A *Pseudomonas aeruginosa* Strain Ikw1 Produces an Unusual Polymeric Surface-Active Compound in Waste Frying Oil-Minimal Medium

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Abstract: A Gram-negative bacterium isolated from the sub-surface water of Ikang River, Niger Delta region, Nigeria produced an unusual biosurfactant in waste frying oil-minimal medium. Cultural and biochemical characterizations as well as 16S rRNA sequencing identified the bacterium as a strain of *Pseudomonas aeruginosa* with 100% sequence homology with *Pseudomonas aeruginosa* strain HNYM41. Biochemical characterizations, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and Fourier transform-infrared (FT-IR) spectrometry identified the active compound as a glycolipopeptide (peptidoglycolipid) composed of 40.36% carbohydrates, 20.16% proteins and 34.56% lipids. The biosurfactant reduced surface tension of water from 72.00 to 24.62 dynes/cm at a critical micelle concentration (CMC) of 20.80 mg/L indicating excellent effectiveness and efficiency properties. Commendable oil-washing property (79.92% oil recovery) with an elution rate of 0.68 mL/min at 70°C, foaming and foam stability, excellent emulsification activity in kerosene, crude oil and palm oil and a significant ($P = 0.000$; $R^2 = 0.9901$) oil solubilization property indicate excellent oil recovery, detergency and remediation potentials of the biosurfactant. Oil displacement, emulsifying and antimicrobial activities of the compound were relatively stable at relevant temperatures, pH and NaCl levels suggesting suitability for applications in hydrophobic compound remediation, emulsion stabilization and preservation of formulations.

Keywords: *Pseudomonas aeruginosa* strain IKW1, glycolipopeptide, enhanced oil recovery, emulsification, stability

1. Introduction

Surface active compounds, otherwise called surfactants, are amphiphilic molecules with hydrophilic and hydrophobic domains which reduce the free surface enthalpy per unit area [1] at air/water interfaces and the interfacial tension at oil/water interfaces [2]. They are about the most sought-after process chemicals worldwide. Their applications range from agriculture and environment [3] [4] to industries including food, cosmetics and pharmaceuticals not to mention petroleum [5]. These applications derive from their nature and the physico-chemical properties which determine their various types including glycolipids, lipopeptides, neutral lipids, phospholipids and the polymeric types [6]-[8].

Synthetic chemistry is at the forefront of production of these molecules derived predominantly from petrochemicals which make them cheap and commercially available [9]. Nevertheless, the bulk applications of these chemicals especially in environmental bioremediation [10] [11] and to a lesser extent, the industries, whose effluents still end up in the environment, have been a source of environmental concern owing to their toxicity and environmental incompatibility [7].

Green surfactants or biologically-derived surface-active molecules, especially those of microbial origin called biosurfactants, are suitable alternatives to their chemical counterparts by reason of their wider applications, biodegradability, low to non-toxicity and environmental compatibility [12]. Biosurfactant-producing microbial strains from different taxonomic groups especially bacteria [13] [14] and yeasts [15] [16] have been isolated and employed in production. In recent times, a new area, called green chemistry,

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has been developed where biosurfactants are applied in the production of nanoparticles [17].

In the industries, most of the biosurfactants are used as emulsifiers but extensive applications in this respect have been limited by relatively high production and recovery costs [1]. Production economics is frequently encountered as the major drawback of biotechnological processes including biosurfactant production and has greatly limited its commercial applications. However, production costs can be reduced by careful selection of producing strains with improved yield, improved product formation rates and use of cheap (often waste) substrates. Recommended protocol for isolation of biosurfactant-producing bacteria involves a combination of screening concepts [18]. However, multiple substrates are required for successful isolation and selection of a diversity of biosurfactant-producing bacteria with abilities to produce novel biosurfactant types [13]. The successful use of wastes like olive oil mill effluent, dairy waste and waste frying oil as substrates for biosurfactant production has been reported by few researchers and with encouraging results [19]-[21].

Here, we report the isolation of a strain of Pseudomonas aeruginosa from the mesotidal waters of Ikang River, Niger Delta area, Nigeria, with ability to elaborate an unusual surface-active compound when grown on waste frying sunflower oil. A waste frying oil disposal problem currently exists in Calabar, the capital city of Cross River State, Nigeria, where it is emptied into drainages resulting in unbearable stench in the capital city. Government has already shut down a number of fast foods in the city thereby worsening the unemployment problem of the state and country. Utilization of the waste frying oil for biosurfactant production will go a long way to solving waste oil disposal and subsequently unemployment problems. To the best of our knowledge, this is the third time a strain of Pseudomonas aeruginosa has been reported to produce a glycolipopeptide biosurfactant but the first report of that production on waste frying oil.

2. Materials and methods
2.1. Collection of morphologically-distinct bacteria
Morphologically-distinct bacteria were isolated from 20 samples comprising surface-water (5), soil (5), sediment (5), crude oil reservoir-water (3) and Nigerian light crude oil sample (2), on Tryptic Soy agar (TSA) medium by the pour plate technique.

2.2. Screening of bacteria for biosurfactant production
2.2.1. Medium formulation for biosurfactant production

Purified cultures of all morphologically-distinct bacteria were grown in minimal medium supplemented with different carbon sources including glucose, glycerol, rice processing effluent, crude oil and waste frying oil at 1% (v/v or w/v) as sole source of carbon and energy. The minimal medium contained (g/L) KH_2PO_4 1.0; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; $NaCl$ 0.5; NH_4Cl 1.0; $FeSO_4.7H_2O$ 0.01 with pH adjusted to 7.0 using 1 M HCl/IM NaOH. Twenty milliliter volume of minimal medium was dispensed into 100 mL Erlenmeyer flasks. Flasks were sterilized by autoclaving at 121°C for 15 min and inoculated, upon cooling, with 2% (v/v) 18 h-old Luria broth culture of each bacterial isolate. Flasks were incubated at room temperature (28 ± 2°C) on a rotary shaker agitating at 150 rpm for 72 h.

2.2.2. Qualitative screening of isolates for biosurfactant production
Cell-free fermentation broth of each flask corresponding to each bacterium was obtained by centrifugation at 8,000 x g for 10 min followed by membrane filtration with 0.2 µM (Millipore) and subjected to qualitative screening for biosurfactant production by the combination of rapid drop collapse test [13], emulsifying activity test [22], salt aggregation test [23] and oil displacement test [6].

2.2.3. Quantitative biosurfactant screening
The initial quantification of biosurfactant in cell-free broth of bacteria which tested positive to at least 75% of the qualitative screen tests followed the oil-displacement assay. This assay is sensitive enough to detect the presence of 10 µg or 10 nmol of biosurfactant in sample solutions [6]. In this test, 15 µL of crude oil (Nigerian medium crude-Adaax) was added to the surface of 40 mL of distilled water in a Petri dish of diameter 15 cm to form a thin uniform oil layer. Plates were allowed to equilibrate for 1 h. Thereafter, 10 µL of each cell-free fermentation broth was gently placed on the center of the oil layer. The diameter of clear halo visualized under visible light was measured with a meter rule after 30 s and area of clear zone calculated using the formula $A = \pi r^2$; where A is the area of the oil film, r the radius and π a constant of value 3.14. The larger the diameter of clear halo, the larger the area and hence the greater the amount of biosurfactant. All determinations were made in triplicates. Biosurfactant hyper-producing strains were selected by consideration of mean oil-displaced areas of their cell-free fermentation broth.

2.3. Identification of biosurfactant -producing bacterium
The selected bacterium was identified morphologically and biochemically using the MICROGEN ID Kit (Microgen Bioproducts Limited, UK) in conjunction with Microgen identification system software as well as by 16S rRNA sequencing.

The sequencing protocol made use of primers 271 and 1492R and utilized a sequence mix composed of NZYTaq 2 x Green Master Mix (Nzytech). Amplification was done by denaturation at 94°C for 3 min, cooling to 5°C for 1 min and raising the temperature again to 72°C. This was repeated through 30 cycles and then held at 72°C for 10 min. The polymerase chain reaction (PCR) products were purified with the JETQUICK PCR purification Spin Kit and used as template for the sequencing reaction. Sequences were compared to GenBank sequences by using standard nucleotide basic local alignment search tool (BLAST). The bacterium was deposited at the University of Calabar collection of Microorganisms (UCCM) and had since been given the collection's code name.

