Cleaner Engineering and Technology 9 (2022) 100521

Contents lists available at ScienceDirect

Cleaner Engineering and Technology

journal homepage: www.sciencedirect.com/journal/cleaner-engineering-and-technology

Evaluation of the toxicity of a rhamnolipid biosurfactant for its application in the optimization of the bio-electrokinetic remediation of petrochemical contaminated soil

Brian Gidudu *, Evans M.N. Chirwa

Water Utilization and Environmental Engineering Division, Department of Chemical Engineering, University of Pretoria, Pretoria, 0002, South Africa

ARTICLE INFO

Keywords:
Remediation
Biosurfactant
Rhamnolipid
Toxicity
Electrokinetic

ABSTRACT

The combination of bioremediation, biosurfactants and electrokinetic remediation as a hybrid system was evaluated for the possibility of enhancing the removal of petrochemical hydrocarbons from contaminated soil as a sustainable and effective replacement for ineffective conventional remediation methods in an optimized manner. To begin with, the toxicity of the biosurfactant was determined by examining the effect of the biosurfactant on plant growth and microbial growth. The independent effect of biosurfactant concentration, voltage, the distance between electrodes and multifactor interactions on the bio-electrokinetic remediation of petrochemical contaminated soil using a bench-scale electrokinetic system were then evaluated. The results revealed that the rhamnolipid biosurfactant did not have any observable inhibition effects on the growth of the test bacteria in 5 days since the growth under test solutions of 0 mg/L, 10 mg/L, 50 mg/L and 100 mg/L did not have any significant differences. On the other hand, the biosurfactant affected root elongation and germination of the three test vegetables. Still, SDS had more substantial adverse effects represented by extremely low values of germination index as compared to the biosurfactant. The bio-electrokinetic remediation of soil revealed that a combination of the highest voltage of 30 V, the lowest electrode spacing of 185 mm and the highest biosurfactant concentration of 84 g/L had the highest microbial growth of 11.52 CFU/mL and hydrocarbon removal of 92% as compared to other configuration combinations. Microbial growth and hydrocarbon removal were mainly affected by voltage and biosurfactant concentration as the independent variables.

1. Introduction

Surfactants are classified as surface-active and emulsifying agents with the ability to reduce interfacial and surface tension between solids, liquids, and gases allowing them to readily disperse or mix as emulsions (Singh and Cameotra, 2004). Besides offering the ability of organic pollutants to be removed from contaminated media in a fast and cost-effective manner, surfactants also provide the capacity for a large volume of contaminated matrices to be treated (Boulakradeche et al., 2015). Brij 35, Igepal CA-720, Sodium dodecyl sulfate (SDS), Tween 80 and Tergitol are some of the most widely used synthetic surfactants (Cameselle et al., 2013). However, most of these surfactants are derivatives of petroleum (Sarubbo et al., 2013). Sodium dodecyl sulfate (SDS) is a typical anionic surfactant with hydrophilic and hydrophobic parts (Sarubbo et al., 2013). Because of its properties, SDS is widely used in decontamination, foaming processes, emulsification of immiscible substances, and protein denaturant in physiological/biochemical experiments (Liu and Wu, 2018). Unfortunately, the chemical and synthetic components of chemical surfactants have rendered them environmentally unfriendly because of their toxicity and resistance to biodegradation (Gudina et al., 2015; Mulligan et al., 2001). Several studies have shown that SDS has toxic effects on organisms such as bacteria, microalgae, fish, and plants from terrestrial and aquatic environments (Liu and Wu, 2018; Sharma et al., 2014).

On the other hand, biosurfactants have been rendered a better substitution for synthetic surfactants because they can be synthesized from several carbons and have greater environmental compatibility, high biodegradability, high foaming capacity and high selectivity (Abalos et al., 2004; Wang et al., 2007). They are also able to function at extreme temperature, pH and salinity (Abalos et al., 2004; Wang et al., 2007). Much as most biosurfactants have been reported to have low toxicity, some studies have reported that some biosurfactants exhibit strong

* Corresponding author.
E-mail address: briangid38@gmail.com (B. Gidudu).

https://doi.org/10.1016/j.clet.2022.100521
Received 15 July 2021; Received in revised form 5 May 2022; Accepted 9 June 2022
Available online 13 June 2022

antimicrobial activity (Sarubbo et al., 2013). But in general, very little is known about the toxicity of biosurfactants to plants and microorganisms in the environment (Lima et al., 2011; Santos et al., 2017; Sharma et al., 2014).

