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Research article

Isolation of biosurfactant-producing and crude oil-degrading bacterium, *Enterococcus hirae*, from hydrocarbon-polluted soils and characterization of the biosurfactant produced

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Abstract
The spillage of petroleum hydrocarbons into our environment causes a lot of disastrous environmental pollution. The employment of biosurfactant-producing and hydrocarbon-utilizing microorganisms enhances the effectiveness of bioremediation of these toxic pollutants. This study was aimed at isolating biosurfactant-producing and crude oil-degrading bacteria from hydrocarbon-polluted soils and characterization of the biosurfactant produced. The biosurfactant screening techniques employed were haemolysis assay, drop collapse test, oil displacement test, tilting glass test and emulsification index (E_{24}) test. The degradation rate of the most potent crude oil degrader was determined using gravimetric method and Gas Chromatography – Mass Spectroscopy (GC-MS) analysis. The bacterium was identified based on phenotypic, biochemical and molecular analyses. Fourier Transform Infra-Red (FTIR) and GC-MS analyses were used to characterize the biosurfactant produced. A potent bacterium, *Enterococcus hirae* (identified by 16s rDNA sequencing) was isolated from hydrocarbon contaminated soil and it could degrade 77.2% of total petroleum hydrocarbons after two weeks of culture when grown in mineral salt medium (MSM) supplemented with 2% (v/v) crude oil as the sole carbon source. Chromatogram of the treated crude revealed that *E. hirae* could potentially degrade various hydrocarbon contents ($C_{21} - C_{35}$) present in the crude oil. The biosurfactant produced was characterized as Glycolipids (Rhamnolipids) using FTIR and GC-MS analyses. This study demonstrates *E. hirae* as an efficient biosurfactant producer and crude oil degrader. To the best of the researchers' knowledge, this is the first time *E. hirae* is reported as both biosurfactant producers and crude oil degrader.

Keywords: Biosurfactant, crude oil, hydrocarbon-polluted soil, *E. hirae*, Glycolipids, Rhamnolipids.

INTRODUCTION

The abundance of crude oil in any crude oil producing locality is seen both as a blessing and a curse, because most of the crude oil drilling sites and storage facilities are situated around human settlement. During the process of oil exploration, collection and transportation of crude oil from the drilling site, leakages of crude oils tend to contaminate lots of agricultural farmlands and water bodies (Patowary et al. 2016). Crude oil and its derivatives are considered as one of the most pervasive environmental pollutants. In Nigeria, a large amount of crude oil and its products are spilled annually into the environment and about 4,919 oil spills were recorded between 2015 to March 2021 (NOSDRA, 2021) which are inimical to the environment. The spills following entry into the soil environment, their small density, higher viscosity and lower emulsifying ability allow them to be easily absorbed in the soil surface and rapidly bind to the mineral and organic matter (solid phases) via a combination of physical and chemical processes, thereby becoming less bioavailable for microorganisms and difficult to biodegrade (Das et al. 2014). Thus, petroleum hydrocarbon pollutants make the development of a

remediation technology essential for cleaning up polluted sites. As compared to other strategies used in cleaning up crude oil polluted sites, microbial remediation is recognized as one of the most effective, eco-friendly and inexpensive technologies. (Bento et al. 2005). Bacterial genera such as *Achromobacter, Acinetobacter, Alcaligenes , Aeromonas, Arthrobacter, Alcaligenes, Bacillus, Corynebacterium, Pseudomonas, Flavobacterium, Mycobacterium, Nitrosomonas, Nocardia, Rhodococcus, S, Streptomyces* and *Xanthobacter* have been proven to be petroleum hydrocarbon degraders (Singh et al. 2014) while fungal genera, namely, *Amorphoteca, Aspergillus, Cephalosporium, Pencillium, Neosartorya, Talaromyces, Graphium, Candida, Yarrowia, Rhodotorula, Geotrichum, Pichia and Trichosporon* were isolated from petroleum contaminated soil and proved to be the potential hydrocarbon degraders (Singh 2006).

However, a suitable method that can be adopted to speed up the bioremediation of sites contaminated with hydrocarbon is the involvement of biosurfactant producing and hydrocarbon degrading microorganisms due to the role of biosurfactant molecules in enhancing the solubility of these hydrophobic pollutants through emulsification, thereby leading to their better bioavailability for microbial degradation. Recent research findings have confirmed the application of biosurfactant onto hydrocarbon contaminated site to have a biostimulation effect as they tend to increase solubility and bioavailability of such hydrocarbons and also stimulate indigenous microorganisms for enhanced biodegradation (Vijayakumar & Saravanan 2015).

Biosurfactants are amphiphilic compounds that are produced by microbial cell surface or excreted, to reduce surface and interfacial tension (Jorge et al. 2018). They have wide applications in medicine, food, petroleum, cosmetics, pharmaceutical and agricultural industries due to its stabilizing, emulsifying (liquid-liquid mixture), wetting, spreading, foaming and cleaning properties and antimicrobial, antifungal and antiviral activities (Rodrigues & Teixeira 2010; Sachdev & Cameotra 2013; Nasiri & Biria 2020). They can be produced extracellularly by a wide range of microorganisms including bacteria, fungi, and yeasts. Examples are *Bacillus salmalaya*, *Candida lipolytica*, *Pseudomonas aeruginosa* and *Trichosporon ashii* (Chandran & Das 2010; Santos et al. 2014). However, hydrocarbon-degrading microbial communities remain the most suitable environment for widespread capability for biosurfactant production (Nwaguma et al. 2016).

Biosurfactants are grouped based on their microbial origin or chemical composition such as fatty acids, peptides, glycolipids, phospholipids, and lipopeptides (Vijayakumar & Saravanan, 2015). Compared to synthetic surfactants with the same functionality, they have advantages such as being able to work in extreme temperature, pH and salinity conditions, being non-toxic or very low toxicity, and being biodegradable. In addition, since they are of biological origin, they can be produced from renewable substrates and modified structurally by genetic engineering and biochemical methods (Banat et al. 2010; Jahan et al. 2020).

