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Isolation, characterization, and application of biosurfactant by *Klebsiella pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil in Ogoniland, Nigeria

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Abstract

Background/aim: Considerable attention has been given to the use of biosurfactants in recent times because of their potential industrial and environmental applications and ecological friendliness. Hydrocarbon-polluted soils have been major sources of biosurfactant-producing bacteria; resultantly, this study had been aimed at isolating and characterizing biosurfactant produced by *Klebsiella pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil in Ogoniland, Nigeria.

Methodology: The biosurfactant screening techniques employed were emulsification assay, emulsification index (E_{24}), lipase activity, haemolytic assay, oil spreading, and tilted glass slide. The bacterial isolate was identified based on phenotypic, biochemical, and molecular means. Thin-layer chromatography (TLC) and gas chromatography mass spectrometry (GC–MS) analyses were used in the classification and characterization of the biosurfactant produced. The biosurfactant produced was applied on selected hydrocarbons to determine its emulsifying capacity.

Results: The phylogenetic tree analysis of the 16S rRNA gene classified the isolate as *K. pneumoniae* strain IVN51. The sequence obtained from the isolate has been deposited in GenBank under the accession number KT254060.1. The result obtained from the study revealed high biosurfactant activity with a maximum E_{24} of 60 % compared to E_{24} of 70 % by sodium dodecyl sulphate (SDS). In addition, the biosurfactant showed emulsifying activity against the following hydrocarbons: petrol, kerosene, xylene, toluene, and diesel. The optimum cultural conditions (temperature, pH, carbon, nitrogen, hydrocarbon, inoculum concentration, and incubation time) for growth and biosurfactant production by *K. pneumoniae* IVN51 were determined. The biosurfactant was characterized as a phospholipid using TLC, while the GC–MS analysis identified the phospholipid as phosphatidylethanolamine.

Conclusion: This study has demonstrated the capacity of *K. pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil to produce biosurfactant and the effectiveness of the produced biosurfactant in emulsifying different hydrocarbons. Furthermore, the biosurfactant produced was found to belong to the class, phospholipids based on the TLC and GC–MS analyses.

Keywords: Biosurfactant, Hydrocarbon-polluted soil, *Klebsiella pneumoniae* strain IVN51, Phospholipid, Phosphatidylethanolamine

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Background

Microorganisms that produce biosurfactant abound in nature; they inhabit both water (fresh water, groundwater, and sea) and land (soil, sediment and sludge). In addition, they can be found in extreme environments (e.g., oil reservoirs) and thrive at a wide range of temperatures, pH values, and salinity (Chirwa and Bezza 2015). In addition, they can be isolated from undisturbed environments, where they have physiological roles, not involving the solubilisation of hydrophobic pollutants, such as antimicrobial activity, biofilm formation or processes of motility, and colonization of surfaces (Van Hamme et al. 2006). However, hydrocarbon-degrading microbial communities remain the most suitable environment for widespread capability for biosurfactant production.

Hydrocarbon-degrading bacterial populations are generally dominated by a few main genera, namely: *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Klebsiella* and *Actinobacteria* in soils and sediments, and *Pseudoalteromonas*, *Halomonas*, *Alcanivorax,* and *Acinetobacter* in marine ecosystems (Bodour et al. 2003). It is not surprising therefore that a lot of biosurfactant or bioemulsifier producers belong to these same genera. An estimate of the frequency of biosurfactant-producing strains within a microbial population cannot be easily determined, as it depends on the experimental procedures used. It has been reported that 2–3 % of screened populations in uncontaminated soils are biosurfactant-producing microorganisms. This figure increases to 25 % in polluted soils (Bodour et al. 2003). On the other hand, enrichment culture techniques specific for hydrocarbon-degrading bacteria may lead to a much higher detection of biosurfactant producers with estimates up to 80 % (Rahman et al. 2002). Biosurfactants produced by microorganisms are grouped into two different classes based on their chemical composition, viz., low molecular weight surface-active agents called biosurfactants and high molecular weight biosurfactants referred to as bioemulsifiers. Examples of low molecular weight biosurfactants are the glycolipids, lipopeptides and lipoprotein, fatty acids, phospholipids, neutral lipids, particulate biosurfactants, and polymeric biosurfactant while the high molecular weight biosurfactants are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers. The best studied bioemulsifiers are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron 1998). The different classes of biosurfactant find application in different industrial processes.

The attention given to the production of biosurfactants in recent times is mainly due to their potential utilization in food processing, pharmacology, cosmetics, oil exploration and exploitation industries, environmental management, and agriculture (Makkar and Cameotra 2002; Mulligan 2005). One application of biosurfactant that is of interest to environmentalist is in environmental management and bioremediation. Biosurfactants have been successfully applied in the bioremediation of crude oil-polluted sites. Biosurfactant from *Pseudomonas aeruginosa* SB30 was used in the EXXON Valdex oil spill in Alaska with 1 % being enough to remove two times the oil on water at temperatures of 40 °C and 80 °C.

In 1990, a superbug (oil eating bug) was invented in the oil spill clean-up of the state of Texas in the USA. This superbug was earlier engineered by Anand Mohan Chakrabarty (Indian-borne American) in 1979. The bug which was able to grow rapidly and produce surface-active substances that degrade hydrocarbon was a hybrid of *Pseudomonas putida*. Various experiments with laboratory scale of sand-packed columns and field trials have successfully demonstrated the effectiveness of biosurfactants in microbial enhanced oil recovery (MEOR). The use of biosurfactants in MEOR can be implemented in two different ways as either an ex situ biosurfactant injection or in situ biosurfactant production to achieve an increase in oil recovery from subsurface reservoirs (Banat et al. 2010). Both of them require that the biosurfactants and their producing microorganisms are able to tolerate the harsh environmental conditions, such as high salinities, temperatures, and pressures.