2.4. Determination of the effectiveness of biosurfactants by surface tension reduction measurement

The surface tensions of sterile clear amber cell-free biosurfactant solutions of four isolates were determined by the ring method [20] at room temperature with a tensiometer (CSC Du-Nouy Tensiometer) fitted with a platinum ring. A volume of 70 mL of each cell-free biosurfactant broth was dispensed into 100 mL beakers and placed on the seat of the tensiometer. The seat was then raised by an adjustment knob until contact was made between the surface of biosurfactant liquid and the platinum ring. The liquid film produced beneath the ring was stretched as attempt was made to bring the ring out of the liquid by means of the adjustment knob. The force needed to break the ring free of the liquid to the surface was read off a scale calibrated in dynes/cm. This was repeated 5 times for each of the biosurfactant solutions and mean determinations obtained and presented as surface tension of biosurfactants from the bacterial strains tested.

2.5. Recovery and characterization of biosurfactant

2.5.1 Biosurfactant recovery

Most effective biosurfactant was recovered from the sterile clear amber liquid (obtained by centrifugation of fermentation broth at 8,000 x *g* for 10 min followed by sterilization by membrane filtration) by acidification of 5 mL sterile biosurfactant solution to pH 2.0 with 6 N HCl [24]. Thereafter, acidified biosurfactant treatments were allowed to stand for 10 h at 4ºC after which equal volumes of the acid-treated biosurfactant and chloroform-methanol mixture (2:1) were prepared in separatory funnels The preparations were allowed to stand for 30 min and the bottom layer (organic phase) separated and subjected to rotary evaporation at 35°C. The brown oily substance that ensued was weighed and expressed as g/L.

2.5.2. Structural characterization of biosurfactant

2.5.2.1. Biochemical characterization

Total carbohydrate in the crude biosurfactant solution was determined by the phenol-sulfuric acid method [25] using glucose (Merck) as standard. Total protein was determined by the Bradford method [26] using bovine serum albumin (Research Organics, USA) as standard. Total lipids was detected and quantified as described by the method of Folch et al. [27].

2.5.2.2. Biosurfactant analysis by Thin Layer Chromatography (TLC)

The crude biosurfactant isolated in sub-section 2.4.1 was dissolved in chloroform and spotted on TLC plates made of silica gel. The developing solvent was a mixture of chloroform: methanol: water (65:25:4). Retention factors were calculated after spots were noted and visualized under UV lamp. The plates were then sprayed with different reagents for the detection of different chemical molecules. Some plates were sprayed with H2SO4 :Methanol (5:85) mixture [13] for the detection of carbohydrates followed by heating at 125°C. Some were steamed in iodine vapour for the detection of lipids (fatty acids) and some sprayed with ninhydrin reagent containing 0.5 g ninhydrin in 100 mL anhydrous acetone for the detection of peptides (proteins). Negative ninhydrin tests prompted digestion of biosurfactant with 6N HCl followed by heating to 105°C for 24 h for the detection of free amino acids [28] and the ninhydrin test repeated. Spot colour was noted. Surface active fractions were confirmed by oil displacement activity of needle-point scrapings from the plates.

2.5.2.3. Biosurfactant analysis by high performance liquid chromatography (HPLC)

The active fractions of crude biosurfactant obtained from analytical TLC were subjected to HPLC analysis using a Gemini C_{18} column (100 x 4.6 mm) with a particle diameter of 5 µM accompanied with a Varian 335 diode array detector to facilitate UV detection over a wavelength range of 200 and 220 nM. Sample analysis followed a linear gradient with 85% eluent A comprising of 0.1% ortho-phosphoric acid and 15% eluent B made of acetonitrile at 0 min, only to increase eluent B to 100% after 40 min at an eluent flow rate of 1.0 mL/min [29].

2.5.2.4. Biosurfactant analysis by Fourier transform-Infrared (FT-IR) spectrometry

The infrared spectra of the partially-purified biosurfactant were recorded on a Fourier transform-infrared system (Spectrum BX-Perkin Elmer) in the 4000-400 cm^{-1} spectral region at 2 cm^{-1} resolution. The sample was spread on 0.23 mm KBr cell and cell inserted into the IR transforming system. The spectra were displayed on a connecting computer monitor after Fourier-transformation.

2.6. Evaluation of biosurfactant effectiveness and efficiency

The effectiveness of the crude biosurfactant, given by surface tension reduction, was determined by the ring method [20] at room temperature with a tensiometer (CSC Du-Nouy Tensiometer) fitted with a platinum ring. The efficiency of the biosurfactant, given by its critical micelle concentration, CMC was determined by measuring surface tension values of increasing concentrations of the biosurfactant. The log-transformed biosurfactant concentrations were regressed on their respective surface tension values using a second-order polynomial function. The CMC was defined as the minimum biosurfactant concentration above which no further reduction in surface tension occurred.

2.7. Activity characterization of biosurfactant

2.7.1. Biosurfactant-enhanced oil recovery (washing activity) by the sand pack method

Biosurfactant enhanced recovery of Bonny light crude oil was conducted using the modified sand pack column technique [30]. One hundred and fifty grams (150 g) of 140 µM size sand particle was packed into a glass column of 20 mm x 25 mm x 85 mm dimensions with a 100 µM pore size sieve. The column was fitted with a tap outlet at the bottom to permit escape of recovered oil. A volume of 100 mL of Nigerian medium crude oil was poured into the column and allowed to stand for 3 h. Then 100 mL of sterile biosurfactant solution was added to the column and the column incubated at 30, 50 and 70°C to ascertain temperature influence on biosurfactant-enhanced recovery. The experiment was set up in triplicates with 50 mL of Milli-Q water serving as control. Volume of oil released by biosurfactant enhancement was measured at 30 min interval for 3 h at the different temperatures and compared with that of the control. Oil recovery rate was determined and expressed as mL/min.

2.7.2. Biosurfactant-enhanced solubilization of Nigerian medium crude

Crude oil solubilization test was performed following a batch solubilization technique [31]. Twenty-five milliliters (25 mL) of Nigerian medium crude oil (ADAAX Oil Nigeria) and different concentrations of crude biosurfactant (0, 50, 100, 200, 300, 500, 700, 1000, 1300, 1650 and 2000 mg/L) solutions were mixed in equal volumes in glass-stoppered 250 mL Pyrex separatory funnel. The funnels were placed on a rotary shaker agitating at 200 rpm, at 30°C for 36 h. A 12 h settling period was allowed followed by withdrawal of the aqueous phase from the bottom of the funnel with minimal disturbance. Samples, now called water soluble fractions (WSF), were analyzed for total petroleum hydrocarbons (TPH) by the n-hexane method. Absorbances of n-hexane extracts from the different treatments were taken at 450 nm with HACH DR 300 Spectrophotometer and amounts determined gravimetrically from a standard curve. Regression statistics (Excel 2007) was used to analyze data obtained to show relationship between biosurfactant concentration and solubilization of crude oil at 95% confidence limit.

2.7.3. Assessment of antimicrobial activity of biosurfactant

Assessments were conducted by the agar disc diffusion method [28] in Muller-Hinton agar for bacteria and Potato dextrose agar for fungi. Discs of 6 mm size were punched from filter paper (Whatman No 3; Millipore) and sterilized by autoclaving in capped bottles. Aliquots of 20 µL (50 µg/mL) of crude biosurfactant solution were absorbed by each disc and allowed to equilibrate for 24 h. Equilibrated discs were aseptically introduced onto the center of Muller-Hinton and Potato dextrose agar plates pre-seeded with appropriate dilutions of test cultures/spores. Cell/spore densities of test cultures were as follows (cfu/mL); 10^6 for *Bacillus subtilis* UCCM 0006, 10^7 for *Micrococcus* sp. UCCM 0011, *Rhodotorula mucilaginosa* UCCM 0014 and *Candida* sp. UCCM 0013, 10^8 for *Serratia* sp. UCCM 0003, *Chromobacterium violaceum* UCCM 0001, *Rhizopus stolonifer* UCCM 0010 and *Penicilium frequentans* UCCM 0015. Antimicrobial potential of biosurfactant was indicated by the appearance of inhibition zone within 48 h. Zone diameters were measured with meter rule and expressed in mm. All preparations were made in triplicates and mean zone diameters calculated.