One of the major limitations of microbial degradation of crude oil is its hydrophobicity, Therefore, the search for hydrocarbon degrading bacteria with the ability to produce biosurfactants is widely recommended for fast degradation of crude oil and its products. The application of microbes posing capacity to degrade hydrocarbons along with the production of biosurfactants can effectively expedite the bioremediation of hydrocarbon polluted environment (Kumar et al. 2006).

Although, the physical and chemical properties of some biosurfactant classes are well-studied, it's very important to characterize the biosurfactant produced during hydrocarbon degradation process as such type of study is very sparse (Chandankere et al. 2014). Therefore, the aim of this research was to isolate the most potent biosurfactant-producing and crude oil-degrading bacteria from hydrocarbon-polluted soils and characterization of the biosurfactant produced. Understanding the role of biosurfactant in hydrocarbon degradation process can provide a new dimension in the field of biosurfactant mediated bioremediation of hydrocarbon pollutants.

MATERIALS AND METHODS

Crude oil, Soil samples and Culture media

The crude oil (1000mL) that was used for this research was collected once in sterile sampling bottles during the harmattan season (December) from Kaduna Petrochemical and Refining

Company, Kaduna, Nigeria. Soil samples for bacterial isolation were collected from some of the diesel-contaminated generator sites powering street light within Kano metropolis [11°59'22"N and 8°29'19"E; 11°59'15"N and 8°28'53"E; 11°59'41"N and 8°30'59"E; 12°00'55"N and 8°30'16"E; 11°58'51"N and 8°31'18"E]. For each soil source, diesel-contaminated soil samples were randomly collected from different points at depths between 0 and 15 cm using a hand-held soil auger and then bulked to get a composite sample. The samples were then transported aseptically sin sterile polythene bags to the laboratory and stored at 4℃ immediately for further use.

The culture media that were used include nutrient agar, nutrient broth, blood agar and minimal salt medium. The minimal salt medium (MSM) contains the following ingredients (g/L): KH_2PO_4, 1.4; Na_2HPO_4, 2.2; $(NH_4)_2SO_4$, 3; $MgSO_47H_2O$, 0.6; NaCl, 0.05; yeast extract, 1; $FeSO_4$ $7H_2O$, 0.01 and $CaCl_2$ $7H_2O$, 0.02; and 1 mL of trace solution containing (in 1 L of distilled water) $ZnSO_4$, 0.29g; $CaCl_2$, 0.24g; $CuSO_4$, 0.25g and $MnSO_4$, 0.17g (Elazazzy et al. 2015). The trace element solution was added after autoclaving the production media, prior to inoculation by filtering it through 0.2µm membrane filters. Sterilization of the culture media were carried out by autoclaving at 121℃ for 15 mins.

Isolation Of Crude Oil Utilizing Bacteria

One-gram (1g) of hydrocarbon contaminated soil samples was inoculated in conical flasks containing mineral salt medium (MSM) with 2% (v/v) crude oil as a carbon source. The pH of the medium was adjusted to 7.0 ± 0.2 using either HCl acid. The conical flasks were then incubated at 35℃ at 150 rpm for 7 days. After 7 days, 1 ml from each conical flask was used for serial dilution followed by spreading of 100 µl from 10^{-4} -10^{-6} diluted samples on nutrient agar plates and incubation of the plates at 35°C for 24hours. Bacterial colonies of different morphology were selected and separately streaked on nutrient agar plates so as to obtain pure culture of the bacterial isolates (Patowary et al. 2017). The isolates were maintained on nutrient agar slant and preserved at 4°C.

Screening Of Biosurfactant-Producing Bacteria

Drop Collapse Assay

Qualitative drop collapse test was performed using crude oil as hydrocarbon substrate by following a method described by Bodour & Miller-Maier (1998). A single drop of crude oil was placed on a glass slide, following which a single drop of 48-h-grown culture broth was dropped onto the crude oil drop and drop collapse activity will then be observed. The shape of drop on oil surface will be observed after 1 minute. The culture supernatant that make the oil drop collapse (i.e. flat shape) was considered as positive result and the drops that remain beaded (i.e. spherical shape) was scored as negative which was examined with distilled water as control.

Haemolytic Activity

This is a qualitative-screening test for the detection of biosurfactant producers (Satpute et al. 2010). After incubation for 36 h, the culture broth was spread on a blood agar plate and cultured in an incubator at 35°C overnight. The indications of yellow transparent zones around colonies suggest biosurfactant production. No change in the colour on the blood agar plates indicates the absence of haemolysis.

Oil-Displacement Technique

The oil displacement test was done by adding 50 ml of distilled water to a petri dish with a diameter of 15 cm. After that, 20 µl crude oil was dropped onto the surface of the water, followed by the addition of 10 µl of cell culture supernatant. The diameter and the clear halo visualized under visible light was measured after 30 seconds (Rodrigues et al. 2006). A colony surrounded by an emulsified halo will be considered positive for biosurfactant production (Morikawa et al. 2000).

Tilting Glass Slide Test

Isolates was grown for 24 h on nutrient agar plates. A sample colony was then be mixed with a droplet of 0.85 % NaCl at one end of the glass slide. The slide was tilted and droplet observed. Biosurfactant producers were detected by the observation of droplet collapsing down (Satpute et al. 2010).