Due to the extensive use of petrochemicals globally, regular contamination of soil by petrochemicals as a result of poor disposal or accidental spillages is commonly reported (Ossai et al., 2020). The carcinogenic, mutagenic and toxic properties of petrochemical constituents such as phenols, petrochemical hydrocarbons, xylene, benzene, and alkanes require their immediate removal from the environment (Caravaca and Roldán, 2003; Souza et al., 2014). In the containment of petrochemical spillages and remediation of petrochemical contaminated media, conventional methods of remediation such as bioremediation, biostimulation, oil containment, oil isolation, bioventing, chemical treatment, and thermal treatment have been reported to be ineffective (Gidudu and Chirwa, 2020c; Karthick et al., 2019; Ossai et al., 2020). Long periods of treatment, alteration of soil properties, the need for the use of auxiliary chemicals, and the failure to eliminate the contaminant rather than contain it are some of the issues associated with these conventional methods (Gidudu and Chirwa, 2020c; Karthick et al., 2019; Ossai et al., 2020).

In the previous years, Electrochemical methods have attracted a lot of attention because of their potential to remove organic compounds from soil (Ammami et al., 2015; Gidudu and Chirwa, 2020c). Unlike conventional remediation methods, electrokinetic remediation hardly produces post-treatment waste, has minimal soil disruption and can effectively remove contaminants in low hydraulic conductivity zones and heterogenous fine-grained contaminated soils (Alshawabkeh, 2009; Ammami et al., 2015; Ossai et al., 2020). Advanced studies have suggested that the combination of bioremediation and electrokinetic remediation is most likely to produce a clean, sustainable and efficient method of remediation (Wu et al., 2020). The combination of electrokinetic remediation and bioremediation may lead to the stimulation of microbial growth by electrochemical oxidation, which together with electrophoresis, electroosmosis, electromigration, and electro-demulsification may lead to the efficient removal of pollutants from contaminated media especially in the initial stages of the process (Gidudu and Chirwa, 2020c; Wu et al., 2020). But prolonged treatment using electrokinetic remediation has been reported to be ineffective as current intensity, electroosmotic flow, electrophoresis, and electromigration decrease (Wu et al., 2020). The combination of bio-electrokinetic remediation with biosurfactants may further improve the contaminant removal process by increasing solubility of the contaminants, improving the bioavailability of recalcitrant hydrocarbons to the microbes, improving the demulsification of oil-solid-water emulsions, and increasing the adsorption of the contaminants by reduction of surface tension (Batista et al., 2006; Gomes et al., 2012). But the effect of voltage, the distance between electrodes, biosurfactant concentration and multifactor interactions on the bio-electrokinetic remediation of contaminated soil is unknown much as the optimizing the bio-electrokinetic remediation process should be based on the independent effects of these factors and their interactions (Alshawabkeh, 2009; Mena et al., 2016; Tang et al., 2018).

Therefore, the purpose of this study is to evaluate the toxicity of a bacterial biosurfactant and determine the independent effect of voltage, biosurfactant concentration, the distance between electrodes, and the interactions between these factors on the hybrid bio-electrokinetic remediation of petrochemical contaminated soil. In previous work done by the same research group, the studies focused on the preliminary studies aimed at the possibility of combining bioremediation, electrokinetic remediation and biosurfactants without evaluating the toxicity of the biosurfactant and the influence of each of the factors on the hybrid system (Gidudu and Chirwa, 2020b, 2020c). In this study, we focused on evaluating the toxicity of the biosurfactant and identifying the most significant factors that should be prioritized in the optimization of the combined process of remediation. To meet the objectives of the study, the toxicity of the biosurfactant to plants and bacteria was evaluated and compared to the toxicity of SDS. The response surface methodology (RSM) in the design of experiment (DoE) was then used to generate an experimental design for the biosurfactant enhanced removal of hydrocarbons from petrochemical contaminated soil. Experimental data was used for optimization purposes and to discover the effects of each of the independent variables and their interactions on the response variables.

2. Materials and methods

2.1. Microbiological culture, growth medium and biosurfactant production

The production of the biosurfactant was done using a great biosurfactant producing strain (*Pseudomonas aeruginosa* PA1) already identified in our previous studies (Gidudu and Chirwa, 2020a). The growth medium was composed of 2 mL of trace elements and 4.43 g KH_2PO_4; 7.59 g $Na_2HPO_4 \times 2H_2O$; 0.4 g $MgSO_4 \times 7H_2O$; 6.0 g $(NH_4)_2SO_4$; 0.4 g $CaCl_2 \times 2H_2O$ dissolved in 1 L of type II distilled water (Trummler et al., 2003). The solution of trace elements was composed of 0.18 g L^{-1} $ZnSO_4 \times 7H_2O$, 0.10 g L^{-1} $MnSO_4 \times H_2O$, 0.16 g L^{-1} $CuSO_4 \times 5H_2O$, 0.18 g L^{-1} $CoCl_2 \times 6H_2O$ and 16 g L^{-1} $FeCl_3 \times 6H_2O$, 20.1 g L^{-1} EDTA (Trummler et al., 2003). The mineral salt growth medium (MSM) was always autoclaved at 121 °C for 15 min before use.