2.7.4. Measurement of emulsifying activity of biosurfactant

The emulsifying property was measured as described in Asitok and Antai [22] and results expressed as per cent E24 (% E24). Kerosene, Nigerian medium crude oil and palm oil served as hydrophobic compounds.

2.7.5. Assessment of foaming property of biosurfactant

The foam power and stability of the glycolipopeptide was evaluated according to the method of Chen et al. [32] with slight modifications. A volume of 200 mL of sterile biosurfactant solution was allowed to flow through a burette from a height of 90 cm into a 500 mL measuring cylinder. The turbulence generated foam whose height was noted immediately and then after 5 min. Foam heights were also measured every 1 h, then every 24 h. The foam height at time 0 min was considered the foam power while the R5, defined as the ratio of the foam height at time 5 min to that at time 0 min, was considered an indication of foam stability.

2.8. Stability characterization of biosurfactant

2.8.1. Temperature stability

To determine the thermal stability of a 5 g/L crude biosurfactant solution, the test biosurfactant preparation was maintained at temperatures of 20, 40, 60, 80, 100, 120, 130 and 140°C for 15 min, cooled at room temperature and oil displacement, emulsification and antimicrobial activity tests of the glycolipopeptide performed as described by Abouseoud *et al*. [33].

2.8.2. pH stability
The effect of pH on biosurfactant activity was performed by adjusting the pH of 5 mL of 0.5% (w/v) crude biosurfactant solution with 1M HCl/1M NaOH through a range from 4 to 11 and held for 15 min. Oil displacement, emulsification and antimicrobial activity tests of the glycolipopeptide were conducted as described previously [33].

2.8.3. Salinity (NaCl) stability
Crude biosurfactant concentration of 0.5% (w/v) was prepared by dissolving 0.5 g of crude biosurfactant in 100 mL of different concentrations of NaCl solution. The concentrations used included 5, 10, 15, 20 and 25% at a pH of 7.0. The preparations were held at 30°C for 15 min to determine the effect of salt concentrations on activities of the glycolipopeptide. Oil displacement, emulsification and antimicrobial activity tests were performed as previously described [33].

3. Results
3.1. Substrate-specific isolation of biosurfactant-producing bacteria
The results of isolation of biosurfactant-producing bacteria from the 20 samples analyzed in our study, as mediated by the different substrates, are presented in **Table 1**. The table reveals that a total of 858 morphologically-distinct bacteria were isolated from all 20 samples with Ifondo water (IFW) harboring the highest number of distinct bacteria of 69. The table also reveals that a total of 141 (16.43%) biosurfactant-producing bacteria were selected using different substrates with waste-frying oil contributing 50 (35.46%). Generally, miscible substrates selected fewer biosurfactant-producing bacteria than hydrophobic substrates. A two-way analysis of variance revealed significant ($P < 0.05$) influence of substrates on the isolation of biosurfactant-producing bacteria.

3.2. Quantitative screening for industrially-relevant biosurfactant-producing bacteria
Table 2 presents the results of the quantitative screening of primarily positive biosurfactant-producing bacteria from the three major kinds of samples. The results show that only 28 (19.59%) of the total biosurfactant-producing bacteria were truly positive for biosurfactant production by the oil displacement assay method. Mean oil-displaced areas

of 4 best isolates were IKW1 (66.5 cm^2) > IFW (55.3 cm^2) > OB6 (52.8 cm^2) > R15B (50.3 cm^2). Results also show very clearly that 3 (75%) of the best isolates were obtained from water samples.

3.3. Identification of biosurfactant-producing bacteria
A summary of the results of characterization tests leading to the identification of the best 4 biosurfactant-producing bacteria is presented in Table 3. The table reveals that isolate IKW1 was *Pseudomonas aeruginosa* with a 100% sequence homology with *Pseudomonas aeruginosa* strain HNYM41 with a GeneBank accession number of JN999891A. Isolate R15B, on the other hand, was identified as *Bacillus cereus* with a 100% sequence homology with *Bacillus cereus* strain F2 with an accession number of JQ579629A. The table also reveals that 3 (75%) of the 4 best biosurfactant-producing bacteria were Gram-negatives.

3.4. Surface tension reduction (effectiveness) potentials of biosurfactants
Investigation into the effectiveness potentials of surface-active compounds of *Pseudomonas aeruginosa* strain IKW1, *Pseudomonas flourescens* strain IFW3, *Pseudomonas* sp. strain OB6 and *Bacillus cereus* strain R15B revealed that *Pseudomonas aeruginosa* IKW1 biosurfactant was the most effective and could reduce surface tension of water from 72 dynes/cm to 24.62 dynes/cm while the least effective was that from *Pseudomonas* sp. strain OB6 which only reduced surface tension from 72 dynes/cm to 35.58 dynes/cm.

3.5. Recovery and identification of *Pseudomonas aeruginosa* Strain IKW1 biosurfactant
Among the 5 organic solvents and their mixtures employed for biosurfactant recovery after precipitation by acidification, the solvent mixture of chloroform-methanol (2:1) recovered the highest biosurfactant amount of 4.39 g/L. Biochemical characterizations of the biosurfactant revealed that the surface-active compound was composed of carbohydrates, proteins and lipids with percent compositions of 40.36, 20.16 and 32.56% respectively. This chemical composition was confirmed by the results of the analytical thin layer chromatographic analysis which revealed 3 active fractions from the chloroform:methanol:water (65:25:4) mixture, their activities of which were confirmed by oil displacement assay. The retention factors (Rf) of the fractions F1, F2 and F3 were 0.45, 0.33 and 0.73 respectively. F1 and F3 stained yellow upon spraying with H_2SO_4:methanol (5:85) mixture confirming the presence of carbohydrates in these fractions. F2 fraction stained red when the plates were heated in iodine vapour confirming the presence

of lipids in the fractions. Original ninhydrin spray was negative as no colour developed from any of the fractions suggesting the presence of blocked N-terminal. Digesting the biosurfactant with HCl and repeating the TLC procedure, caused the F2 fraction with Rf = 0.33 to turn red when ninhydrin was sprayed on it indicating the presence of free amino acids.

The high performance liquid chromatography (HPLC) analysis of preparatory thin layer chromatographic fractions revealed just one peak indicating that the biosurfactant was purified to 95% purity level.

Figure 1 is the spectrum of Fourier transform-infrared (FT-IR) spectrometry of the surface-active compound of *Pseudomonas aeruginosa* strain IKW1. The figure reveals the presence of C-H/O-H stretch bands at wave number between 3765 and 3367 cm^{-1} indicating the presence of aldehydes in carbohydrates. C-H asymetric vibrations of CH_2 and CH_3 were observed at 2926 cm^{-1} and 2868 cm^{-1} suggesting the presence of alkanes in lipids. This was confirmed by the presence of deformation vibrations at 1456 cm^{-1}. The characteristic stake-shaped band for N-H/C=O bond was observed at 1712cm^{-1} indicating the presence of amino and carboxylic groups. These characteristics, in combination with biochemical and chromatograhic results strongly suggest carbohydrate (**glyco**), lipid (**lipo**) and protein (**peptide**) composition for this biosurfactant. The surface-active compound could therefore be safely referred to as a glycolipopeptide (or a peptidoglycolipid).

3.6. Determination of glycolipopeptide efficiency with the critical micelle concentration (CMC)

The efficiency of the glycolipopeptide biosurfactant, given by its critical micelle concentration (data not shown) revealed that the glycolipopeptide has a CMC of 20.80 mg/L. The second order polynomial used to fit the regression model revealed a significant goodness-of-fit (P = 0.000; R^2 = 0.9683) of the model.