Emulsification Stability (E_{24}) Test

The E_{24} was determined as described by Nitschke & Pastore (2004). Two millilitres (2 mL) of crude oil was added to the same amount of cell-free broth in different 15 ml test tube. The mixtures

were vortexed at a high speed for 2 min. The test tubes were placed vertically at room temperature without disturbance for 24 h. After 24 h, the height of the stable emulsion layer was measured. E_{24} index is defined as the percentage of the height of emulsified layer divided by the total height of the liquid column multiplied by 100.

$$E_{24} \quad (\%) = \frac{\text{total height of the emulsified layer} \times 100}{\text{height of the liquid layer}}$$

Selection of Most Efficient Crude-oil Degrading Bacterial Isolate

Among all the biosurfactant producing isolates, the most efficient crude oil degrader strain was selected depending on their growth in crude oil enriched condition. The screening was done as per the method described by Rahman et al. (2002). For the screening, 5 mL seed culture of each bacterium was aseptically inoculated into 100 mL of sterilized mineral medium enriched with 2% (v/v) crude oil prepared in 500 mL Erlenmeyer flasks and kept in a shaking incubator for 7 days at 35℃ and 200 rpm. A set of flasks containing the same composition of culture media was also maintained in same conditions as control where no inoculum was added. The bacterial growth in the medium of each flask at 0 and 7th day was estimated by taking optical density at 600 nm by UV-Vis spectrophotometer. The bacterial isolate showing maximum growth in crude oil containing media was selected for further studies.

Identification of Biosurfactant Producing and Crude Oil Degrading Isolates

Morphological characterization (i.e. colony, cell and spore morphology), Gram-reaction, and some biochemical test were carried out on the most effective biosurfactant-producing and crude-oil degrading isolate using Bergey's Manual of Determinative Bacteriology as a guide (Nwaguma et al. 2016).

DNA of the most potent bacterial isolate was extracted as follows: Pure culture of the isolate was inoculated in nutrient broth and cultivated at 37^0C for overnight. By using 5ml of cultivation, cells were harvested and added to 100 µl of lysozyme and incubated for 30 mins which was then followed by addition of 700 µl cell lysis buffer (SDS, Tris-EDTA etc.). The vial was gently mixed by inverting for 5 mins until white DNA strands were seen. The DNA was precipitated by ethanol from the aqueous layer. The DNA pellet was dried and dissolved in 50 µl of 1x TE buffer. The DNA quality was checked with 0.8% agarose gel stained with ethidium bromide (0.5 µg/µl). Single intensive DNA was seen which was extracted to use in order to amplify 16s rDNA gene as a template DNA (Zhang & Stewart 2000).

The 16S rDNA was amplified using universal primer set 27F (5^1-AGA GTT TGA TCC TGG CTC AG-3^1) and 1492R (5^1GGT TAC CTT GTT ACG ACTT3^1). The PCR reaction was carried out in 25 µL volumes containing 12.5 µL of the Master Mix, 0.4 µL of each primer, and mixed with 5 µL of the DNA template. Sterile nuclease-free water of volume, 6.7 µL, was added. The PCR reaction conditions consisted of initial denaturation at 94^oC for 5 mins followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, extension at 72℃ for 45℃, and a final extension at 72℃ for 7 min. PCR products were analyzed on 1% agarose gel and examined by electrophoresis. The PCR product were set for 16S rDNA sequencing. The sequences were input in the NCBI site for BLAST, and the most homologous strains were identified (Patowary et al. 2017; Wuyang et al. 2018).

Degradation of crude oil by the selected bacterium

Degradation Rate Determination by Using Gravimetric Method.

First, the bacterium was inoculated to the 100 mL of MSM containing 2% (v/v) crude oil in a 250 mL flask. The degradation conditions were at 35°C and 180 rpm for two weeks. After 14 days of incubation, the crude oil was extracted as follows: the culture broth was mixed with petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently to break the emulsification, which resulted in 3 layers: top layer (which is a mixture of Petroleum ether, Crude oil and acetone), middle layer (clumping cells) and bottom aqueous layer (the culture broth without microbial cells and crude oil).

The lower two layers were separated out while the top layer was taken out in a clean beaker. The extracted oil was passed through anhydrous Sodium tetraoxosulphate (VI) (Na_2SO_4) to remove

moisture while petroleum ether and acetone were evaporated on a 70°C -water bath. The quality of the crude oil before and after degradation was weighed by the precision balance. The formula for the degradation rate is as followed as described by Wuyang et al. (2018):

Degradation rate = $(m_0 - m_i) / m_0 \times 100\%$
m_0 is the quality of crude oil before degradation; m_i is the quality of crude oil before degradation.

Gas Chromatography–Mass Spectrometric (GC-MS) Analysis

Extracted crude oil sample was analyzed by GC-MS to confirm the degradation efficacy of the strain by following the procedure given by Patowary et al. (2016). 1ml of control crude oil and extracted residual crude oil degraded by the selected bacterium were analyzed through a triple quadruple Gas Chromatograph-Mass Spectrometer equipped with an auto-injector. For the detection of various petroleum hydrocarbons, the GC program was optimized and all analyses were carried out with the split ratio of 20:1. Helium was used as the carrier gas with a flow rate of 1.0mL min^{-1}, maintaining an injection temperature of 300°C. The column oven temperature was set at 60 °C with a hold time of 5 mins and was subsequently increased to 280°C with a ramp of 8 °C min^{-1} with the final hold of 37 mins. The mass spectrometric data were acquired in electron ionization mode (70eV). The ion source temperature and interface temperature for MS were set at 230°C and 310°C respectively. The mass range (m/z) was selected as 45-600 for the entire analysis. The chromatograms were analyzed with GC-MS solution software (version 4) and the compounds identification was performed using NIST 11 library database.

Characterization of biosurfactant

Fourier Transform Infra-Red (FTIR) Analysis

This analysis was used for the detection of functional groups and chemical bond type present in the biosurfactants produced. It was carried out on a FTIR spectrophotometer by the KBr pellet method (Chandran & Das, 2010). Approximately 2 mg of the lyophilized biosurfactant was grinded with 100 mg of KBr in a mortar and pressed for 30 seconds with a load to obtain translucent pellets. The scan wavelength range was from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

Gas Chromatography–Mass Spectrometric (GC-MS) Analysis

GC-MS analysis of the biosurfactant was carried out according to the method of Chandran & Das (2010). The purified biosurfactant (10mg) was mixed with 5% HCl-methanol reagent (1ml). After the reaction was quenched with 1ml of water, the sample was extracted with n-hexane and injected into the GC-MS machine equipped with a capillary inlet and mass selective detector set to scan from m/z 45 to m/z 800 at scan rate of 1.2 scans per second. The oven temperature was programmed from 130℃ to 230℃ at 2℃ min^{-1}. The carrier gas was Helium at a flow rate of 1ml min^{-1} and a split ratio of 50:1.