Although there is surprisingly dearth of information regarding the application of phospholipid biosurfactants, few studies have reported their application in environmental management. Wiącek and Adryańczyk (2015) showed that the nature of biosurfactant, ethanol concentration, and proportion of the oil-to-water phase are the most important factors for processing and stabilizing phosphatidylcholine-based emulsions. Phospholipids are known to form major components of microbial membranes. Wiącek (2012) was the first study that explored the effects of both electrolyte ions and ethanol molecules on 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) hydrolysis by phospholipase. When certain hydrocarbon-degrading bacteria or yeasts are grown on alkane substrates, the level of phospholipid increases greatly. For instance, using hexadecane-grown *Acinetobacter* sp. HO1-N, phospholipids (mainly phosphatidylethanolamine) rich vesicles were produced (Youssef et al. 2005). Phospholipids have been quantitatively produced from *Thiobacillus thiooxidans* that are responsible for wetting elemental sulphur necessary for growth (Martinez-Toledo et al. 2015). Phosphatidylethanolamine produced by *Rhodococcus erythropolis* grown on *n-alkane* resulted in the lowering of interfacial tension between water and hexadecane to less than 1 mN m^{-1} and CMC of 30 mg L^{-1} (William 2014).

This study investigated the isolation, characterization, and application in hydrocarbon emulsification of biosurfactant by *Klebsiella pneumoniae* strain IVN51 isolated from hydrocarbon-contaminated soil in Ogoniland, Nigeria.

Methods

Sample collection

The soil samples used for bacterial isolation were obtained from the Kporghor community of Tai Local Government Area (Ogoniland), in the Niger Delta region of Nigeria.

For each soil source, 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 transported aseptically in sterile polythene bags to the laboratory for the analysis. The samples were stored at ambient temperature for further use (Deepika and Kannabiran 2010).

Physicochemical analysis of soil sample

The physicochemical parameters analysed were pH, temperature, and total petroleum hydrocarbon (TPH). Gas chromatographic analyses were carried out as described by Chikere et al. (2015).

Total petroleum hydrocarbons (TPH)

Dried soil samples were powder sieved and cold-extracted in conical flask for a total of 2 h in each case using 100 % dichloromethane. The solvent from the resultant solution was removed by means of rotary evaporator under vacuum (pressure not greater than 200 mbar) and finally by a flow nitrogen at not more than 30 °C to yield the extracted organic matter (EOM).

The semi-volatile compounds were introduced into the GC–MS by injecting the sample extract into a gas chromatograph (GC) equipped with a narrow-bore-fused-silica capillary column. The GC column was temperature-programmed to separate the analytes, which were then detected with a mass spectrometer (MS) connected to the gas chromatograph.

Analyte eluted from the capillary column was introduced into the mass spectrometer via a jet separator. The identification of target analytes was accomplished by comparing their mass spectra with the electron impact spectra of authentic standards. Quantitation was accomplished by comparing the response of a major (quantitation) ion relative to an internal standard using an appropriate calibration curve for the intended application.

Condition

The GC–MS system comprised of Agilent 6890GC (Agilent Technologies, Wilmington, USA) with 5975B MSD and MSD chemstation (version D. 03.00). Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min at a pressure of 75 kPa. The injector temperature was set at 250 °C. The program used was 2 min hold time, ramp to 240 °C at 7 °C/min, and a final ramp to 285 °C at 12 °C with an 8 min hold time. Column—30-m × 0.25-mm ID × 0.25 µm film thickness silicone-coated fused-silica capillary column.

MSD condition

Solvent delay: 4 min, Mode-Scan at 3.54, Solvent delay: 3 min, Quard temp: 150 °C, Source temp: 230 °C, Transfer line temp: 280 °C, Sampling: 2, Low mass: 45.0 amu, High mass: 450 amu, and Threshold: 150.

Isolation of bacteria

Serial dilution was performed according to the method described by (Nandhini and Josephine 2013). Nine millilitres (9 mL) of normal saline (0.85 % NaCl in distilled water) was first dispensed into each clean test tube, sterilized in an autoclave at 121 °C (15 psi) for 15 min and allowed to cool. To prepare stock solution, 10 g of the dry soil sample was dissolved in 90 mL of sterile normal saline; from this stock solution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were made.

Hundred microliters (100 µl/0.1 mL) of 10^{-3}, 10^{-5}, and 10^{-6} dilutions were spread-plated on the modified mineral salt medium (MSM) described by Techaoei et al. (2011), containing the following ingredients (in 1 L distilled H_2O): glycerol, 5 g; asparagine, 1 g; K_2HPO_4, 1 g; $MgSO_4 \times 7H_2O$, 5 g; KCl, 1.0 g; agar powder, 15 g; and 1 mL of trace solution containing (in 1 L of distilled water) $MgSO_4 \times 7H_2O$, 0.5 g, $CuSO_4 \times 5H_2O$, 0.16 g, and $FeSO_4 \times 7H_2O$, 0.015 g and incubated at 30 °C for 72 h. Morphologically distinct colonies were identified and purified. The total viable cell count (TVC) was determined. The bacterial isolates were stored in MSM slants and kept under refrigerated condition (4 °C) for further studies.

Screening of biosurfactant-producing bacteria

The bacterial isolates were subjected to different screening methods to obtain biosurfactant-producing strains. Haemolytic activity, oil-spreading technique, lipase activity using tributyrin clearing zone (TCZ), emulsification stability (E_{24}) test, emulsification assay, and tilted glass slide test were employed. The selection of the biosurfactant producer was based on the ability of a given strain to give positive results in all the screening tests performed.

Haemolytic activity

This is a qualitative-screening test for the detection of biosurfactant producers (Satpute et al. 2010). Nutrient

agar (NA) supplemented with 5 % (*v/v*) fresh blood was used according to Banat (1993) and Carrillo et al. (1996). The plates were incubated at 37 °C for 24 h. After incubation, the plates were then observed for the presence of clear zone around the colonies.

Oil-spreading technique

This is one of the best methods used in detecting the presence of biosurfactant (BS) producers (Satpute et al. 2010). Twenty microliters (20 µl) of crude oil was added to 40 mL of distilled water (DW) in a petri plate. To oil-coated water surface, 10 µl culture supernatant was added. A colony surrounded by an emulsified halo was considered positive for BS production (Morikawa et al. 2000). The diameter of the cleared zone on oil surface was visualized and measured after 30 s. Huy et al. (1999) reported that this observed emulsified halo correlates with surfactant activity and is known as displacement activity.