3.7. Enhanced oil recovery (washing activity), solubilization, emulsfication and foaming activities of glycolipopeptide

Results of the sand pack experiment demonstrating the oil recovery potential of the glycolipopeptide (data not shown) revealed that highest recovery rate of crude oil of 0.87 mL/min occurred at 50°C at 120th min of incubation. Biosurfactant-enhanced recovery rate of crude oil at 30°C increased linearly with time until it peaked at 0.63 mL/min at 150th min. A near-perfect parabolic curve with a peak of 0.68 mL/min at 90 min was attained when temperature was raised

to 70°C with an oil recovery effectiveness of 79.92%. Only 21.37% of oil was recovered with Milli-Q water (control) with a constantly increasing recovery rate throughout the 3 h holding time.

The results of the solubilization potentials of the glycolipopeptide revealed significant (P < 0.05) positive linear relationship (R^2 = 0.9901); oil solubilization increasing as biosurfactant concentration increased.

Emulsifying activity of the glycolipopeptide was tested with kerosene, crude oil and palm oil as hydrophobic compounds. The results showed that emulsification indices of the surface-active compound were 79.71%, 84.87% and 87.54% in kerosene, crude oil and palm oil respectively.

Results of the foaming property experiment of the biosurfactant revealed that the active compound could produce small densely-packed foam with a height of 7.2 ± 0.3 cm at time 0 min. The foam was stable for > 48 < 72 h. R5 of the foam was 1.

3.8. Antimicrobial potential of glycolipopeptide

The results of the antimicrobial potentials of the glycolipopeptide showed that the biosurfactant could most inhibit *Bacillus subtilis* UCCM 0006 to a zone diameter of 34 mm. However, the surface-active compound showed no inhibitory activity against *Serratia* sp.UCCM 0003. Overall spectrum of antimicrobial coverage of the glycolipopeptide was narrow.

3.9. Stability characterization of glycolipopeptide activities

The results of the effect of temperature, pH and NaCl on the oil displacement activity of the glycolipopeptide are presented in **Figure 2**. **Figure 2A** reveals that oil displacement activity of the glycolipopeptide was stable up to 80°C after which it dropped gradually. The glycolipopeptide was observed, in **Figure 2B**, to be stable at alkaline pH levels with maximal stability of oil displacement activity at pH between 7 and 9. The oil displacement activity of the surface-active compound in **Figure 2C** was decreased gradually with increase in NaCl concentrations. NaCl concentration of up to 10% still demonstrated commendable displacement activity.

Results of the effect of temperature, pH and NaCl on the emulsifying activity of the glycolipopeptide are presented in **Figure 3**. **Figure 3A** shows that the emulsifying activity of the glycolipopeptide increased as temperature increased. An activity of 79.71% at 30°C in kerosene increased to 91.43% at 140°C while that of 82.87% in palm oil increased to 95.49% at 140°C. **Figure 3B** shows the influence of pH on the

emulsifying activity of the biosurfactant and reveals that the activity increased as the pH increased from 4 to 7 and stabilized at the alkaline region. The influence of NaCl on the emulsifying activity of the biosurfactant is shown in **Figure 3C** and reveals that 77.21% of the emulsifying activity was retained at 10% NaCl.

Figure 4 is a presentation of the results of the effect of temperature, pH and NaCl concentrations on the antimicrobial activity of the glycolipopeptide. **Figure 4A** shows that the antimicrobial activity was retained up to 60°C and reduced after that until 140°C. **Figure 4B** shows that the antimicrobial activity of the surface-active compound was high at acidic and extremely alkaline pH but was moderate at neutral and weakly alkaline pH (8 to 9). The influence of NaCl concentrations on the antimicrobial activity of the biosurfactant is presented in **Figure 4C** and reveals that the activity remained stable up to 10% NaCl concentration but gradually decreased at higher NaCl concentrations.

4.   Discussion

The results of this study are very emphatic on the influence of carbon substrates on the isolation of biosurfactant-producing bacteria as opposed to its influence on microbial selection for an industrial process. Our results show significant ($P < 0.05$) influence of substrates on isolation or distribution of biosurfactant-producing bacteria from/in the samples but non-significant ($P > 0.05$) influence of nature of samples on the same property. This infers that whatever the nature of sample, a tendency exists to isolate a biotechnologically-relevant biosurfactant-producing organism if the substrate in the screening medium appropriate for the organism. These results ratify the suspicions of Bodour *et al*. [13] that nature of carbon substrates is a major reason for the difficulty encountered in the many attempts at isolating biosurfactant-producing bacteria from samples.

The substrate-dependence of biosurfactant elaboration by bacteria in particular and microbes in general is borne out of the different physiological roles to which the producing organism puts the surface-active agent post-synthesis. It would be strange for a bacterium to elaborate biosurfactants for solubilization when a highly soluble substrate like glucose is the carbon source in the screening medium. If the organism did, then the biosurfactant would be for a purpose other than solubilization. The dominant selection of 29 (54.72%) biosurfactant-producing strains when waste frying sunflower oil served as carbon substrate in the screening medium suggests its better nutrient level and/or mix which directs biosurfactant synthesis in most of the bacteria. Successful production of biosurfactants in waste

frying oil has been covered in the review by Makkar and colleagues [34]. Only few reports exist in the use of waste frying oil for biosurfactant production and even fewer for isolation of biosurfactant-producing bacteria in nature.

True biosurfactant-producing bacteria were confirmed in our study from a combination of four qualitative tests in addition to the rapid drop collapse test. The ability of an organism to test positive for biosurfactant-production in one screening method but fail in another suggests diversity in physiological roles of biosurfactants and their very chemical nature. For instance, diffusion restraints in blood agar may not allow high molecular weight biosurfactants to be detected by blood cell hemolysis [20]. Mabrouk et al. [18] combined four different methods to be able to isolate the glycolipopeptide-producing Bacillus sp. E34. The need for multiple substrates for successful isolation of biosurfactant-producing bacteria underscores both the tedion involved in biosurfactant bio-prospecting and the unpredictability of appropriateness of selection protocols in industrial organism search.

The results of the quantitative screening assessed by the method of oil displacement present a rather interesting trend. The method was selected by reason of the significant ($P = 0.000$; $R^2 = 0.9997$) linear relationship between area of displaced oil and amount of surface-active compound demonstrated by sodium dodecyl sulphate (SDS). This strong relationship had earlier been reported by Satpute *et al*. [14]. Apart from ES2 isolated from Eketai sediment sample, the immediate 9 most efficient biosurfactant-producing bacteria were obtained from water or crude-oil samples suggesting the physiological need for bulk production of the surface-active agents in liquid media/systems which compensates naturally for the dilution effect of the liquid environment.

The best 4 biosurfactant-producing bacterial species namely *Pseudomonas aeruginosa* Strain IKW1, *Pseudomonas* sp. Strain OB6, *Pseudomonas fluorescens* Strain IFW3 and *Bacillus cereus* Strain R15B are not new to biosurfactant research and have been copiously documented [35]. The 100% sequence homology of Pseudomonas aeruginosa strain IKW1 and Bacillus cereus Strain R15B with Pseudomonas aeruginosa Strain HNYM41 and Bacillus cereus Strain F2 respectively, suggests that these strains are the same but there has been no report on the biosurfactant-producing potentials of these bacteria.

The effectiveness of a biosurfactant is given by its measure of surface/or interfacial tension reduction. Pseudomonas aeruginosa Strain IKW1 surface-active compound reduced the free surface enthalpy per unit

area of water from 72 dynes/cm to 24.62 dynes/cm thus capable of bringing 65.44% of hydrophobic substances into solution to form emulsion. The second best surface-active compound obtained from Bacillus cereus Strain R15B was able to reduce surface tension from 72 dynes/cm to 29.40 dynes/cm. The most powerful biosurfactant, surfactin, was reported to reduce the surface tension of water to 27.50 mN/m [7] thus, making the active compound from Pseudomonas aeruginosa Strain IKW1in this study a more effective biosurfactant.