Biosurfactant production started with the inoculation of a pure strain of *P. aeruginosa* in 200 mL of sterile nutrient broth contained in an Erlenmeyer flask. The cells were left to grow for 24 h at 250 rpm, temperature of 35 °C, and pH of 7. The cells were then harvested for 10 min at 4 °C and 10,000 rpm. For massive production of the biosurfactant, the harvested cells were moved to 1000 mL of MSM supplemented with 3% sunflower oil (v/v) in 3 L Erlenmeyer flasks. The flasks were incubated for 96 h at 35 °C, 250 rpm and pH of 7.

2.2. Biosurfactant recovery and purification

The biosurfactant was recovered by acid precipitation using aliquots of the supernatant according to Noparat et al. (2014). This was done by removing the cells from the broth by centrifugation for 20 min at 12,000 rpm and 4 °C. The biosurfactant precipitate was obtained by adding 6 N HCl to adjust the pH to 2. This was followed by centrifugation for 20 min at 12,000 rpm and 4 °C. The biosurfactant was extracted by adding chloroform and methanol (2:1) to the extract and left in the vacuum for the solvents to evaporate. The residue left after evaporation was dissolved in methanol and filtered through a filter (0.22 mm, Millipore). The crude biosurfactant obtained was purified through a column of silica gel to remove impurities. The crude biosurfactant was eluted through methanol and chloroform in 20:80 v/v, then 65:35 v/v to remove the remaining impurities. The biosurfactant was now ready for analysis.

2.3. Identification of the biosurfactant using ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

The analysis was done using an Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometer at the LC-MS Synapt Facility of the Department of Biochemistry at the University of Pretoria. The UPLC was calibrated using sodium formate clusters in a mass range of 100–3000 Da in ESI mode and to obtain ions in negative and positive mode. The instrument was configured to collect high energy (ramp: 20–40 V) for structure elucidation and low energy precursor (4 V) product spectra by operating the instrument in MS^E mode. The spectrometry was done by injecting 5 μL of the analyte into a Waters C_{18} BEH 1.7 μm (2.1 × 100 mm) column together with water and acetonitrile containing 0.1% formic acid. Acetonitrile and water were used as the mobile phase run with a 20 min gradient at a flow rate of 0.4 μL/min. The 20 min gradient of acetonitrile: water started with a run time of 5 min for a volume of 30% v/v followed up with 8 min for 30–100% v/v, 2

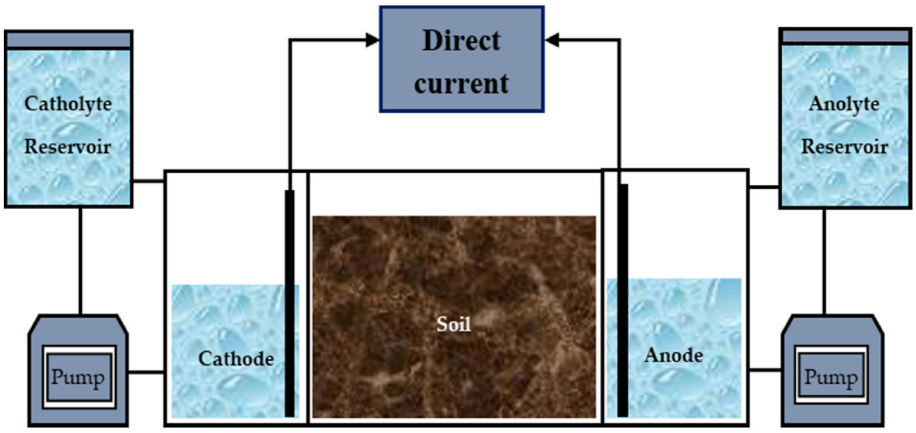

Fig. 1. Illustrative view of the electrokinetic reactor.

min for 100% v/v, 1 min for 100–30% v/v and 4 min for 30% v/v. At a constant flow rate of 5 mL/min, the solution of leucine enkephalin (2 ng μL) was used as the lock mass. The ion modes were obtained at a capillary voltage of 2.8 KV, source temperature of 100 °C, cone voltage of 30 V with can gas of 100 L/h, the scan time of 0.5 s, and desolvation temperature of 300 °C with desolvation gas of 500 L/h.