Lipase activity by tributyrin clearing zone (TCZ)

Lipolytic activity was observed directly by changes in the appearance of the substrate, tributyrin, and triolein, which were emulsified mechanically in various growth media poured into petri dish. The isolates were screened for lipolytic activity on mineral salt agar containing 1 % tributyrin (*w/v*). The pH of the medium was adjusted to 7.3 ± 0.1 using 0.1 M of HCl and incubated at 35 °C for 3 days. The plates were examined for zones of clearance around the colonies, as described by Gandhimathi et al. (2009).

Emulsification stability (E_{24}) test (emulsification index)

The emulsification index (E_{24}) provides a rapid and reliable measure of the quantity of biosurfactant. The E_{24} was determined as described by Nitschke and Pastore (2004). Two millilitres (2 mL) of kerosene were added to the same amount of cell-free broth. The mixture was vortexed at a high speed for 2 min. After 24 h, the height of the stable emulsion layer was measured. E_{24} index, defined as the percentage of the height of emulsified layer divided by the total height of the liquid column (Nitschke and Pastore 2004), was determined. In this study, sodium dodecyl sulphate (SDS) and water were used as positive and negative controls, respectively: $E_{24}(\%) = \frac{\text{total height of the emulsified layer}}{\text{height of the liquid layer}} \times 100.$

Emulsification assay

Culture broths were centrifuged at 10,000 rpm for 15 min/RT. Three millilitres of supernatant were mixed with 0.5 mL hydrocarbon and vortexed vigorously for 2 min. This was left undisturbed for 1 h to separate the aqueous and hydrocarbon phases. Un-inoculated broth was used as blank. The absorbance of the aqueous phase was measured with a spectrophotometer at 400 nm (Patil and Chopade 2001).

Tilting glass slide test

This technique is effectively a modification of the drop collapse method (Satpute et al. 2010). Isolates were grown for 24 h on nutrient agar plates. A sample colony was 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).

Optimization of cultural conditions for enhanced biosurfactant production

The effect of different cultural conditions (incubation time, pH, temperature, nitrogen source, inoculum concentration, and carbon source) on the growth of the selected bacterial isolates, and the ability of the strain to produce biosurfactant was determined. The inoculum for the optimization used was first standardized using MacFarlane's standard.

The optimum incubation time for growth and biosurfactant production by the selected strain was studied by varying the incubation time (24, 48, 72, 96, 120, 144, and 168 h) of the culture medium. The culture medium was inoculated with a 24 h culture broth containing a total viable cell count (TVC) of 8.7×10^6 cfu/mL of the selected isolate and incubated at 35 °C for 48 h in a rotary shaker incubator. Biosurfactant production was measured using $E_{24,}$ while growth was determined using a spectrophotometer.

The bacterial isolate was incubated at different temperatures (25, 30, 35, 40, and 45 °C) for 48 h, after which the biosurfactant production and growth of the strain were determined. The optimum pH for growth and biosurfactant production by the bacterial isolate was studied by varying the pH (5, 6, 7, 8, 9, 10, and 11) of the culture medium. After 48 h of incubation, biosurfactant production and growth were determined. The bacterial isolate was incubated with different carbon sources (dextrose, fructose, glucose, glycerol, starch, and sucrose) for 48 h, after which biosurfactant production and growth were determined. The bacterial isolate was incubated with different nitrogen sources (asparagine, NH_4NO_3, peptone, urea, and yeast extract) for 48 h, after which biosurfactant production and growth were determined.

Production of biosurfactant

The optimized parameters were used in setting up the biosurfactant production media. The production was carried out in a 500 mL Erlenmeyer flask containing 200 mL of the production.

Bacterial identification

Biochemical and phenotypic characterization was carried out on the positive biosurfactant-producing isolate using Bergey's Manual of Determinative Bacteriology as a guide (Buchanan and Gibbons 1974).

Bacterial genomic DNA extraction was done using the ZR Soil Microbes DNA Mini-Prep extraction kit (Zymo Research Corporation, South Africa). The quantity and purity of the extracted genomic DNA bacterial isolates were analysed using an ND-1000 spectrophotometer (Thermoscientific, Inqaba Biotech, South Africa) and agarose gel electrophoresis. The genomic DNA was stored at −20 °C. The amplification of the 16S rRNA gene of the isolates was carried out using primer set 27F (5^1AGAGTTTGATCMTGGCTCAG-3^1) and 1492R (5^1GGGTTACCTTGTTACGACTT3^1). The PCR reaction was carried out in 25 µL volumes containing 12.5 µL of the Master Mix (Zymo 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 following PCR conditions were used: initial denaturation at 95 °C for 5 min, denaturation (95 °C for 30 s.), annealing 52 °C for 30 s, extension (72 °C for 45 s.), and final extension step at 72 °C for 3 min was cooled for 4 °C. Five microliters (5 µL) of the amplified products were run on agarose gel electrophoresis at 120 V for 15 min to determine the quality of the products. The amplified products were also purified using the DNA clean and concentrator (DCC) kit (Zymo research institute, South Africa) before being made ready for sequencing. PCR products of the bacterial DNA were sequenced using the Sanger method of sequencing with 3500 ABI genetic analyser, at Inqaba Biotechnical Industries, South Africa. The sequences generated by the sequencer were visualized using Chromaslite for base calling. BioEdit was used for sequence editing; Basic Local Alignment Search Tool (BLAST) was performed using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded and aligned with ClusterW and phylogenetic tree drawn with the MEGA 6 software.

Preliminary classification of the biosurfactant

The following analyses: CTAB/methylene-blue agar test (Siegmund and Wagner 1991; Kayganich and Murphy 1991); Biuret test (Feigner et al. 1995); and phosphate test (Okpokwasili and Ibiene 2006) were carried out to determine the class of the biosurfactant produced.

Thin-layer chromatography

The detection of phospholipids was done using the phospholipid-specific spray method described by Goswami and Frey (1971). Metallic copper (0.08 g) was placed in a solution of 0.25 g ammonium molybdate in 1 mL of distilled water. The mixture was chilled and 1 mL of concentrated sulphuric acid added; the deep blue solution was then shaken. The reaction mixture was kept for 2 h at room temperature with occasional shaking. Forty millilitres (40 mL) of distilled water were thereafter added and the content shaken; a colour change from deep blue to light brown was observed and noted. The copper metal was then removed, and 3.2 mL of concentrated sulphuric acid was added; the resulting solution remained light brown.