The efficiency of a biosurfactant is, on the other hand, given by its critical micelle concentration; defined as the minimum concentration of the biosurfactant above which no further reduction in surface tension occurs and supramolecular structures like micelles and bi-layers form. In this study, the critical micelle concentration (cmc) of the surface-active compounds of Pseudomonas aeruginosa Strain IKW1 and Bacillus cereus Strain R15B were 21 and 12 mg/mL respectively. Different surface tension reduction and cmc values of biosurfactants have been reported by various authors and biosurfactants with surface tension reduction up to 35 dynes/cm and cmc between 1 and 150 mg/L are reported to be good biosurfactants [7]. This indicates that the best two bio-molecules in our study are effective and efficient and therefore suitable for their desired applications.

Different solvents and/or their mixtures have been adopted for biosurfactant isolation depending on the chemical nature of the biosurfactant. In this study, acidification, followed by solvent extraction with chloroform:methanol recovered 4.39 g/L of crude biosurfactant. The successful recovery of biosurfactants from cell-free fermentation broth by this method had earlier been reported by Onbasli and Aslim [36].

Biochemical characterizations, thin layer chromatography, high performance liquid chromatography and Fourier transform-infrared (FT-IR) spectrometry identified surface-active compound by Bacillus cereus strain R15B as a lipopeptide (data not shown) and that from Pseudomonas aeruginosa strain IKW1 as a polymeric compound composed of 40.36% carbohydrates, 20.16% proteins and 32.56% lipids. As far as we know, this is the third report on the ability of a strain of Pseudomonas aeruginosa to produce a glycolipopeptide (or peptidoglycolipid) biosurfactant. The first appearance of this report came from Koronelli et al. [37] while the second came from Ilori and Amund [38]. This therefore contradicts the speculation that Pseudomonas aeruginosa only produces rhamnolipid (glycolipid) type of biosurfactant. Two decades before the brilliant review of Desai and Banat [7] on biosurfactants, a strain of Pseudomonas aeruginosa [39] was reported to produce a protein-like activator PA for n-alkane oxidation. Thavasi et al. [21] recently isolated a strain of Pseudomonas aeruginosa from salt-water that produces a lipopeptide biosurfactant. The implication of these is that different strains of Pseudomonas aeruginosa obtained from different samples at different climatic and ecological conditions would produce surface-active compounds of differing chemical nature and composition to facilitate their competitiveness in those environments.

The commercial applications of biosurfactants are intrinsically related to their activities and a number of such activities were investigated in our study. The washing activity of the glycolipopeptide investigated by the sand pack method at different temperatures was targeted at demonstrating the oil recovery potential of the biosurfactant. The dual influences of holding time and temperature on the recovery rate of Nigerian medium crude oil at a constant biosurfactant concentration were significant (P < 0.05). Our results of 79.92% recovery of the oil at 70°C with the highest elution rate of 0.68 mL/min occurring at 90 min suggests sublime efficiency of the glycolipopeptide to recover crude oil from producing and abandoned oil reservoirs.

The ability of hydrophobic organic compounds to be solubilized and transported into the immediate vicinity of bacterial cells capable of metabolizing (degrading) them is potentially the rate-limiting step in bioremediation **[40].** The underpinning activities of microorganisms able to degrade hydrophobic organic compounds include solubilization; which makes the compound bio-available, and emulsification; which makes the compound bio-accessible, to the bio-remediating microorganisms. The solubilization ability of our study glycolipopeptide was excellent increasing with glycolipopeptide concentration and suggesting significant (P = 0.000) influence of biosurfactant on the amount of oil solubilized. The linear regression model used to fit the data revealed that 99.01% of the solubilized oil was due to the biosurfactant and that 0.99% of the dissolved oil would be due to other factors especially water and temperature. The glycolipopeptide in this study was able to emulsify kerosene, crude oil and palm oil to emulsification indices of 79.71%, 84.87% and 87.54% respectively. Our results are in agreement with those of a glycolipopeptide produced by *Bacillus* sp.E34 [18] with similar emulsification index of 77% in kerosene and 84.5% in crude oil. Our results of soil washing experiment as well as those of solubilization and emulsification indicate the potentials for application of our glycolipopeptide in enhanced oil recovery, remediation of environments polluted with petroleum and petroleum products as well as treatment of palm oil effluents.

The usefulness of biosurfactants with antimicrobial properties in bioremediation of hydrocarbon-impacted ecosystems has been a research question in our labouratory for some time. Inhibitory activity of our glycolipopeptide against *Bacillus subtilis* UCCM 0006; a most potent crude oil degrader in the University of Calabar Collection of Microorganisms (UCCM) goes to suggest the non-suitability of this surface-active compound for bioremediation when the major remediating organism is *Bacillus subtilis*. Interestingly, the biosurfactant was metabolized by *Serratia* sp. Strain DW2 (100% sequence homology with *Serratia* sp. Strain ZJ-I- JQ954966A) as sole source of carbon and energy and so demonstrated no antimicrobial activity against it. This indicates the biodegradability potential of the biosurfactant and suggests its suitability for applications in environments dominated by this bacterium. The antimicrobial potentials of our biosurfactant suggest the need for microbiological characterization of hydrocarbon-polluted sites before biosurfactant-enhanced remediation attempt.

A property that is immediately visible upon agitation of a biosurfactant solution is foaming and this is characteristic of all detergents. The glycolipopeptide under study was found to demonstrate excellent foaming power and therefore excellent detergency. The small densely-packed foam ensured strong inter-molecular forces responsible for the attraction of the foam molecules together ensuring good activity and stability; a property that facilitates emulsion formation and stabilization of the surface-active compound. The foam power and its stability for more than 48 h suggest suitability for applications in the detergent and food industries.

The oil displacement activity of a biosurfactant is a property that ensues as the contact angle at the oil-water interface is changed by a surface-active compound and varies directly with the amount of biosurfactant in the sample. The more the active compound at the oil-water interface the more its pressure; the more the pressure the lower the contact angle and the greater the solubilization of the hydrophobic component in the solvent. This activity is therefore a rapid indirect assessment of the solubilization potential of a biosurfactant and its stability to the influences of temperature, pH and NaCl is essential for oil recovery and bioremediation exercises. The oil displacement activity of the glycolipopeptide in this study was significantly stable to temperatures up to 80°C and pH from 7 to 9 but decreased linearly with increase in NaCl concentrations. These, in consonance with its oil-washing activity, suggest the suitability of the surface-active compound for biosurfactant-enhanced recovery of crude oil.

On the other hand, the emulsification activity of the glycolipopeptide increased with increase in temperature indicating the thermal stability of the bonds involved in the formation of the polymer. The emulsions formed at high temperatures, with kerosene as the hydrophobic compound, were stable for more than 4 months at the ambient conditions of our labouratory. The emulsifying ability of the glycolipopeptide was stable over a wide range of pH values (6-10 for kerosene and 7-10 for palm oil) but decreased gradually with increasing NaCl concentrations. Only 23.47% of the emulsifying activity of the biosurfactant was lost at 10% NaCl concentration which underpins the suitability of the compound for tertiary recovery of oil.

The antimicrobial activity of the glycolipopeptide was stable up to 60°C and decreased as temperature increased. The loss of antimicrobial activity at this temperature and the stability or improvement of oil displacement and emulsifying activities suggests the probable loss of the peptide moiety of the glycolipopeptide and its possible non-requirement for emulsifying activity. This is at variance with the hypothesis that protein moiety is responsible for the emulsifying activity of biosurfactants [41]. If this was so, then rhamnolipids would have no emulsifying activities. pH and NaCl significantly ($P < 0.05$) affected the antimicrobial property of the glycolipopeptide, nevertheless, the activity was stable at alkaline pH and NaCl concentrations below 10%. Acidic pH values have been shown to influence the electrostatic interactions between polar head groups of biosurfactant molecules **[42].** This suggests the applicability of the surface-active compound for antimicrobial coverage in preparations of food and pharmaceutical formulations where emulsion developments are desirable.