2.4. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in a static test. The static test was based on root elongation and seed germination of *Pisum sativum*, *Phaseolus vulgaris* and *Brassica napus* var. *napus* following the method described by Santos et al. (2017). Toxicity was determined in sterilized Petri dishes (1 × 10 cm) containing cotton wool. Six seeds were inoculated in each Petri dish with 5 mL of the test solution at 0 mg/L (control), 10 mg/L, 50 mg/L and 100 mg/L of either the biosurfactant or SDS at 27 °C. Seed germination and root elongation (≥5 mm) were determined after 5 days of incubation in the dark. Relative seed germination, relative root length and germination index were then determined as seen below;

Table 1
Components of the rhamnolipid biosurfactant.

[M+H] (m/z)	Rhamnolipid components	Molecular formula	Mass defect (±)
Mono-rhamno-mono-lipidic congeners			
302.158776	Rha-$C_{8:2}$	$C_{14}H_{22}O_7$	−0.0110
334.227676	Rha-C_{10}	$C_{16}H_{30}O_7$	−0.0285
358.22756	Rha-$C_{12:2}$	$C_{18}H_{30}O_7$	−0.0284
386.296976	Rha-$C_{14:2}$	$C_{20}H_{34}O_7$	−0.0666
Mono-rhamno-di-lipidic congeners			
502.6653	Rha-C_{10}-$C_{10:1}$	$C_{26}H_{46}O_9$	−0.3511
518.377276	Rha-C_{10}-C_{10}-CH_3	$C_{27}H_{50}O_9$	−0.0318
532.42746	Rha-C_{10}-C_{12} or Rha-C_{12}-C_{10}	$C_{28}H_{52}O_9$	−0.27954
Di-rhamno-di-lipidic congeners			
650.571776	Rha-Rha-C_{10}-C_{10}	$C_{32}H_{58}O_{13}$	−0.1840
664.465176	Rha-Rha-C_{10}-C_{10}-CH_3	$C_{33}H_{60}O_{13}$	−0.0618
678.488776	Rha-Rha-C_{10}-C_{12} or Rha-Rha-C_{12}-C_{10}	$C_{34}H_{62}O_{13}$	−0.0697
735.5540	Rha-Rha-C_{12}-C_{14} or Rha-Rha-C_{14}-C_{12}	$C_{38}H_{70}O_{13}$	0.03937

$$\text{Relative seed germination } (\%) = \frac{\text{number of seeds germinated in the extract}}{\text{number of seeds germinated in the control}} \times 100\% \quad (1)$$

$$\text{Relative root length}(\%) = \frac{\text{mean root length in the extract}}{\text{mean root length in the control}} \times 100 \quad (2)$$

$$\text{Germination index} = \frac{\%\text{ of root growth} \times \%\text{ of seed germination}}{100\%} \quad (3)$$

2.5. Toxicity of the biosurfactant and SDS to bacteria

The toxicity of the biosurfactant and SDS to the microbes was evaluated by determining the effect of the test solutions at different concentrations of 0 mg/L (control), 10 mg/L, 50 mg/L and 100 mg/L on microbial growth. The test was done on *P. aeruginosa* PA1 and *Serratia marcescens* SA1 hydrocarbon-degrading strains already identified using16s rRNA in our previous studies (Gidudu and Chirwa, 2020a; Gidudu et al., 2020). Samples were collected from the reactors after every 12 h of exposure of the test solutions to the microbes to determine colony forming units (CFU) as previously described by APHA (2005).

2.6. Source and properties of the petrochemical contaminated soil

The soil contaminated with petrochemicals was previously characterized by particle size, initial organic content, porosity, soil type, conductivity and elemental composition (Gidudu and Chirwa, 2020c). The petrochemical contaminated soil used was composed of 150 mL of oil/kg of soil. Sulfinyl sulfone, toluene, 2-hexyl-1-octanol, pentane-1-butoxy, 2, 2-dimethyl propane, n-hexadecane and n-eicosane were the hydrocarbons and hydrocarbon derivatives identified in the contaminated soil (Gidudu and Chirwa, 2020a).

