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Application of biosurfactants and pulsating electrode configurations for enhanced electrokinetic remediation of petrochemical contaminated soil

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Section S1: Methods and Materials

Chemicals and reagents. Most of the chemicals used where obtained from Merk, Germany. The mineral salt medium (MSM) sterilized by autoclaving at 121 °C for 15 min was used for the growth and production of biosurfactants. The medium was prepared as was reported by Trummler *et al.* [1] by dissolving in 1 L of distilled water: 6.0 g $(NH_4)_2SO_4$; 0.4 g $MgSO_4 \times 7H_2O$; 0.4 g $CaCl_2 \times 2H_2O$; 7.59 g $Na_2HPO_4 \times 2H_2O$; 4.43 g KH_2PO_4; and 2 mL of trace element solution. Plate count agar, nutrient agar and nutrient broth were prepared by dissolving the amounts indicated on the bottle in distilled water followed by autoclaving at 121 °C in order to sterilize for 15 min. The agar was poured on to the agar plates between 40-50 °C. The trace elements solution consisted of, 20.1 g L^{-1} EDTA (Disodium salt), 16 g L^{-1} $FeCl_3 \times 6H_2O$, 0.18 g L^{-1} $CoCl_2 \times 6H_2O$, 0.18 g L^{-1} $ZnSO_4 \times 7H_2O$, 0.16 g L^{-1} $CuSO_4 \times 5H_2O$ and 0.10 g L^{-1} $MnSO_4 \times H_2O$.

Microbial culture, media and growth conditions. Strain PA1 was obtained from an API tank sludge in South Africa by Selective enrichment to obtain efficient hydrocarbon degraders in 250 mL Erlenmeyer flasks according to Trummler *et al.* [1]. The enrichment was done by inoculating 5 g of sludge into 100 mL of MSM enhanced with 5% (v/v) sunflower oil as a carbon source and energy source at 30 °C and 120 rpm for 7 d. A total of 6 subsequent enrichments was done to isolate the petroleum hydrocarbon degrading consortium by transferring 10 mL of enriched culture into another flask containing 100 mL of freshly sterilized MSM with 5% (v/v) sunflower oil and incubated. The isolation and purification of the consortium was done by spreading and streaking on Luria-Bertani agar. 100 µL of culture dilutions were spread on the LB agar plates and incubated at 37 °C for 72 h. The isolates were later subjected to 16S rRNA gene sequencing analysis basing on colony morphology. Strain PA1 was selected for use in this research due to its effective biosurfactant production capabilities.

Characterization and Identification of Microbial species. Pure cultures of biosurfactant producing isolates were characterized using the 16S rRNA genotype fingerprinting method. This was achieved by extracting the DNA from the pure cultures according to the protocol described in the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA). The 16S rDNA region was amplified by PCR using the primers 8F and 907R. The amplified genome was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Amplifications were performed in a GeneAmp PCR System 9700-Thermocycler from PE Applied Biosystems. PCR products were analysed together with a molecular weight ladder. The DNA sequence for each pure colony was then uploaded to the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed from the identified 16S rRNA sequences using the neighbor-joining method in the MEGA Version 6.

Biosurfactant production, recovery and purification. To produce biosurfactant, a pure strain of PA1 was inoculated in Erlenmeyer flasks containing 200 mL of sterilised nutrient broth in a sterile environment. The flask was then incubated at 35 °C, pH = 7 and 250 rpm for 48 h. The cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 minutes. The cells were then transferred to larger erlenmeyer flasks containing 1,000 mL of mineral salt medium supplemented with 3% oil (v/v)

and incubated at 35 °C, pH = 7 and 250 rpm for 2 weeks. To obtain a cell free supernatant after growth for two weeks the pH was adjusted to 7, cells were then removed by centrifugation (12,000 rpm at 4 °C for 20 min). Crude biosurfactant was precipitated from the supernatant by adding 6 N HCl to pH of 2.0. The acid precipitate was recovered by centrifugation (12,000 rpm at 4 °C for 20 min). The biosurfactant was further extracted with chloroform and methanol (2:1) followed by evaporation of the solvent in a vacuum. The obtained residue was dissolved in methanol and filtered through a 0.22 mm filter (Millipore). The crude extracts were purified through a silica gel column (silica gel 60 (63–200 mesh); Merck KGaA). The impurities were further removed from the extract by eluting with chloroform, and twice using chloroform and methanol in 80:20 v/v (100 mL), then 35:65 v/v (100 mL) respectively. The solvents were finally evaporated from the eluted extract at 40 °C. This was all done according to Bezza and Chirwa [2].