Statistical Analysis

All the parallel experiments were carried out in triplicates. The results were represented as the mean ± standard deviation. Analysis of variance (ANOVA) was used to compare different variables at 95% probability level. All statistical analyses were done using Statistical package for social sciences (SPSS) software.

RESULT AND DISCUSSION

Screening and selection of biosurfactant producing bacteria.

Out of the 35 crude oil utilizing bacterial isolates that were isolated from the diesel-contaminated soils, 15 isolates were selected as biosurfactant producers based on their ability to give positive results to all the screening methods employed. (Table 1 - 5). The 35 crude oil utilizing bacterial isolates were code named according to the sample area name where T represent isolates from Tal'udu site; G represent isolates from Gwammaja site; F represent isolates from FCE site; A represent isolates from Dan Agundi site; E represent isolates from Emir's palace site.

Selection of most efficient crude oil degrading bacteria isolates

From the total of 15 biosurfactant producing bacteria, isolates G_1 and E_2 were selected as the most efficient crude oil degrader based upon their distinguished growth (OD_{600nm}) on 2% (v/v) crude oil enriched condition (Figure 1). Isolate G_1 showed the maximum growth (OD_{600} = 1.008 $\pm$ 0.09) followed by E_2 (0.840 $\pm$0.44) in crude oil containing media after 7^{th} day of incubation. Thus,

from the result obtained isolates G_1 and E_2 were selected for further degradation studies.

Degradation of Crude oil

Isolates G_1 and E_2 were subjected to 14-day degradation of crude oil singly and in consortium. Using gravimetric method, the degradation rate was determined as 77.19% degradation efficiency for G_1, 62.72% for E_2 and 29.31% for their consortium as shown in Table 6. With this result, isolate G_1 was regarded as the most potent crude oil degrader and was selected for further studies.

GC-MS analysis of the residual hydrocarbon extracted from isolate G_1 culture was conducted and compared with an abiotic control under the same conditions. The obtained chromatograms (Figure 2) revealed that the total petroleum hydrocarbons were reduced in the crude oil sample treated with isolate G_1 as compared to the abiotic control sample, thus validating the gravimetric results and suggesting that the isolate was highly effective in degrading different components of the crude oil. The retention time, area (%), names, molecular weight and molecular formula of the different compound present in the chromatograms were presented in Table 7 and 8 respectively.

Table 1: Haemolytic activity of the crude oil utilizing bacteria

Isolate	Haemolytic activity	Isolate	Haemolytic activity
T_1	+	F_4	-
T_2	-	F_5	+
T_3	+	A_1	-
T_4	-	A_2	+
T_5	-	A_3	-
T_6	+	A_4	+
T_7	+	A_5	-
T_8	+	A_6	+
T_9	+	A_7	-
T_{10}	+	A_8	-
G_1	+	E_1	-
G_2	+	E_2	+
G_3	+	E_3	-
G_4	+	E_4	-
G_5	-	E_5	+
F_1	+	E_6	-
F_2	-	E_7	+
F_3	-		

+ = β haemolysis; - = γ haemolysis;

Table 2: Drop collapse test result of the crude oil utilizing bacteria

Isolate	DCT	Isolate	DCT
T_1	+++	F_4	++
T_2	++	F_5	-
T_3	-	A_1	++
T_4	-	A_2	-
T_5	-	A_3	++
T_6	-	A_4	+++
T_7	++	A_5	-
T_8	+++	A_6	++
T_9	++	A_7	-
T_{10}	+++	A_8	+
G_1	++	E_1	+
G_2	+	E_2	+
G_3	++	E_3	-
G_4	++	E_4	+
G_5	-	E_5	++
F_1	++	E_6	+
F_2	++	E_7	++

DCT = Drop collapse test: Completely spherical (-), flat (+), Moderately flat (++) and completely flat (+++)

F_3	+

Table 3: Oil displacement test result of the crude oil utilizing bacteria

Isolate	ODT	Isolate	ODT
T_1	++	F_4	+
T_2	-	F_5	-
T_3	++	A_1	++
T_4	+	A_2	++
T_5	-	A_3	-
T_6	-	A_4	++
T_7	+++	A_5	+
T_8	++	A_6	++
T_9	++	A_7	+
T_{10}	++	A_8	+
G_1	+++	E_1	-
G_2	+	E_2	+++
G_3	++	E_3	-
G_4	+++	E_4	++
G_5	-	E_5	++
F_1	++	E_6	-
F_2	++	E_7	++
F_3	-		

ODT = Oil displacement test: No displacement (-), <3 - <5cm (+), >5 - <7cm (++), >7 - <9cm (+++)

Table 4: Tilting glass slide test result of the crude oil utilizing bacteria

Isolate	TGS	Isolate	TGS
T_1	+	F_4	+
T_2	+	F_5	+
T_3	+	A_1	+
T_4	-	A_2	-
T_5	-	A_3	+
T_6	+	A_4	+
T_7	+	A_5	+
T_8	+	A_6	+
T_9	+	A_7	-
T_{10}	+	A_8	+
G_1	+	E_1	+
G_2	+	E_2	+
G_3	+	E_3	-
G_4	+	E_4	-
G_5	+	E_5	+
F_1	+	E_6	+
F_2	-	E_7	+
F_3	+		

TGS = Tilting Glass Slide

Table 5: Emulsification index (E_{24}) result of the crude oil utilizing bacteria