The solutions to be tested were applied on pre-coated thin-layer-plate silica gel (F-254 of 0.25 mm thickness) and sprayed with the reagent. The plate was then kept in an oven at 65–70 °C for 5 min; it was removed and again sprayed with the reagent and kept for an additional 5–6 min in the oven. Phospholipids stained blue against a light blue background; all other compounds did not give any colour. Overheating produced a pink coloration of the cholesterol, which ultimately turned greenish grey against a light blue background. The plate was developed with chloroform–methanol–water 65: 24: 4 (*v/v/v*), air dried, and then sprayed with the reagent. This procedure can detect as little as 1 µg of phospholipids (Goswami and Frey 1971).

GC–MS analysis

The partially purified phospholipid-biosurfactant fractions (10 mg) for the GC/MS analysis were saponified with 1 M NH_4OH, mixed with d8-AA and d5-DHA (internal standards), esterified with pentafluorobenzyl bromide in N, *N*-diisopropylethylamine, and extracted into isooctane. Thereafter, 1 µl of the extracted solution was injected into Agilent 7890A GC–MS (Agilent Technologies, US), which was set to scan from *m/z* 50 to *m/z* 760 at a scan rate of 1.2 scans per second. The capillary column used was an Agilent J&W DB-35 ms Ultra Inert (30 m × 0.25 mm inner diameter; 0.25 µm film thickness) GC column. The oven temperature was programmed from 130 °C to 230 °C at 2 °C min^{-1}. Meanwhile, the temperature of the injector port was 230 °C, while the transfer line temperature was 290 °C. Helium was used as the carrier gas, with a constant flow rate of 0.8 mL/min.

Application of the biosurfactant on hydrocarbon emulsification

The biosurfactant produced was applied on different hydrocarbons (xylene, petrol, diesel, kerosene, and toluene) and the ability to emulsify these hydrocarbons determined using E_{24}-index.

Statistical analysis

The results were compared by the one-way analysis of variance (one-way ANOVA) and multiple range tests to

find the differences between the measurement means at 5 % (0.05) significance level using IBM® SPSS® Statistics Version 20.0 (Gailly and Adler, US) (Ezebuiro et al. 2015).

Results and discussion

Baseline physiochemical analyses of the soil sample

The physiochemical characteristics of the soil sample are presented in Table 1. The hydrocarbon-polluted soil had a pH of 5.7 ± 0.1. The temperature of the soil was 28.5 ± 0.4 °C. The soil types ranged from humus soil to humus soil mixed with crude oil, and the TPH (mg/kg) value of the soil was 9419.

Screening and selection of the biosurfactant producers

Out of the 29 bacterial isolates screened, four isolates were selected as biosurfactant producers based on their ability to give positive results to all the screening methods employed. From the four biosurfactant-producing bacteria, the best isolate IVN-51 was chosen (Table 2).

Optimization of cultural conditions for enhanced biosurfactant production

From the results obtained, the optimum incubation time for both growth and biosurfactant production was 48 and 120 h with the OD (optical density) reading of 1.7600 ± 0.014 and E_{24} value of 20.00 ± 1.41 %, respectively. The result of the effect of incubation time on growth and biosurfactant production is presented in Fig. 1a.

The effect of different incubation temperatures on growth and biosurfactant production showed the optimum incubation temperatures as 35 and 30 °C for growth and biosurfactant production by the bacterium, respectively (Fig. 1b).

The effect of different pH values on growth and biosurfactant production showed the optimum pH as 9 and 7 for growth and biosurfactant production, respectively. The optimum pH OD reading was 0.5855 ± 0.004, while the optimum pH for biosurfactant production had E_{24} of 28.0 ± 1.41 %. Figure 1c shows the results of the pH optimization for growth and biosurfactant production.

Figure 1d shows the effect of different carbon sources on the growth of the bacterial isolate and ability to produce biosurfactant. The result obtained shows that glycerol had the highest effect on bacterial biomass. Meanwhile, dextrose had the best effect on the production of biosurfactant by the bacterial strain with E_{24} of 23.20 ± 1.41 %.

Figure 1e shows that NH_4NO_3, as a nitrogen source, had the best effect on the production of biosurfactant by the bacterial strain, while asparagine had the highest effect on bacterial growth with the OD reading of 1.2040 ± 0.014.

Identification of the isolate

Phenotypic and biochemical characterization placed the isolate (IVN-51) in the genus *Klebsiella* belonging to the phylum, proteobacteria; class, gammaproteobacteria; order, enterobacteriales, and family, enterobacteriaceae (Table 3).

The phylogenetic analysis based on the 16S rRNA gene of the sequence generated from the isolate classified the isolate as *Klebsiella pneumonia* strain IVN51 (Figs. 2, 3) The sequence has been deposited under the accession number, KT254060.1.

Characterization of biosurfactant produced

The preliminary analyses of the biosurfactant placed it in the class phospholipids (Tables 4, 5). Furthermore, the result of the thin-layer chromatography showed that the biosurfactant produced, belonged to the class phospholipids (Figs. 4, 5), whereas the GC–MS analysis identified

Table 1 Physiochemical properties of the soil samples

Parameter	Hydrocarbon-polluted soil (HPS)
pH	5.7 ± 0.1
Temperature (°C)	28.5 ± 0.4
Types of soil	Humus soil mixed with crude oil
TPH (mg/kg)	9419

Table 2 Comparison of screening characteristics of isolate IVN-51 with other biosurfactant-producing bacterial isolates

Isolate codes	Source of sample	Lipase test (mm)	Emulsification assay (@400 nm)	Emulsification index (E_{24}) %	Tilting glass slide test	Haemolytic assay (mm)	Oil-spreading test (mm^2)[a]
IVN-02	HPS	13.0 ± 2.0	0.5045 ± 0.0025	11.1 ± 2.1	+	3.0 ± 2.0	28.3 ± 0.79
IVN-45	HPS	8.0 ± 2.0	0.5085 ± 0.0015	40.0 ± 1.0	+	12.0 ± 2.0	19.6 ± 0.78
IVN-51	HPS	11.0 ± 1.0	0.4020 ± 0.002	47.8 ± 1.0	+	6.0 ± 2.0	78.5 ± 0.79
IVN-67	HPS	16.0 ± 2.0	0.3220 ± 0.001	24.0 ± 2.0	+	2.0 ± 0.5	176.6 ± 3.14