2.7. Bio-electrokinetic remediation

The electrokinetic reactor was constructed from acrylic glass material to have two electrode (cathode and anode) compartments (160.5 mm × 150 mm × 150 mm) and one medium compartment (90 mm × 150 mm × 150 mm) to accommodate the contaminated soil during treatment as seen in Fig. 1. Graphite electrodes with 20 mm diameter and 100 mm length were connected to the DC power supply (0–3 RS-IPS

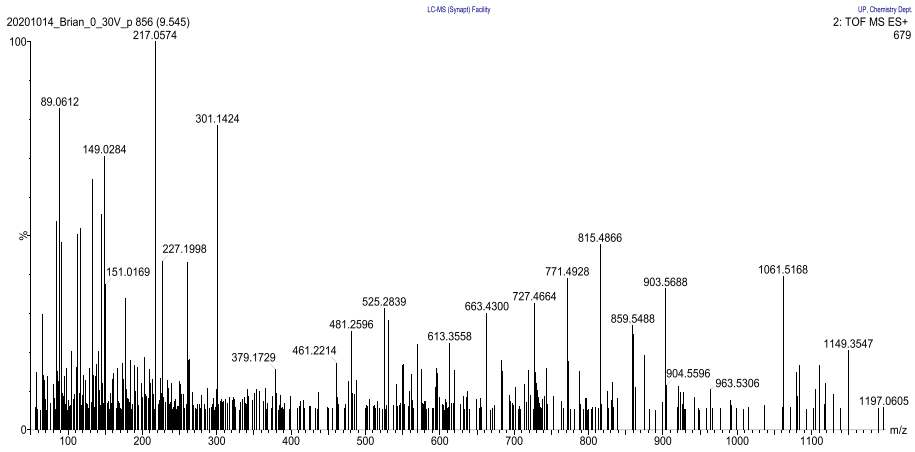

Fig. 2. Mass-Charge spectrum of the biosurfactant obtained by liquid chromatography tandem mass spectrometry.

303A, 0–30 V) and positioned in the two electrode compartments to be at either 185 mm, 260 mm and 335 mm as per the combinations of the experimental design. The electrode compartments were filled with deionized water, and electrode-medium compartment interfaces were sealed with filters (Whatman microfiber Grade GF/A: 1.6 mm) to allow electroosmotic flow, electrophoresis and movement of bacteria across the reactor. The voltage applied was varied from 30 V to 20 V–10 V as per the capacity of the DC power supply following the combinations of the experimental design. To achieve bio-electrokinetic remediation, 2 kg of petrochemical contaminated soil mixed with 300 mL of different biosurfactant concentrations (28 g/L, 56 g/L, 84 g/L) and 30 g of bacterial cells grown for 24 h were introduced into the medium compartment. The critical micelle concentration of the biosurfactant was 156 mg/L (Gidudu and Chirwa, 2020c). The factor combinations considered in terms of voltage applied, biosurfactant concentration, and electrode distance for each of the experiments were obtained using the design of experiments in the Design-Expert software. The central composite design (CCD) employed in the response surface methodology was used in the optimization process for 20 experimental runs with 8, 6 and 6 factorial, center and axial points respectively. Three numerical factors of biosurfactant concentration, voltage, and the distance between electrodes were used as the independent variables. Biosurfactant concentration had the range of 28 g/L-84 g/L, voltage had the range of 10 V–30 V and the distance between electrodes ranged from 185 mm to 335mm (Table 1). These were chosen as per the capabilities of the electrokinetic reactor design. Therefore, all the points were required to be face centered to fit within the limitations of the reactor design. Bacterial growth (CFU/mL) and Hydrocarbon removal (%) were used as the two main response variables in the model. The quality of fit of the model was evaluated using analysis of variances (ANOVA) for the experimental data to fit the model in Eq. (4) (where Y is the independent variable representing either hydrocarbon degradation or bacterial growth, β_0 is the intercept value, β_0, β_1 and β_3 are the first order coefficients, β_4, β_5 and β_6 are interaction coefficients and β_7, β_8 and β_9 are coefficients of the quadratic terms) to describe all the effects and interactions of the variables. Electroosmotic flow was also monitored and determined as the electrolyte volume that moved and accumulated in either of the electrode compartments (Dastgheib et al., 2008; Gidudu and Chirwa, 2020c).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 AB + \beta_5 AC + \beta_6 BC + \beta_7 A^2 + \beta_8 B^2 + \beta_9 C^2 \quad (4)$$