Isolate	E_{24} (%)	Isolate	E_{24} (%)
T_1	61.20 ± 0.62	F_4	51.33 ± 1.03
T_2	50.67 ± 0.63	F_5	41.17 ± 0.85
T_3	51.17 ± 0.85	A_1	52.62 ± 0.72
T_4	47.62 ± 0.27	A_2	44.64 ± 0.63
T_5	50.50 ± 0.41	A_3	45.65 ± 0.25
T_6	47.96 ± 0.46	A_4	61.64 ± 0.50
T_7	51.33 ± 1.03	A_5	41.17 ± 0.85
T_8	56.01 ± 0.38	A_6	55.92 ± 0.93
T_9	56.35 ± 0.60	A_7	41.35 ± 1.04
T_{10}	51.00 ± 0.82	A_8	51.33 ± 1.15
G_1	55.42 ± 0.65	E_1	50.50 ± 0.81
G_2	52.54 ± 0.82	E_2	66.33 ± 0.96
G_3	50.50 ± 0.42	E_3	40.83 ± 1.93
G_4	51.63 ± 0.78	E_4	45.53 ± 1.05
G_5	46.25 ± 0.6	E_5	52.67 ± 0.72
F_1	55.33 ± 0.62	E_6	45.62 ± 0.74
F_2	52.54 ± 0.82	E_7	56.27 ± 0.59
F_3	50.50 ± 0.41		

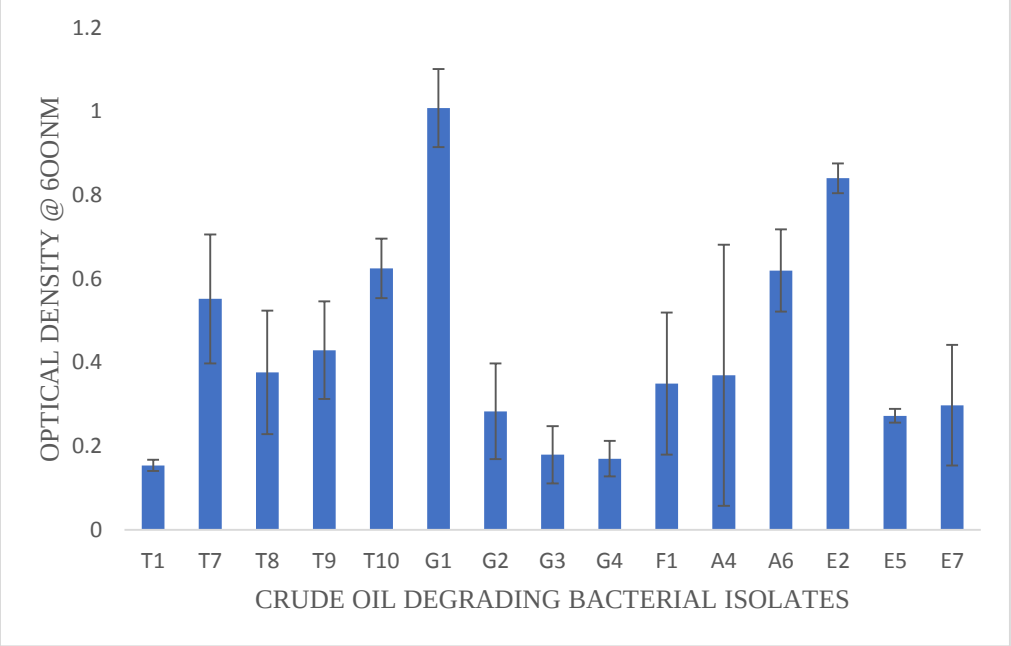
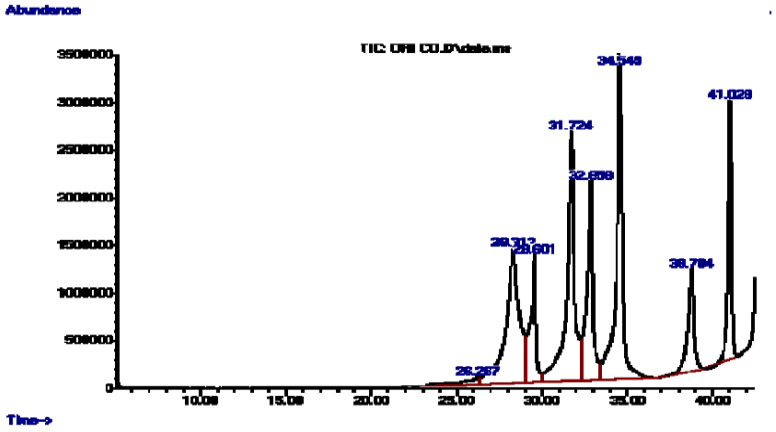


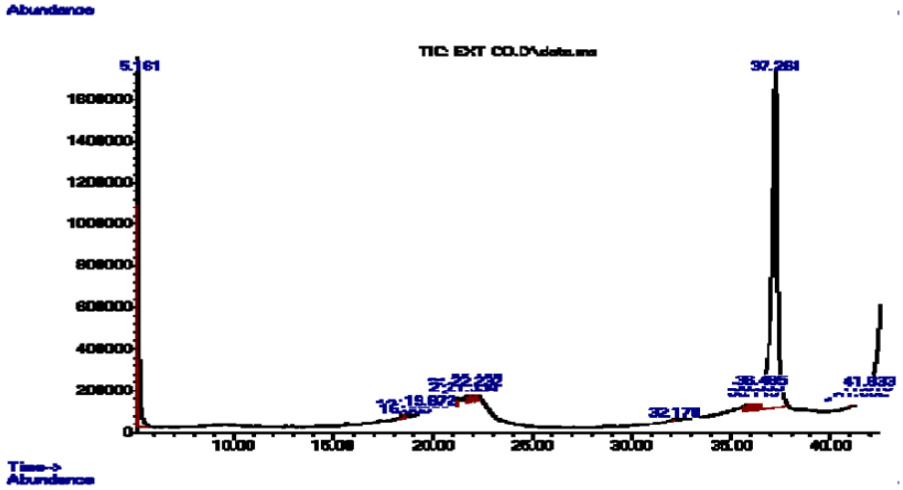
Figure 1: Selection of most potent crude oil degrader