All values are mean ± SD for triplicate cultures

HPS hydrocarbon-polluted soil;+ positive; DH_2O distilled water

[a] Surface area

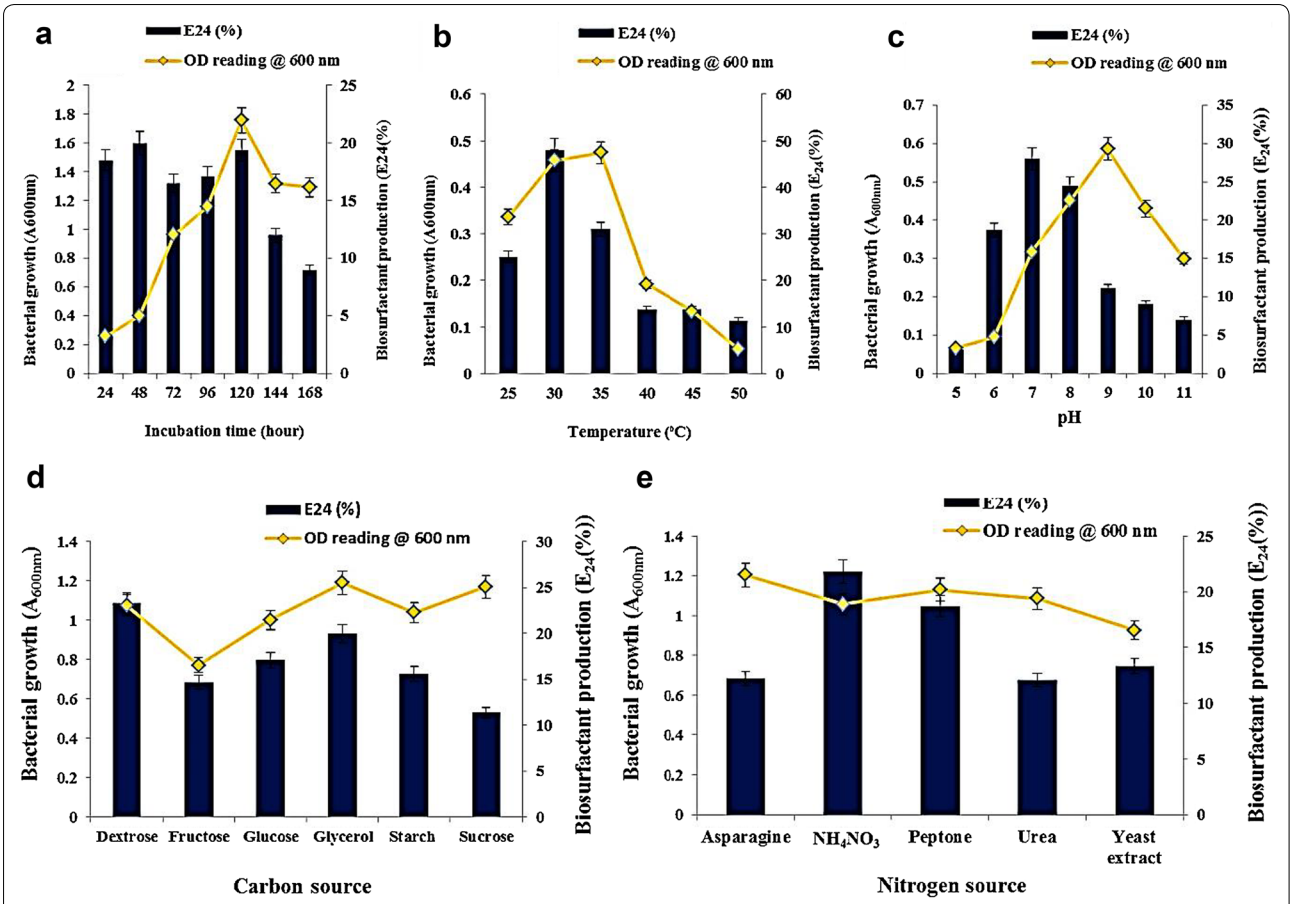

Fig. 1 Effect of cultural conditions on bacterial growth and biosurfactant production (**a** effect of incubation time; **b** effect of temperature; **c** effect of pH; **d** effect of different carbon sources; **e** effect of different nitrogen sources)

the phospholipid, phosphatidylethanolamine ([(2R)-2-octadecanoyloxy-3-tetradecanoyloxypropyl] 2-(trimethylazaniumyl) ethyl phosphate with molecular weight (MW) 734 as the most abundant component (Fig. 5). The components of the cell-free broth are presented in Table 6, and they include: phosphate, phosphatidylethanolamine, with amino acids, such as arginine, leucine, and glycine, while the fatty acid contents included palmitic acid and oleic acid.

Application of the biosurfactant on hydrocarbon emulsification

When the biosurfactant produced was applied on different hydrocarbons, it showed varying degrees of emulsification. In addition, the biosurfactant-producing bacterium was able to grow on the different hydrocarbons. The highest emulsification was observed with kerosene, while the least emulsification was observed with xylene (Fig. 6). Furthermore, the hydrocarbon that supported the growth of the isolate mostly was petrol, while diesel had the least support for the growth of the isolate (Fig. 7).

Discussion

This study evaluated the isolation, characterization, and application of phospholipid-biosurfactant by *K. pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil in Ogoniland, Nigeria. Baseline physicochemical parameters of the soil sample from which the biosurfactant-producing bacterium was isolated revealed a hydrocarbon-contaminated soil. Many studies have reported the isolation and distribution of biosurfactant-producing bacteria in hydrocarbon-polluted sites (Bodour et al. 2003; Saravanan and Vijayakumar 2012; Zou et al. 2014). Although biosurfactant-producing bacteria are ubiquitous in nature, they are mostly found in hydrocarbon-contaminated environments.