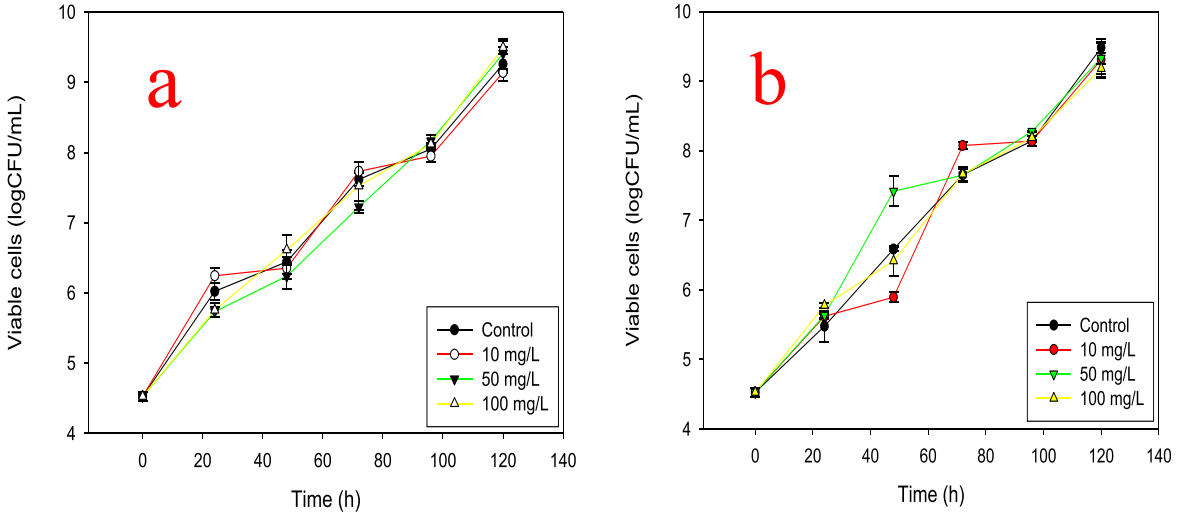

Fig. 3. The effect of the biosurfactant to microbial growth of *P. aeruginosa* (a) and *S. marcescens* (b).

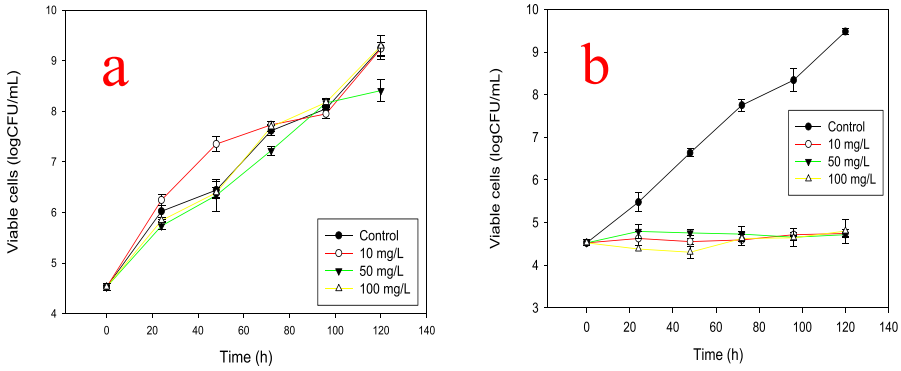

Fig. 4. The effect of SDS to microbial growth of *P. aeruginosa* (Fig. 4 (a)) and *S. marcescens* (Fig. 4 (b)).

3. Results and discussion

3.1. Identification of the biosurfactant produced by the bacteria using ultra-performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

The biosurfactant was identified using Liquid Chromatography-Mass Spectrometry before determining its toxicity and potential for enhancing remediation. The chromatographs obtained were analyzed using the MassLynx V4.1 (Waters) software (Fig. 2). The LC-MS/MS spectrometry of the biosurfactant revealed the presence of a rhamnolipid biosurfactant with four di-rhamnolipid congeners and seven mono-rhamnolipid congeners (Table 1). The seven mono-rhamnolipids produced were composed of four mono-rhamno-mono-lipidic congeners and three mono-rhamno-di-lipidic congeners while the di-rhamnolipids were mainly composed of di-rhamno-di-lipidic congeners. These