Table 6: Crude oil degradation rate by isolates G_1 and E_2, singly and in combination

Parameters	G_1	E_2	Consortium
Weight of Original Crude oil	2.0533g	2.0533g	2.0533g
Weight of Residual Crude oil	0.4684g	0.7654g	1.4514g
Amount of Crude oil degraded	1.5849g	1.2879g	0.6019g
% of CO degraded	77.19%	62.72%	29.31%



(a) Chromatogram of original Crude oil before degradation



(b) Chromatogram of Residual Crude oil after 7 days degradation

Figure 2: Chromatograms of Crude oil before (a) and after 7 days (b) degradation by Isolate SG_1

Table 7: Details of components of the Original crude oil

S/N	RT	Area %	Library ID	M.Wt	Mol. Form.
1	26.267	1.38	17-Pentatriacontene	490.93	$C_{35}H_7O$
2	28.312	19.78	Aspidospermidin-17-ol, 1- acetyl-19,21-epoxy-15,16-dimethoxy-	414.49	$C_{23}H_{30}N_2O_5$
3	29.601	7.96	Triacontyl pentafluoropropionate	584.83	$C_{33}H_{61}F_5O_2$
4	31.724	20.15	i-propyl 11-octadecanoate	340.58	$C_{22}H_{44}O_2$
5	32.858	12.08	Undec-10-ynoic acid, heptadecyl ester	420.71	$C_{28}H_{52}O_2$
6	34.549	22.65	1-Hexacosene	364.69	$C_{26}H_{52}$
7	38.784	16.00	n-propyl 11-octadecenoate	324.54	$C_{21}H_{40}O_2$

RT = Retention time; M.Wt = Molecular weight; Mol. Form. = Molecular Formula

Table 8: Details of components of the Residual crude oil

S/N	RT	Area %	Library ID	M.Wt	Mol. Form.
1	5.161	22.53	9-octadecenoic acid (Z)-, 2-hydroxy ethyl ester	326.51	$C_{20}H_{38}O_3$
2	18.483	0.32	Acetic acid, chloro-,octadecyl ester	347.00	$C_{20}H_{39}ClO_2$
3	18.589	0.08	Butyl 9-tetradecenoate	282.50	$C_{18}H_{34}O_2$
4	19.447	0.18	2-Methyl-Z,Z-3,13-octadecadienol	280.50	$C_{19}H_{36}O$
5	19.535	1.77	9-octadecenoic acid, (E)-	282.50	$C_{18}H_{34}O_2$
6	19.714	0.27	5-Eicosene, (E)-	280.53	$C_{20}H_{40}$
7	19.872	0.07	1-Nonadecene	266.50	$C_{19}H_{38}$
8	21.060	1.03	1-Octadecene	252.50	$C_{18}H_{36}$
9	21.138	0.61	1-Pentadecene	210.40	$C_{15}H_{30}$
10	21.694	0.08	E-11-Tetradecenoic acid	226.35	$C_{14}H_{26}O_2$
11	21.934	4.79	Oleic acid	282.46	$C_{18}H_{34}O_2$
12	22.185	0.95	Cis-Vaccenic acid	282.50	$C_{18}H_{34}O_2$
13	36.311	0.19	13-Octadecenal, (Z)-	266.50	$C_{18}H_{34}O$
14	37.261	67.03	n-propyl 11-octadecenoate	324.50	$C_{21}H_{40}O_2$
15	41.546	0.05	9-octadecenal, (Z)-	266.50	$C_{18}H_{34}O$
16	41.749	0.02	Cis-13-Octadecenoic acid	282.50	$C_{18}H_{34}O_2$

RT = Retention time; M.Wt = Molecular weight; Mol. Form. = Molecular Formula

Identification of the isolate

Phenotypic and biochemical characterization placed isolate G_1 in the genus *Enterococcus* belonging to the phylum *Bacillota*; class *Bacilli*; order *Lactobacillales*; family *Enterococcaceae* (Table 9). The 16S rRNA gene partial sequence of the isolate G_1 was submitted to the NCBI GenBank database under accession number 1439. BLAST search was conducted to compare the sequences with existing sequences. Sequence similarity to the closest sequence of *Enterococcus hirae* [accession number (OR 206274)] was found to be 99.80%. Therefore, the identity of isolate G_1 was confirmed as *Enterococcus hirae.*

Characterization of the biosurfactant produced

The FTIR spectrum of the purified biosurfactant revealed important bands at 3 350, 2 121, 1 737, 1 640, 1 242, 1 100 and 1 048cm^{-1} (Figure 3). Due to presence of hydrogen bonding, the appearance of a strong and broad band of the hydroxyl group (-OH) free stretch was observed at 3 350 cm^{-1}. The peak at 2 121 cm^{-1} indicate the stretching bond of alkyne molecules (C≡C). Peaks corresponding to C=O stretch was obtained at wave numbers 1 737 and 1 704 cm^{-1}. The absorption peak 1 640 cm^{-1} confirm the presence of primary amine (N-H bend) while peaks corresponding to C-O were obtained at wavelengths 1 242, 1 100 and 1 048 cm^{-1}indicating the presence of alcohols, carboxylic acids and ethers in the biosurfactant. Therefore, from the above interpretation, it can be summarized that chemical structure of the biosurfactant produced is identical to those of previously reported rhamnolipid (a glycolipid type of biosurfactant) which comprises of rhamnose ring attached with long hydrocarbon chains.

The GC-MS analysis of the biosurfactant identified 9-octadecenoic acid (z)-, 2-hydroxy-1,3-propanediyl ester with molecular weight of 631g ($C_{39}H_{72}O_5$) as the most abundant component as shown in Figure 4. Its presence confirms the rhamnolipid nature of the biosurfactant.