Screening for biosurfactant production. The isolated cultures were screened for biosurfactant production using the drop collapse method and the oil spreading test. In the drop collapse method, 2 L of mineral oil was added to each well of a 96-well micro titer plate. The plate was equilibrated for 1 h at room temperature, and then 5 µl of the culture was added to the surface of oil [3]. The shape of the drop on the surface of oil was inspected after 1 min. The result was negative If the drop remained beaded while the result was positive If the drop collapsed. Cultures were tested in triplicate. Oil spreading test was done as described by Morikawa *et al*. [4] in which 50 mL of distilled water was added to a large petri dish (25 cm diameter) followed by the addition of 20 µl of oil to the surface of the water. 10 µl of culture were then added to the surface of oil. The diameter of the clear zone on the oil surface was measured and related to the concentration of biosurfactant. Mineral salt medium and distilled water without cells were used as controls for both screening tests.

Biosurfactant characterization. *Thin Layer Chromatography (TLC).* 10 mg of the extract dissolved in methanol was applied near the bottom edge of the TLC plates in small spots. Biosurfactants were characterized by thin layer chromatography on silica gel 60 plates (F254; Merck). *Chromatograms.* The plates were developed with chloroform: methanol: water (65:15:4, v/v) as the solvent system. Spots were revealed by spraying with 0.35% (w/v, in acetone) ninhydrin for detection of compounds with free amino groups. The reagents were sprayed, and the plates were heated at 110 °C for 5 min until the appearance of the respective colors [2].

Fourier transform infrared spectroscopy (FTIR). To identify the chemical bonds and the functional groups present in the chemical structures the Perkin Elmer 1600 Fourier Transform Infra-Red (FTIR) spectroscopy equipped with an Attenuated Total Reflectance (ATR) Crystal Accessory (Perkin Elmer, Connecticut, USA) was used. The sample was prepared by mixing 1 mg of crude biosurfactant with 100 mg of KBr and pressed with a load for 30 s, to obtain translucent pellets. The IR scan was performed over 400-4000 cm^{-1} with a resolution of 2 cm. The reflectance spectra were recorded and averaged over 32 scans, using the total internal reflectance configuration with a Harrick™ MVP-PRO cell consisting of a diamond crystal. Spectra were viewed and analyzed by Spectrum 10™ Software (Perkin Elmer) [2].

Determination of surface tension and critical micelle dilution (CMD). The surface tension of the biosurfactant supernatant was determined using the KrüssTensiometer (K11 model – Germany) equipped with a 1.9 cm platinum ring based on the Du Nouy ring method [5]. The measurements were done at room temperature in triplicates to present values as derivatives of averages of independent measurements. Surface tensions of diluted biosurfactants (10 different dilutions) were determined to express the concentrations of the biosurfactants in critical micelle dilution. The dilutions were made using a phosphate buffer solution (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) followed by surface tension measurements as described above.

Evaluation of demulsification potential of the biosurfactants. W/O model emulsions were prepared according to the following protocol [6]. The ME (W/O) were prepared by mixing 0.2% Tween 80 and either kerosene, hexane or toluene at 1:2 (v/v) at 24,000 rpm for 2 min in a Turrax-type agitator (Marconi) to produce three different emulsions. O/W emulsions of Tween-Triton-kerosene

were obtained by producing a stock solution of kerosene prepared by mixing 2.5 mL of triton with 100 mL kerosene on a stir plate, and a stock solution of Tween-80–water was prepared by adding 200 μL of tween in 100 mL de-ionized water. Tween-Triton-kerosene was finally obtained by mixing 0.9 mL Tween-80–water and 2.1 mL triton-kerosene solutions at 24,000 rpm for 2 min. The emulsion type was then verified by the Oil Red O-test [6]. Demulsifcation evaluations were done by adding 300 μL of biosurfactant supernatant in tubes of known dimensions containing 2 mL of emulsion and sealed. This was followed by agitation in a vortex for 2 min at 13,800 rpm to achieve complete mixing. The tubes were kept undisturbed in an upright position in a water bath at 35 ^{o}C. The changes in the volume of the oil phase (top), water phase (bottom), and emulsion phase (in-between) were recorded at 24-hour time intervals for up to 5 d. Demulsification of emulsions was based on the volume of separated emulsion compared to the original volume (EV) and expressed as the demulsification percentage [6] % Demulsification = [(initial volume (2 mL) - final emulsion volume at interphase (mL))/initial volume (mL) × 100]. All experiments were carried out in triplicates.