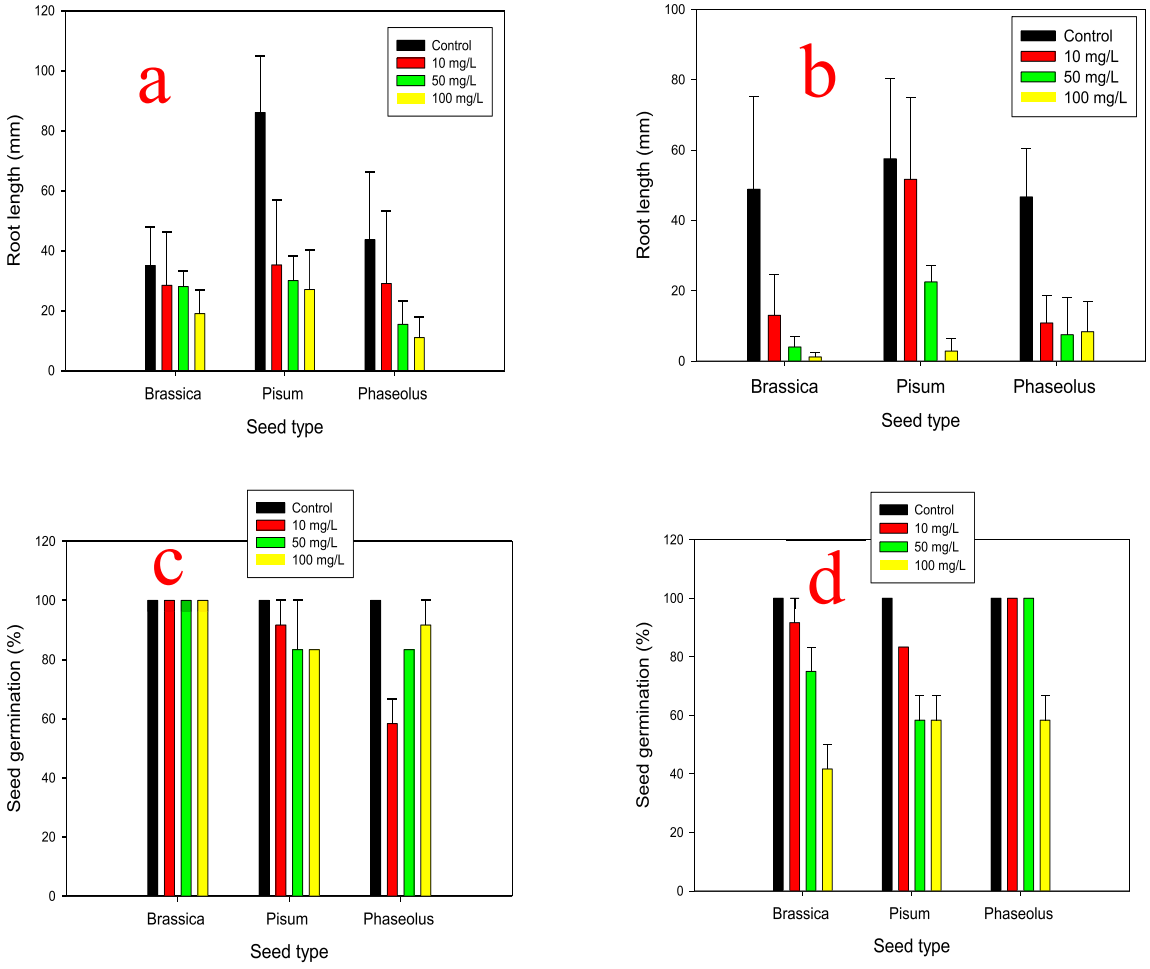

Fig. 5. The effect of biosurfactants and SDS on root length and seed germination. Fig. 5 (a) and Fig. 5 (c) are for biosurfactants, while Fig. 5 (b) and Fig. 5 (d) are for SDS.

observations are similar to the reports made when the same strain was used to produce a rhamnolipid biosurfactant using a different carbon source (Gidudu and Chirwa, 2021).

3.2. Effect of sodium dodecyl sulfate and the biosurfactant on microbial growth

When *P. aeruginosa* cells were exposed to the biosurfactant, there was an increase in viable cell count from 4.523 ± 0.048 CFU/mL to 6.023 ± 0.085 CFU/mL for the control, 6.024 ± 0.075 for 10 mg/L, 5.734 ± 0.051 for 50 mg/L and 5.841 ± 0.018 for 100 mg/L in 24 h (Fig. 3 (a)). The same was observed for *S. marcescens* whose cell count increased from 4.324 ± 0.037 CFU/mL to 5.478 ± 0.159 CFU/mL for the control, 5.619 ± 0.025 for 10 mg/L, 5.636 ± 0.024 for 50 mg/L and 5.776 ± 0.026 for 100 mg/L (Fig. 3 (b)). After 120 h, the viable counts were higher than 9 CFU/mL for both strains at all concentrations of the test solutions. When the control is compared to when the strains are exposed to the biosurfactant, minor differences in the number of viable cells for the 5 days of exposure are observed.