Table 9: Morphological and biochemical characteristics of Isolate G_1

Test	Result
Colony form	Irregular
Colony colour	Yellow
Colony surface	Smooth
Cell shape	Cocci
Grams reaction	+
Motility	-
Beta haemolysis	+
Citrate utilization	-
Gelatin hydrolysis	+
Starch hydrolysis	-
Catalase	-
Oxidase	-
Esculin hydrolysis	+
Voges Proskauer	+
Metabolism with	
D-fructose	+
D-Glucose	+
Glycogen	-
Lactose	+
Maltose	+
Mannitol	-
D-mannose	+
Rhamnose	-
Ribose	+
Sorbitol	-
Sucrose	+
Trehalose	+
Xylose	-
Genus	Enterococcus

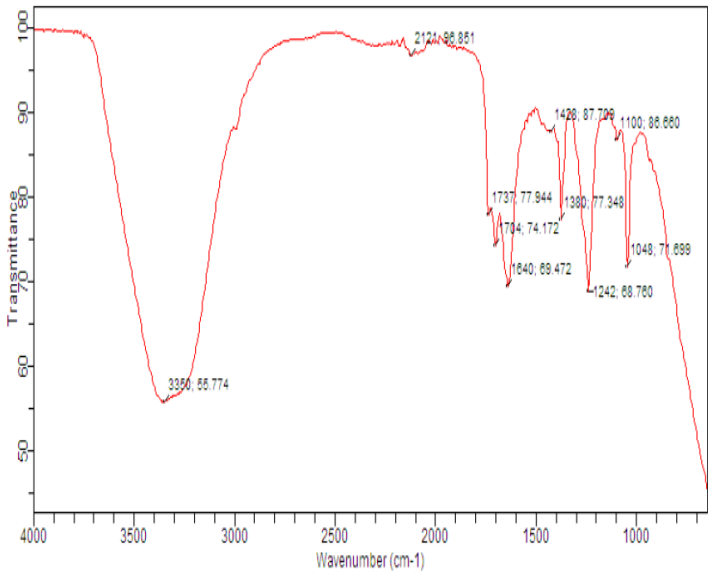


Figure 3: Spectrum of produced biosurfactant by *Enterococcus hirae*

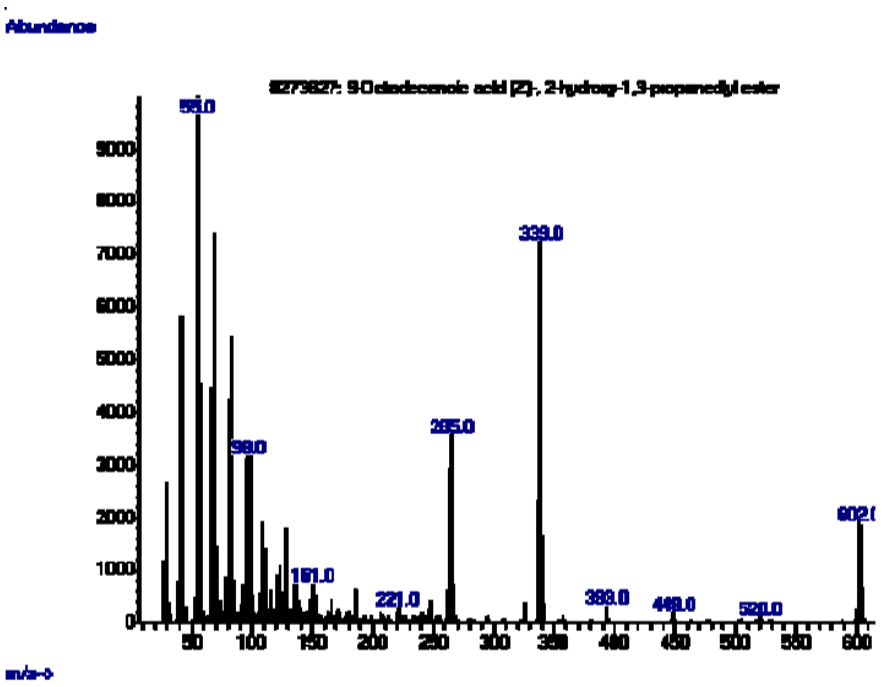


Figure 4: Chromatogram of produced biosurfactant by *Enterococcus hirae*

DISCUSSION

This study evaluated the isolation of *Enterococcus hirae*, a biosurfactant-producing and crude-oil degrading bacterium, from diesel contaminated soil and characterization of the biosurfactant produced. Many studies have reported the isolation and distribution of biosurfactant producing bacteria in hydrocarbon contaminated sites (Bodour et al. 2003; Saravanan & Vijayakumar, 2012; Zou et al. 2014). Although biosurfactant producing bacteria are ubiquitous in nature, they are mostly found in hydrocarbon contaminated environments (Nwaguma et al. 2016).

The screening methods employed were haemolytic assay, oil displacement test, drop collapse test, tilting glass slide test and emulsification index. These methods have been previously reported for the identification of biosurfactant producing bacteria (Banat, 1993; Carillo et al.1996; Haba et al. 2000; Patil & Chopada, 2001; Ellaiah et al. 2002; Bodour et al. 2003; Satpute et al. 2008; Chandran & Das, 2011). The isolates screened in this study showed varying results for the different screening methods and the biosurfactant producers were selected based on their ability to give positive results in all the screening methods. Haemolytic assay, drop collapse test and tilting glass slide test are qualitative screening techniques while emulsification index and oil displacement test are both qualitative and quantitative techniques (Satpute et al. 2010). The use of these techniques is similar to the report of Satpute et al. (2008) who used the combination of oil displacement test, drop collapse test, tilting glass slide test and E_{24} index to select biosurfactant producers. He further suggested that a single method is not suitable to identify all the types of biosurfactant and recommended the combination of methods.