5.   Conclusion
Pseudomonas aeruginosa Strain IKW1 was isolated from the mesotidal waters of Ikang River, Niger Delta area, Nigeria and demonstrated commendable ability to elaborate a rare but effective and efficient surface-active compound identified as a glycolipopeptide. The active compound demonstrated excellent properties of oil washing, solubilization, emulsification, foaming and antimicrobial action. These activities were stable at moderately high temperatures, alkaline pH and NaCl concentrations below 10%. The bacterium is recommended for large-scale production of the biosurfactant on waste frying oil as a restaurant waste management option and for applications in tertiary oil recovery, bioremediation of hydrocarbon-impacted environments and the development of detergent, food and pharmaceutical preparations where emulsion development, stabilization and preservation are

desired.

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Table 1. Distribution of biosurfactant-producing bacteria in samples as assessed by the rapid-drop collapse test

S/N	Samples	nCAHB	gMSM	glMSM	rpeMSM	coMSM	wfoMSM	Total
1	DW	38	0	1	0	2	3	6
2	DS	67	0	0	1	5	2	8
3	FSW	43	0	0	0	0	1	1
4	FSS	38	0	0	2	0	3	5
5	EW	46	3	2	2	2	2	11
6	ES	63	4	2	4	1	2	13
7	MW	33	2	0	1	1	1	5
8	MS	45	1	1	2	0	2	6
9	SCW	36	0	0	3	2	5	10
10	SCS	39	1	1	2	1	2	7
11	IKW	65	0	0	1	3	1	5
12	IFW	96	0	1	0	4	1	6
13	GS-A	50	1	1	2	0	5	9
14	GS-B	69	3	0	0	0	0	3
15	WA	23	0	1	0	3	3	7
16	WB	29	0	0	0	2	6	8
17	LPS	32	1	3	1	4	1	10
18	R15	23	0	0	0	6	2	8
19	R4	16	1	0	0	0	6	7
20	COS	7	2	1	0	1	2	6
	TOTAL	858	19	14	21	37	50	141

nCAHB-number of culturable aerobic heterotrophic bacteria; R15-Reservoir 15; R4-Reservoir 4; gMSM-glucose + mineral salts medium; glMSM-glycerol + mineral salts medium; rpeMSM-rice processing effluent + mineral salts medium; coMSM-crude oil + mineral salts medium; wfoMSM-waste frying oil + mineral salts medium; DW-Douglas creek water; DS-Douglas creek sediment; FSW-Flare station water; FSS-Flare station sediment; EW-Eketai water; ES-Eketai sediment; MW-Mkpanak water; MS-Mkpanak sediment; SCW-Stubbs creek water; SCS-Stubbs creek sediment; IKW-Ikang water; IFW-Ifondo water; GSA-Garden soil A; GSB-Garden soil B; WA-Mechanic workshop soil A; WB-Mechanic workshop soil B; LPS-Lubricating oil polluted soil; R15-Reservoir 15 oil formation water; R4-Reservoir 4 oil formation water; COS-crude oil sample

A *Pseudomonas aeruginosa* Strain Ikw1 Produces an Unusual Polymeric Surface-Active Compound in Waste Frying Oil-Minimal Medium

Table 2. Quantitative screening of biosurfactant-producing bacteria from samples as assessed by the oil displacement assay

Water		Sediment		Soil	
Isolate	Amount (cm^2)	Isolate	Amount (cm^2)	Isolate	Amount (cm^2)
IKW1	66.5 ± 1.8	ES2	31.2 ± 0.6	WA3	24.6 ± 1.1
IFW3	55.3 ± 1.2	SS1	17.3 ± 0.7	LPS1	23.7 ± 0.6
OB6[a]	52.8 ± 1.2	MS2	17.3 ± 1.2	WA4	22.0 ± 0.5
R15B	50.3 ± 2.2	DS2	15.2 ± 1.3	WB1	21.2 ± 1.1
DW2	40.2 ± 0.7	ES4	13.8 ± 0.7	LPS2	21.2 ± 0.8
EW2	38.1 ± 0.5	SS2	13.8 ± 0.2	WA6	18.1 ± 0.9
SW1	32.0 ± 0.4	MS5	10.7 ± 0.9		
R15A	30.2 ± 0.6				
OB3[a]	28.9 ± 1.3				
MW2	21.2 ± 0.3				
OB1[a]	16.6 ± 0.4				
EW5	16.6 ± 1.2				
DW1	13.2 ± 1.2				
EW3	11.9 ± 0.8				
EW6	11.3 ± 1.5				

DW-Douglas creek water; DS-Douglas creek sediment; EW-Eketai water; ES-Eketai sediment; IKW-Ikang water; IKS-Ikang sediment; MW-Mkpanak water; MS-Mkpanak sediment; OB-Crude oil bacteria; R15-Reservoir 15 water; SW-Stubbs creek water; SS-Stubbs creek sediment; WA-mechanic workshop soil A; WB-Mechanic workshop soil B; WC-Mechanic workshop soil C; LPS-Lubricating-oil polluted soil, [a]-crude oil sample bacteria. Values are means of triplicate determinations ± standard deviation.

Table 3. Identities of selected biosurfactant-producing bacteria

S/N	Isolate code	Gram reaction	Identity	Close relative with % similarity and accession number	Isolating carbon source
1	DW2	-	*Serratia* sp.	100% *Serratia sp.* ZJ-I (JQ954966A)	Crude oil and waste frying oil
2	IKW1	-	*Pseudomonas aeruginosa*	100% *Pseudomonas aeruginosa* strain HNYM41 (JN999891A)	Glycerol, crude oil and waste frying oil
3	IFW3	-	*Pseudomonas flourescens*.	96% *Pseudomonas fluorecens*-25[b]	Crude oil and waste frying oil
4	OB6	-	*Pseudomonas* sp.	100% *Pseudomonas* sp. RPT-52 (JF756593A)	Glycerol, rice processing effluent and waste frying oil
5	R15B	+	*Bacillus cereus*	100% *Bacillus cereus* strain F2 (JQ579629A)	Glucose, crude oil and waste frying oil
6	LPS1	+	*Bacillus* sp.	99% *Bacillus* sp. ABO1 (JX094300A)	Glycerol, crude oil and waste frying oil