Section S2: Evaluation of the Demulsification potential of the biosurfactants in O/W and W/O emulsions

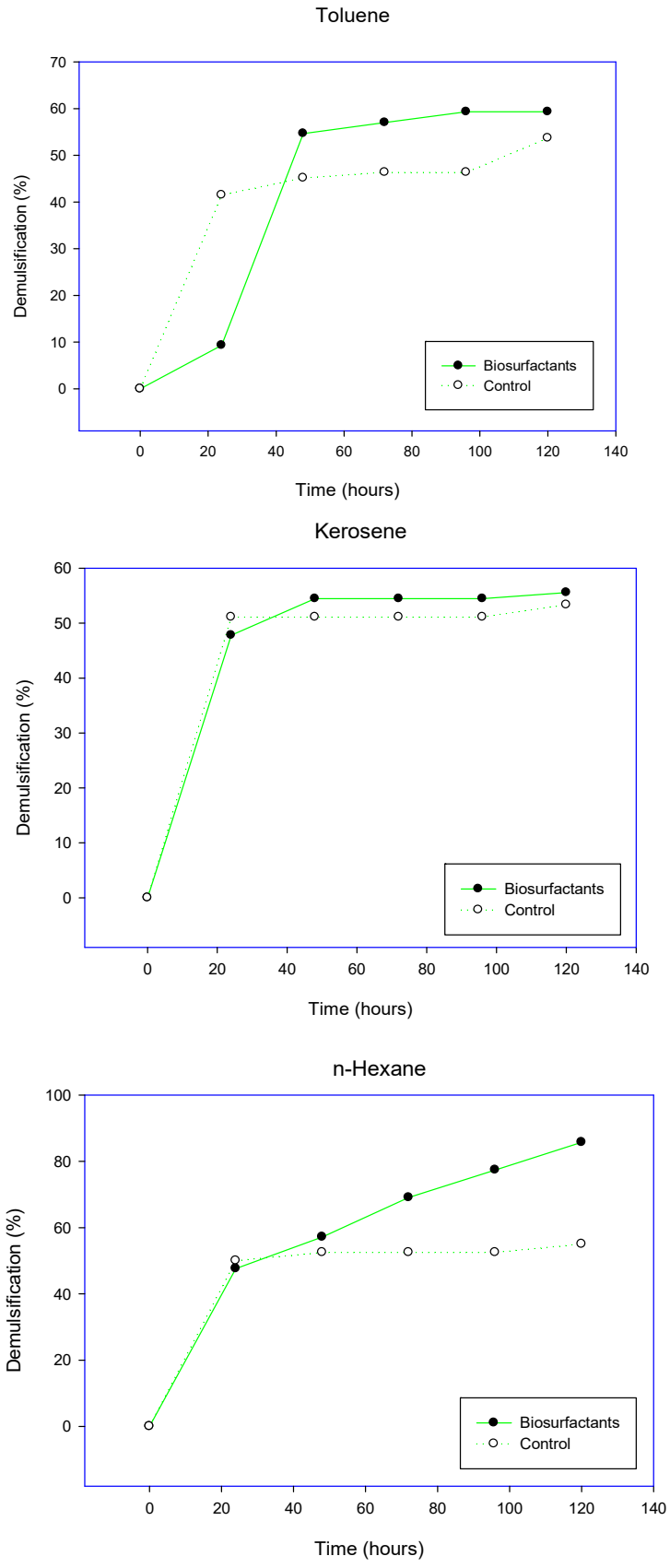

Figure S1. Demulsification effect of the biosurfactant supernatant on W/O emulsions of Toluene, Kerosene and n-Hexane.

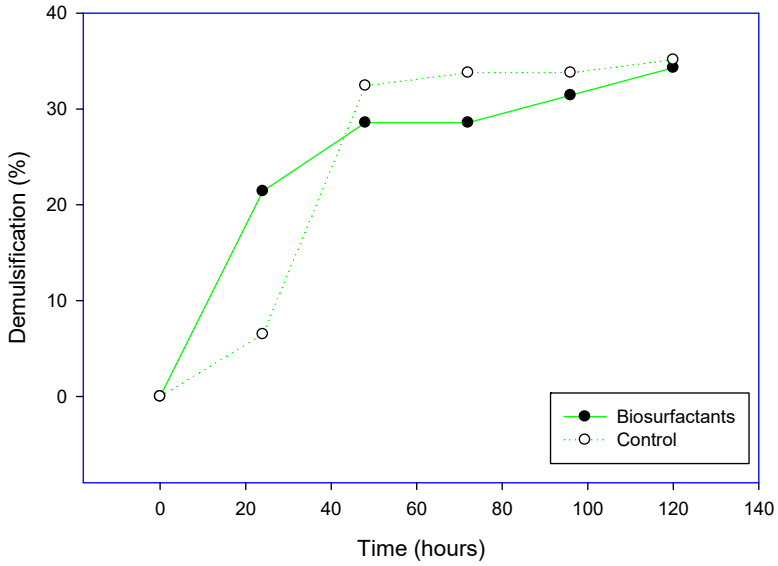

Figure S2: Demulsification effect of the biosurfactant supernatant on O/W emulsions of Tween-Triton-Kerosene

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