The screening methods employed were emulsification assay, emulsification index (E_{24}), lipase activity, haemolytic assay, oil spreading, and tilted glass slide. These methods have been previously reported for the identification of biosurfactant-producing bacteria: tilted glass slide (Bodour and Miller-Maier 1998; Satpute et al. 2008), haemolytic assay (Banat 1993; Carrillo et al. 1996),

Table 3 Biochemical characteristics of the biosurfactant-producing isolate

Isolate code	IVN-51
Gram's stain	−(rods)
Citrate	+
Motility	−
Oxidase	−
Catalase	+
Indole	−
Urease	+
MR	−
VP	+
TSI	
Slant	A
Butt	A
H_2S	−
Starch hydrolysis	+
Gelatin hydrolysis	–
Sugar fermentation	
Maltose	+/A
Glucose	+/A
Lactose	+/A
Mannitol	+/A
Sucrose	+/A
Probable genus	*Klebsiella*

+ positive; − negative; *K* alkaline; *A* acid; *MR* methyl red; *VP* Vogues Proskauer; *TSI* triple sugar iron

emulsification assay (Patil and Chopade 2001), lipase activity (Satpute et al. 2010), oil spreading (Satpute et al. 2008; Chandran and Das 2011), and emulsification index (Haba et al. 2000; Ellaiah et al. 2002; Chandran and Das 2011). The isolates screened in this study showed varying results for the different screening methods.

The biosurfactant-producing bacterium was selected based on its ability to give positive results to all the screening methods. Haemolytic assay, tilting glass slide, and lipase are qualitative-screening techniques, while emulsification index and oil-spreading technique 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 spreading, drop collapse, tilted glass slide, and emulsification index to select biosurfactant producers. Satpute et al. (2008) suggested that a single method is not suitable to identify all the types of biosurfactants, and recommended the combination of methods. In addition, Chandran and Das (2011) used different screening methods, such as emulsification capacity, oil-spreading assays, hydrocarbon overlaid agar, and modified drop collapse methods to detect biosurfactant production. Deepika and Kannabiran (2010) reported the confirmation of biosurfactant production by the conventional screening methods, including haemolytic activity, drop collapsing, and lipase production activity.

The effect of incubation time (24, 48, 72, 96, 120, 144, and 168 h) on the ability of the test isolate to grow well and produce biosurfactant was investigated in this study. The optimum biosurfactant production (20.00 ± 1.41 %) was observed after 48 h (2 days) of incubation time. The value (20.00 ± 1.41 %) obtained for biosurfactant production after 48 h was similar with that obtained after 120 h (5 days) of incubation. However, the optimum growth (1.7600 ± 0.014) was observed after 120 h (5 days) of incubation. This result is similar to that obtained by Patil et al. (2014) who reported optimum growth and biosurfactant production after 96 h of incubation with *Pseudomonas aeruginosa* F23.

Optimization of the cultural temperature of *K. pneumoniae* IVN51 showed the highest biosurfactant production (48.0 ± 2.83 %) and growth (0.4740 ± 0.006) at temperatures 30 and 35 °C, respectively, after 48 h of incubation. Similar results have been reported by several authors. Patil et al. (2014) reported maximum biosurfactant production at the temperature of 30 °C for *Pseudomonas aeruginosa* F23 isolated from oil contaminated soil sample. At temperatures less than or greater than 30 °C, the isolate showed lower biosurfactant-producing ability. Different bacteria species produce biosurfactant

Table 4 Physicochemical characterization of the biosurfactant produced by *K. pneumoniae* IVN51

Material	Surface tension (mN/m)	Emulsification assay (@400 nm)	Emulsification index (E_{24}) %	Tilting glass slide test	Oil-spreading test (mm^2)[a]
DH_2O	71 ± 0.9	–	–	–	–
Partially purified biosurfactant	31 ± 0.4	0.4363 ± 0.001	60 ± 2.0	+	254.3 ± 4.2
SDS	35 ± 0.8	0.6113 ± 0.004	70.0 ± 1.6	+	283.4 ± 3.14

All values represent mean ± SD for triplicate cultures

SDS Sodium dodecyl sulphate (positive control); + positive; − negative; *DH_2O* distilled water (negative control)

[a] Surface area

Table 5 Preliminary result showing the class of the biosurfactant produced

Biosurfactant test	Biosurfactant aimed at detecting	Result
Biuret test	Lipopeptide biosurfactant	–
CTAB/methylene-blue agar test	Rhamnolipid	–
Phosphate test	Phospholipids	+[a]

+ positive; − negative

[a] Formation of yellow color, which was followed by the slow formation of a fine yellow precipitate, indicated the presence of phospholipid biosurfactant

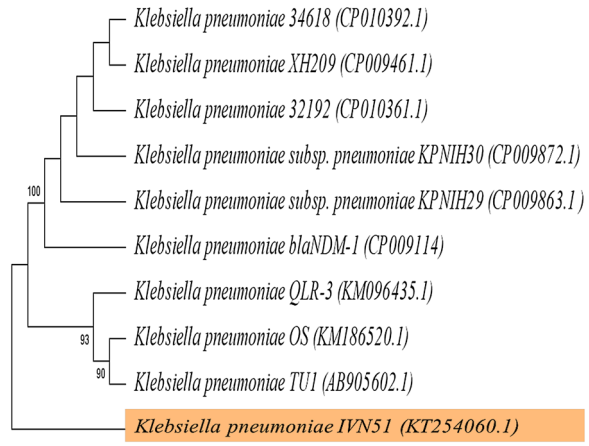

Fig. 2 Neighbor-joining phylogenetic tree of isolate IVN51 made by MEGA 6.0 (Tamura et al. 2013). Bootstrap values of >50 % (based on 1000 replicates) are given in the nodes of the tree. Nucleotide substitution mode used was Jukes and Cantor. NCBI accession numbers are given in parentheses