DW-Douglas creek water; IKW-Ikang water; IFW3-Ifondo water; OB-Crude oil bacteria; R15-Reservoir 15 water; LPS-Lubricating-oil polluted soil. [b] isolate identified with Microgen ID kit, incomplete 16S rRNA sequencing, accession number not yet obtained.
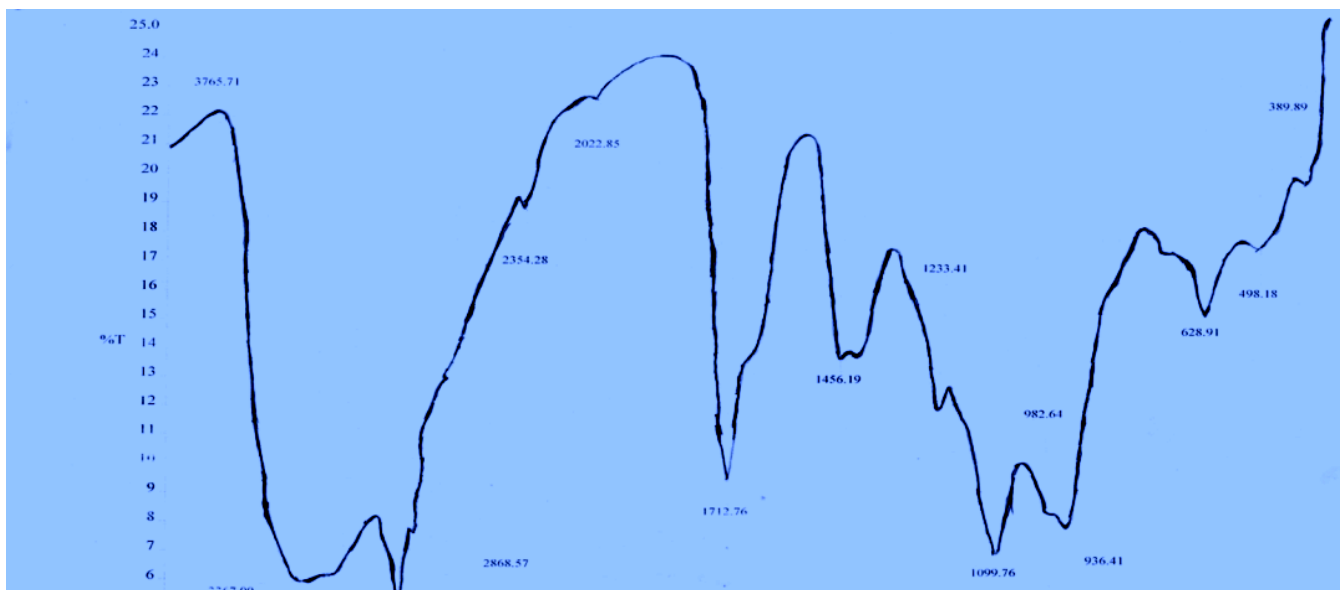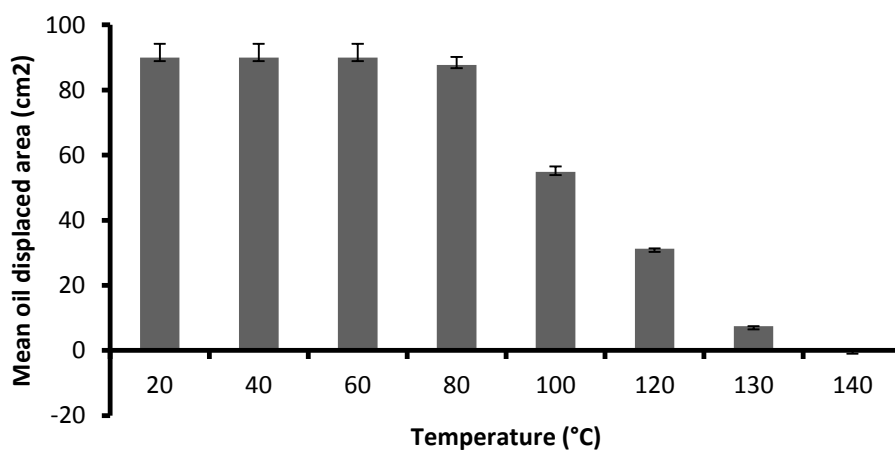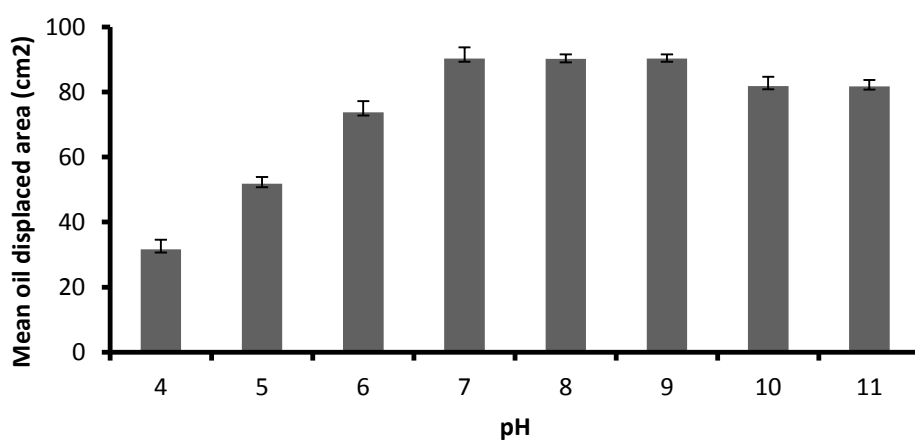
Figure 1. Spectrum of *Pseudomonas aeruginosa* strain IKW1 glycolipopeptide biosurfactant from Fourier-transform infrared (FT-IR) spectrometry obtained by plotting % transmittance (y-axis) against wave numbers in cm^{-1} (x-axis)
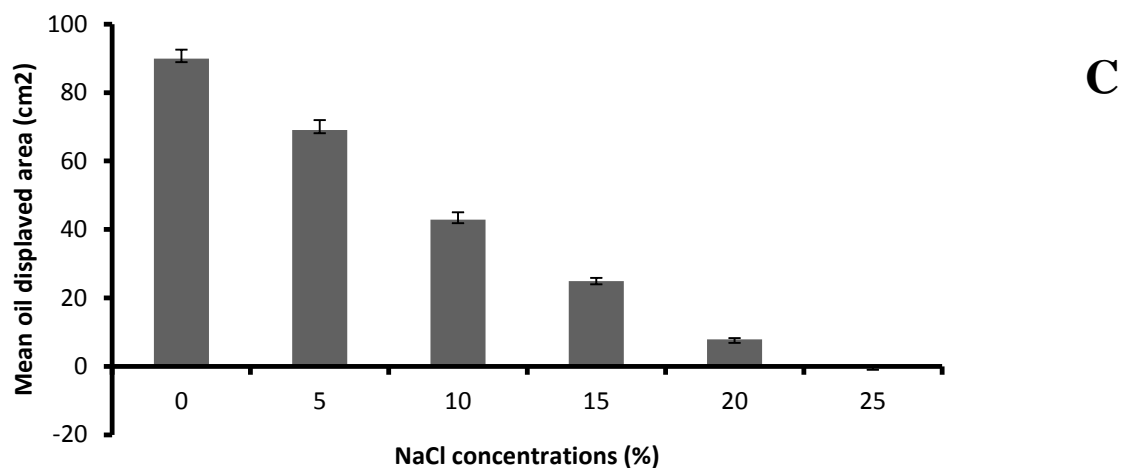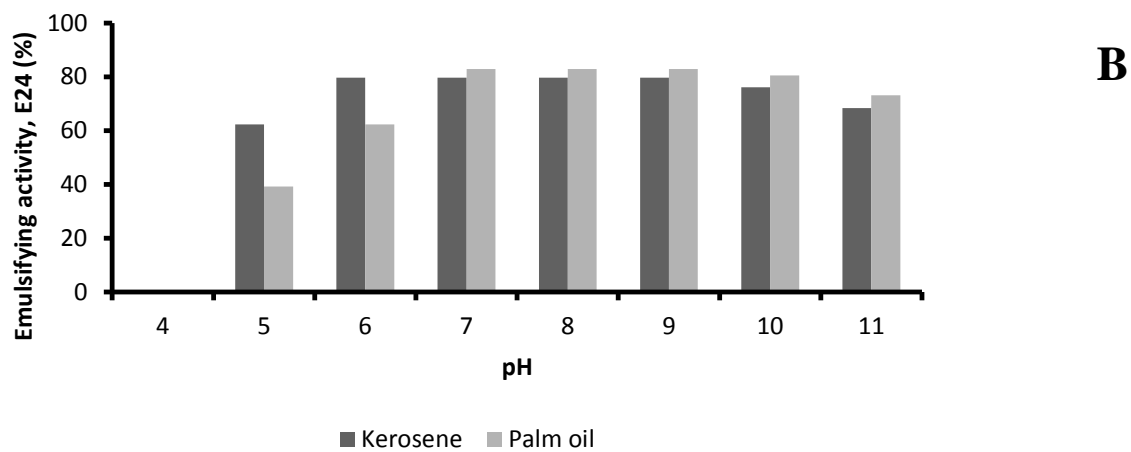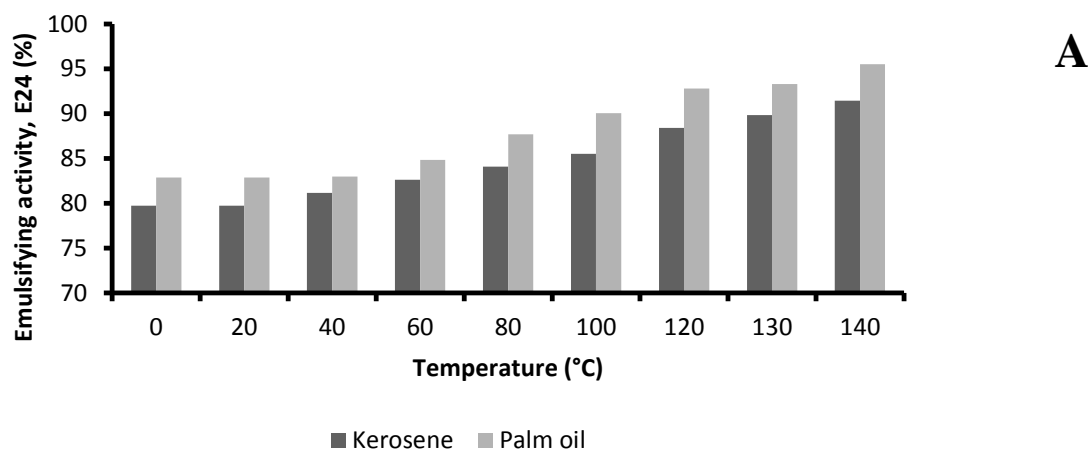