Drop collapse test relies on the basis that as the surface tension between the droplet and the sealing film reduces, the droplets on the film would collapse. So, if the droplets are flat in shape, the reactions are positive while if the droplets are spherical in shape, the reactions are negative (Jain et al. 1991). Biosurfactant have the ability to displace oil, if there are biosurfactant produced, it will form an oil spreading circle on the oil film (Joshi et al. 2008). As for haemolytic assay, the presence of transparent clearing zone in the blood agar is a sign of haemolysis. Therefore, the stronger the haemolysis is, the higher the surface activity of biosurfactant is.

From the fifteen biosurfactant producers, two isolates (G_1 and E_2) showed significant remarkable growth ($p < 0.05$) in a 7-day crude oil degradation and were regarded as the most potent crude oil degrader. These two isolates were further tested, singly and in consortium, in a 14-day degradation of crude oil using gravimetric method. Isolate G_1 had the highest degradation rate of 77.19%, followed by E_2 (62.72%) while their consortium had the least degradation rate of 29.12%. In this investigation, isolate G_1 and E_2 showed a significantly higher degradation of crude oil compared to many recent reports dealing with the microbial degradation of crude oil. Biosurfactant increases the surface areas of sparsely soluble hydrocarbon compounds by reducing surface and interfacial tensions which lead to increased bioavailability and mobility of contaminants, thereby increasing the rate of hydrocarbon bioremediation (Mehanty et al. 2006)

The antagonism of isolate G_1 and E_2, which led to lower crude oil degradation efficiency, could be as a result of a specific strain producing metabolites or changing the environmental or cultural conditions that will then inhibit the growth of other isolate. The antagonism between certain microorganisms has often been reported. Islam et al. 2021 found that antagonism between *P. aeruginosa* and *B. cereus* in a waste water-fed microbial fuel cell inhibited cell growth and power generation. The crude oil degradation result posits isolate G_1 as the most potent crude oil degrader. GC-MS analyses of the residual hydrocarbon extracted from isolate G_1 culture was conducted after two weeks and compared with an abiotic control assayed under the same conditions. From the chromatograms, it was revealed that total petroleum hydrocarbon is reduced in the crude oil sample treated with isolate G_1. Wide ranges of alkanes ($C_{21} – C_{35}$) that were originally present in the abiotic crude oil sample (see Table 7) were degraded to form new compounds in the range of $C_{14} – C_{21}$ in the treated sample with the generation of 19 prominent degradation intermediates forming various esters and acids (see Table 8).

The 16S rRNA sequence of isolate G_1 showed 99.80% similarity to *Enterococcus hirae*. The production of biosurfactant by *E. hirae* was only reported by Soltani et al. 2022. This work has further validated the production of biosurfactant by *E. hirae* and for the first time, *E. hirae* is reported as a biosurfactant producer and crude oil degrader. The molecular composition of the biosurfactant produced by *E. hirae* was evaluated using FTIR and GC-MS analyses. The FTIR analysis revealed the presence of seven spectra at 3 350, 2 121, 1 737, 1 640, 1 242, 1 100 and 1 048cm^{-1}. For interpretation of various functional groups present in the biosurfactant, the FTIR spectra was compared to a standard IR table and from the result, it can be summarized that the chemical structure of this biosurfactant is identical to those of previously reported Rhamnolipid as reported by Patowary et al. (2018) and Pornsunthorntawee et al. (2008). GC-MS profile of the isolated biosurfactant revealed different types of fatty acids. The predominant fatty acid detected in this study, Octadecenoic acid, has been associated with Rhamnolipid type of biosurfactant according to Tuleva et al. (2005), Rahman and Gakpe (2008), Da Rosa et al. (2010), Toribio et al. (2010), Saravanan & Vijayakumar (2012) and Zargar et al. (2022).

Rhamnolipids use has been extensively investigated in the areas of biological environmental remediation where they can facilitate the removal of environmental pollutants such as hydrocarbons and heavy metals. In a study conducted by Liu et al. (2021), they compared various surfactants including the plant surfactant, Saponin and microbial biosurfactants such as Surfactin and Rhamnolipid for their remediation efficiency of oil contaminated soils, Rhamnolipid showed the highest total petroleum hydrocarbons (TPH) removal efficiency which was higher than other surfactants including the selected chemical surfactants (i.e Tween 80 and Triton X-100). They concluded that this result demonstrates the excellent interfacial activity of Rhamnolipid and its great potential for removal of oil contaminants. According to Fenibo et al. (2019), Rhamnolipids have been used for various types of remediation of hydrocarbon spills more than any other biosurfactants due to their important properties that are related to pollutant removal such as solubilization, emulsification, dispersion, foaming, wetting, complexation and the ability to change the surface properties of bacterial cells especially during the remediation of hydrocarbons and their derivatives as well as metal contaminants (Liu et al. 2018).

CONCLUSION AND RECOMMENDATIONS

Fifteen (15) crude oil utilizing bacteria have been screened to produce biosurfactant and degrade crude oil from diesel contaminated soil samples. Through biosurfactant screening experiments and crude oil degradation experiments, it can be concluded that *E. hirae* is the most efficient biosurfactant producer and crude oil degrader. Therefore, the properties of biosurfactant produced and hydrocarbon biodegradation by *E. hirae* were investigated in details. The results showed that:

1. *Enterococcus hirae* exhibited excellent degradation of various crude oil component with a degradation efficiency rate of 77%, thus positioning the bacterium as a promising strain in the remediation of petroleum hydrocarbon contaminations and in microbial enhanced oil recovery.
2. *E. hirae* could biodegrade C_{21} – C_{35} hydrocarbons present in crude oil.
3. The biosurfactant produced is a rhamnolipid type of biosurfactant based on the result obtained from FTIR and GC-MS analyses.

In view of the excellent performance in biosurfactant production and crude oil degradation, *E. hirae* showed a promising application prospect in various environmental applications, particularly in the remediation of petroleum hydrocarbon contamination sites.

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