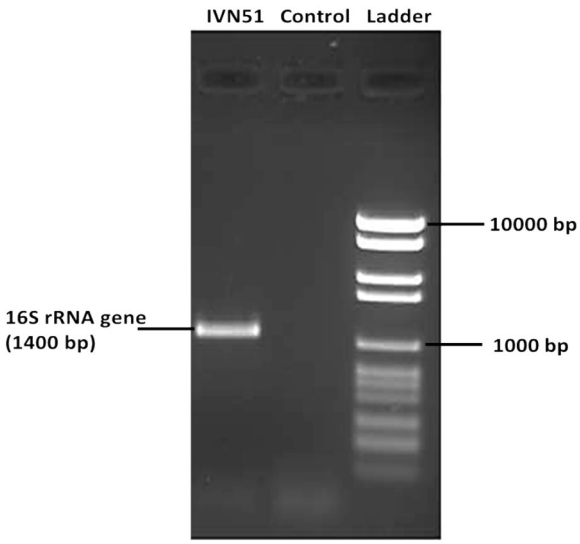

Fig. 3 PCR amplification images of the 16S rRNA gene bands of the biosurfactant-producing bacterium (*Lane 1* 16S rRNA (ribosomal RNA) of the isolate; *Lane 2* control; *Lane 3* DNA maker)

at different temperatures ranges. However, most of them produce at the temperature range of 30–37 °C (Chander et al. 2012). Youssef et al. (2004) reported that a change in temperature can cause alteration in the composition of biosurfactant.

The result of pH optimization for growth and biosurfactant production by *K. pneumoniae* IVN51 is consistent with that obtained by Hamzah et al. (2013). Hamzah et al. (2013) reported maximum biosurfactant production by *Pseudomonas aeruginosa* UKMP14T. In addition, Gumaa et al. (2010) obtained maximum biosurfactant production at pH 8 and maximum biomass at pH 9 with *Serratia marcescens* N3. The result showed that while maximum biosurfactant was achieved at neutral pH, the bacterium grew best at slightly alkaline pH. Studies (Saharan et al. 2011; Saikia et al. 2012; Xia et al. 2012) have reported the effect of pH on biosurfactant production by bacteria. Meanwhile, Mata-Sandoval et al. (2001), Al-Araji and Issa (2004), Rashedi et al. (2005), and Kannahi and Sherley (2012) reported maximum biosurfactant production at pH below 7.

The effect of different carbon sources (dextrose, fructose, glucose, glycerol, starch, and sucrose) on biosurfactant production and the growth of *K. pneumoniae* IVN51 investigated in this study revealed that the maximum biosurfactant production was obtained when grown in a mineral salt medium amended with dextrose; maximum growth (1.1920 ± 0.004) was achieved with glycerol as the carbon source. Although the isolate was able to grow in the presence of other carbon sources, dextrose and glycerol gave the highest result for biosurfactant production and growth, respectively.

Nitrogen plays an important role in the production of surface-active compounds by microorganisms (Mercade et al. 1996). The effect of different nitrogen sources (asparagine, NH_4NO_3, peptone, urea, and yeast extract) on the biosurfactant production and growth of *K. pneumoniae* IVN51 was studied. There are observations that different nitrogen sources can stimulate biosurfactant production by some microorganisms. The result showed maximum biosurfactant production when grown in a mineral salt medium amended with NH_4NO_3 and maximum growth (1.2040 ± 0.014) when grown in a mineral salt medium amended with asparagine. This finding is similar to that obtained by Shekhawat et al. (2014), who reported maximum biosurfactant production and growth of *Bacillus* sp. with NH_4NO_3 as a source of nitrogen. Other researchers have reported maximum biosurfactant production with other nitrogen sources. Hamzah et al. (2013) reported maximum biosurfactant production by *Pseudomonas aeruginosa* UKMP14T with $(NH_4)_2SO_4$

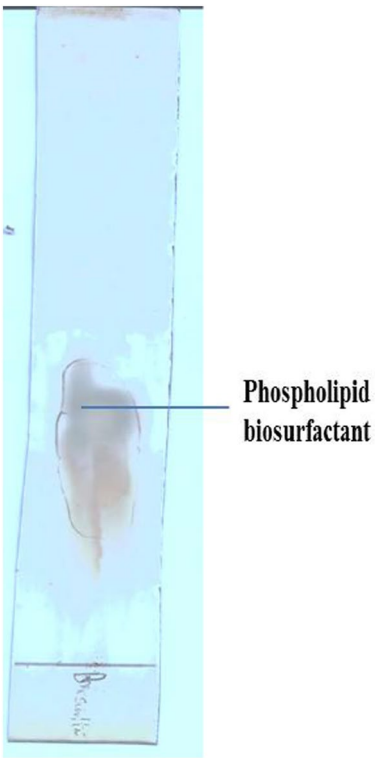

Fig. 4 Phospholipid produced by the *K. pneumoniae* IVN51 on thin-layer plate (*Light brown* colouration visible on the plate is an indication of the presence of phospholipids)

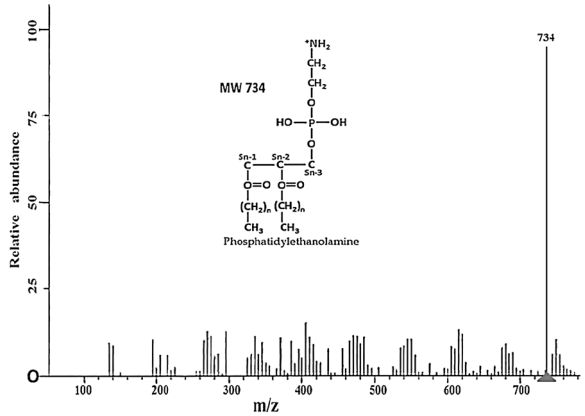

Fig. 5 Mass spectrum of partially purified phospholipid-biosurfactant produced by *Klebsiella pneumonia* IVN51 using silica column chromatography (Phosphatidylethanolamine; MW: 734)

as the nitrogen source. Similar results were obtained by Karkera et al. (2012) for *Pseudomonas aeruginosa* R2, and optimum nitrogen source was found to be NH_4NO_3 (0.4 %). Patil et al. (2014) reported KNO_3 as the optimum nitrogen source for biosurfactant production. The

Table 6 Composition of the cell-free broth

Sample	Amino acids	Fatty acids	Others
Cell-free broth	Arginine Leucine Glycine	Oleic acid Palmitic acid	Phosphate Ethanolamine