A

B

Figure 2. Stability of glycolipopeptide oil displacement activity to (A), temperature (B) pH (C) NaCl
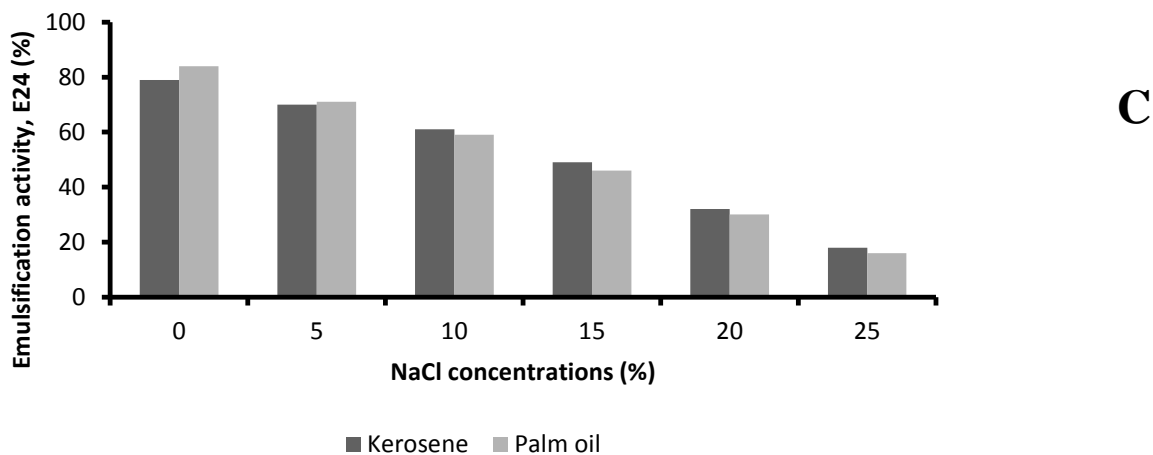
C

Figure 3. Stability of glycolipopeptide emulsifying activity to (A) Temperature (B) pH (C) NaCl

$y = 0.0107x + 4.9293$
$R^2 = 0.9018$

A

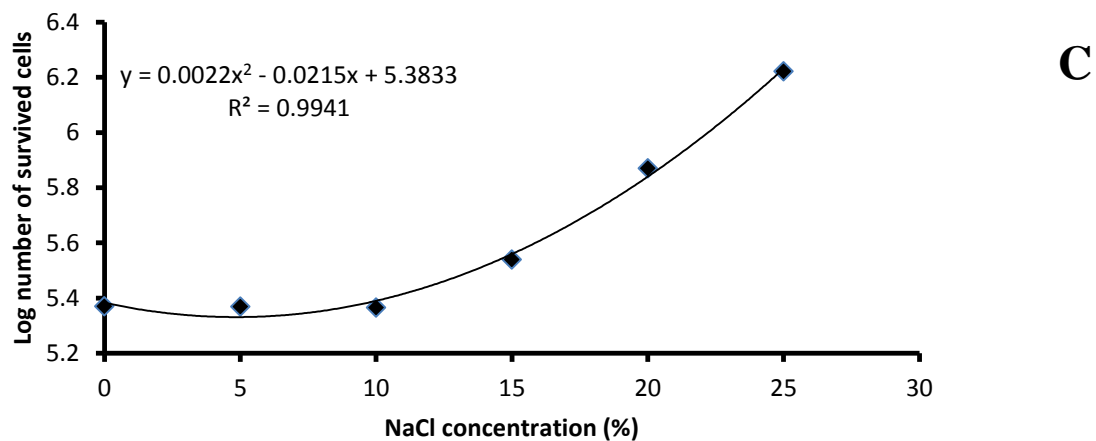$y = -0.256x^2 + 4.0678x - 9.542$
$R^2 = 0.9754$

B
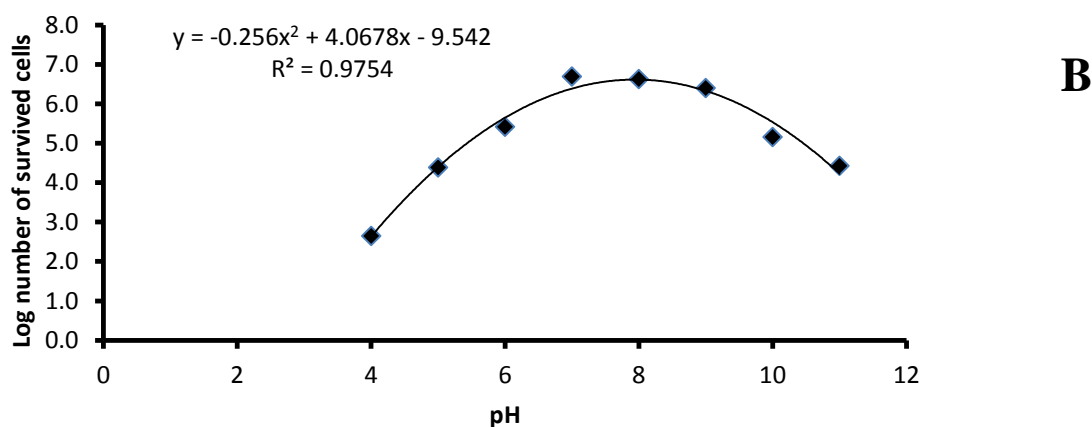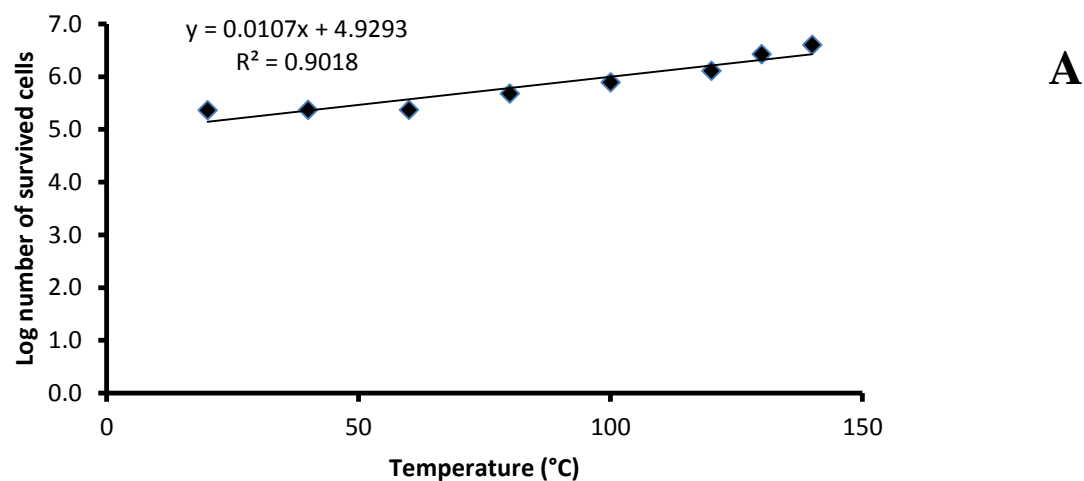
Figure 4. Stability of glycolipopeptide antibacterial activity to (A) temperature (B) pH (C) NaCl