based production processes and offers a potential solution to the global waste management crisis. To fully assess the environmental impact and sustainability of rhamnolipid production, a comprehensive life cycle assessment (LCA) is recommended for future studies. By comparing the LCA of rhamnolipid production to conventional synthetic surfactants, a clearer picture of the overall environmental benefits can be obtained. This analysis would encompass factors such as energy consumption, greenhouse gas emissions, water usage, and waste generation throughout the entire production lifecycle. The non-pathogenic nature of the isolated strains positions them as promising candidates for environmental applications. Their ability to produce rhamnolipids, coupled with the increasing global concern over heavy metal pollution, highlights the potential of these strains for bioremediation strategies in contaminated water bodies.

CRediT authorship contribution statement

Siyabonga Nkosi: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **John O. Unuofin:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Olubusola A. Odeniyi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Samuel A. Iwarere:** Writing – review & editing, Validation, Resources, Investigation, Formal analysis. **Michael O. Daramola:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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