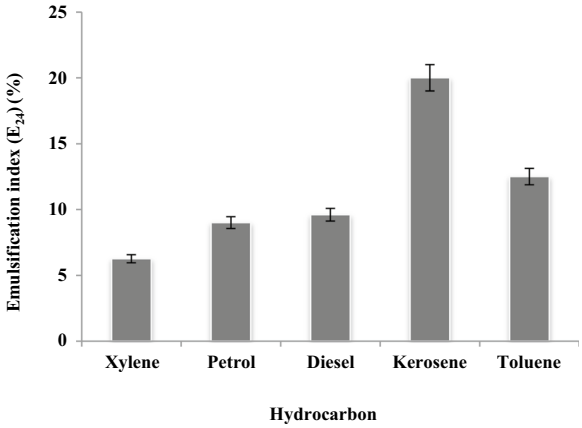

Fig. 6 Emulsification of different hydrocarbons by the biosurfactant produced by *K. pneumoniae* IVN51

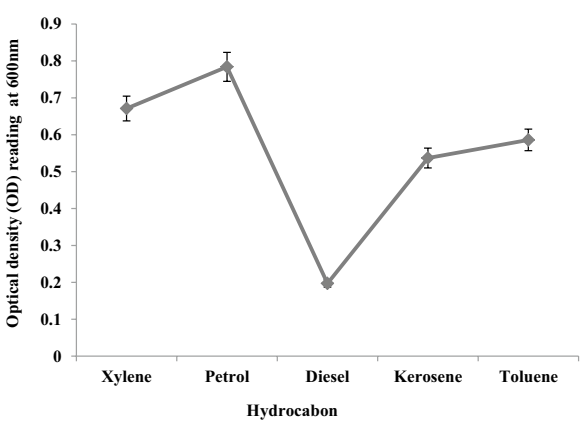

Fig. 7 Effect of different hydrocarbons on the growth of *K. pneumoniae* IVN51

difference observed in the production of biosurfactants when *Klebsiella pneumonia* IVN51 was grown in the presence of different nitrogen sources may be due to the preferential demand for a particular nitrogen source for growth and secondary metabolites production by the bacterium.

Preliminary performance of the biosurfactant carried out, excluded the presence of glycolipids, rhamnolipids and lipopeptide, with a positive result for phospholipids using phosphate test. Phosphate test has been applied

by Okpokwasili and Ibiene (2006) in determining the presence of phospholipid biosurfactants. They reported that the formation of yellow colour, which may be followed by slow formation of a fine yellow precipitate on reaction of 5 % ammonium molybdate and 6 M HNO_3 with the biosurfactant extract, indicated the presence of phospholipid biosurfactant. The thin-layer chromatographic analysis of the crude biosurfactant confirmed that the biosurfactant was of the phospholipid class. The isolation of phospholipids from *K. pneumoniae* has been reported (Jamal et al. 2011); however, they did not identify the type of phospholipid responsible for the biosurfactant activity. This study went further to identify the type of phospholipid-biosurfactant. The GC–MS analysis carried out on the partially purified biosurfactant showed that the phospholipid-biosurfactant produced by the isolate was phosphatidylethanolamine ([(2R)-2-octadecanoyloxy-3-tetradecanoyloxypropyl] 2-(trimethylazaniumyl) ethyl phosphate with molecular weight (MW) 734. Phosphatidylethanolamine is the most abundant membrane phospholipid in many prokaryotic cells. The phospholipid-biosurfactant produced by the isolates showed emulsification properties against a wide range of hydrocarbons. Other researchers have reported phospholipid-biosurfactant production and the effect of certain conditions on the emulsifying capacity (Wiącek 2012; Wiącek and Adryańczyk 2015). The GC–MS analysis of the cell-free broth revealed that it contained the following components: phosphate, phosphatidylethanolamine, with the following amino acids arginine, leucine, and glycine, while the fatty acid contents include palmitic acid and oleic acid. These components have been associated with phospholipid-biosurfactant (Adamu et al. 2015).

The application of the phospholipid-biosurfactant produced in this study against different hydrocarbons showed varying degrees of emulsification against the tested hydrocarbons. There is dearth of information on the emulsification ability of different hydrocarbons by biosurfactants. This study has provided information on the emulsification capacity of the phospholipid-biosurfactant produced. The biosurfactant showed higher emulsification activity against straight chain hydrocarbons when compared with aromatic and cyclo-aromatic hydrocarbons. Moreso, lighter crude oil portions (petrol), supported the growth of the bacterium more than heavier portions (diesel). Emulsification capacity may be important in the bioremediation of crude oil contaminated environments. The use of biosurfactants for the bioremediation of hydrocarbon contaminated soil has been widely studied (Banat et al. 1991; Deschenes et al. 1996; Robinson et al. 1996; Okpokwasili and Ibiene 2006; Satpute et al. 2010).

The isolate *K. pneumoniae* IVN51 used in this study showed similarity with the following Genebank isolates: *K. pneumoniae* 34,618, 97 %; *K. pneumoniae* XH209, 97 %; *K. pneumoniae* 32,192, 97 %; *K. pneumoniae* subsp. *pneumoniae* KPNIH29, 97 %; *K. pneumoniae* subsp. *pneumoniae* KPNIH30, 97 %; *K. pneumoniae* blaNDM-1, 97 %; *K. pneumoniae* QLR-3, 97 %; *K. pneumoniae* OS, 97 %; and *K. pneumoniae* TUI, 97 %. The production of biosurfactant by *K. pneumoniae* has been reported (Jamal et al. 2011), although not widely. This work has, therefore, further validated the production of biosurfactant from *K. pneumoniae.* The safety aspect of the isolate used in this study was taken into consideration.

Conclusion

This study has shown that the bacterium *K. pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil was capable of producing biosurfactant and that the biosurfactant it produced was a phospholipid based on the result obtained from GC–MS analyses. The biosurfactant produced was also found to demonstrate emulsification activity against the following hydrocarbons: xylene, kerosene, petrol, diesel, and toluene, a feature, which is attractive for application in the biodegradation of petroleum hydrocarbons. In addition, it was observed that temperature, pH, incubation time, carbon sources, and nitrogen sources all impacted on the ability of the isolate to produce biosurfactant. The result of the optimization process can be useful in enhancing the production of surface-active agents, making them attractive options for application at industrial level.

Authors' contributions

IVN and GCO conceived the study. IVN carried out the laboratory analysis. IVN, GCO, and CBC participated in the study design and coordination and drafting of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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