When the strains were exposed to SDS, the effect of the test solution to *P. aeruginosa* was hardly observed since the difference in viable counts between the control and the different concentrations of SDS were minor (Fig. 4 (a)). This should have been because of the nature of the strain which gives it the ability to withstand toxic environments considering that Pseudomonads by nature are the greatest hydrocarbon degrading strains (Das and Chandran, 2011). When *S. marcescens* was exposed to the test solution of SDS, the effect of SDS on the strain was observed with a clear and significant difference in viable cell count when compared to the control (Fig. 4 (b)). In fact, the viable cell counts remained below 5 CFU/mL after 24 h to the end of experiments at all concentrations of 10 mg/L, 50 mg/L and 100 mg/L of SDS.

Surfactants affect microbes in two different ways, either by disrupting the cell membranes as a result of the interactions between the surfactant and the lipid components of the cell or the reaction of the surfactant with the cell protein required for cell functioning (Jensen, 1999; Lima et al., 2011). In general, cationic surfactants have been reported to be toxic at pH of 7 while anionic surfactants are known to be more toxic below the pH of 7 (Lima et al., 2011; Volkering et al., 1998). The low toxicity of the biosurfactant produced in the present study is therefore attributed to its anionic nature since the cells were exposed to the test solution at the pH of 7 in all experiments. Similar to our observations, Lima et al. (2011) also reported that the exposure of five different biosurfactants produced by different strains had low inhibition on the bacterial growth of *Acinetobacter baumannii* LBBMA 04, but SDS led to the total inactivation of the same strain at a concentration four times higher than the critical micelle concentration of the surfactant. Inactivation was also observed when the strain was exposed to the biosurfactants with concentrations eight times higher than the critical micelle concentration (Lima et al., 2011). Singh et al. (2020) also reported that the continuous increase in the concentration of biosurfactants led to inhibition of growth for both *Escherichia coli* and *Enterobacter cloacae* strains on agar plates. Hogana et al. (2019) also reported that the biosurfactant produced by *P. aeruginosa* ATCC 9027 was 92% inherently degraded in 30 days by a microbial inoculum in mixed liquor suspended solids while the synthetically produced monorhamnolipid diastereomers were only 34–73% degraded implying that the synthetic surfactants may have antibacterial properties and are recalcitrant to degradation even when exposed to capable degrading microorganisms.

3.3. Effect of surfactants on root length and seed germination

The three main steps involved in the design of biosurfactant-mediated bioremediation processes are the initial characterization of the polluted area, laboratory-scale experiments and field-scale feasibility studies (Ławniczak et al., 2013). During laboratory-scale studies, the assessment of potential biosurfactant-induced toxicity to all environmental components is very important including the toxicity of the biosurfactants to plants, before field-scale studies are undertaken (Ławniczak et al., 2013). This is even more important for the *in situ* application of biosurfactants for remediation purposes where both plants and organisms in the environment could be exposed. The phytotoxicity of the biosurfactant was tested on *P. sativum, P. vulgaris, and B. napus* at different concentrations of 10 mg/L, 50 mg/L and 100 mg/L. The results in Fig. 5 (a) and Fig. 5 (c) show that the biosurfactant had a significant effect on the elongation of the roots. The control had an average length of 48.83 mm for *B. napus*, 57.5 mm for *P. sativum* and 46.67 mm for *P. vulgaris*. The length of the roots reduced with an increase in biosurfactant concentration, where 10 mg/L had an average length of 13 mm for *B. napus*, 51.7 mm for *P. sativum* and 10.83 mm for *P. vulgaris*. The roots became much shorter when the seeds were exposed to 50 mg/L leading to a reduction in root length to 4 mm for *B. napus*, 22.5 for *P. sativum* and 7.5 mm for *P. vulgaris*. The roots were shortest at a concentration of 100 mg/L with 1.16 mm for *B. napus*, 2.83 mm for *P. sativum* and 8.3 mm for *P. vulgaris*.

Inhibition of root elongation in the three test vegetables by SDS was also observed (Fig. 5 (b) and Fig. 5 (d)). There was a further reduction in root length with an increase in SDS concentration. The controls had the longest root lengths, while the seeds exposed to 100 mg/L had the shortest root lengths.

When the seeds of *B. napus* were exposed to the biosurfactant, the seeds that germinated were as many as those that germinated in the control where the biosurfactant and SDS were absent. *P. sativum* test seeds were affected by the biosurfactant since the relative germination was lower than 83.3% compared to the control that had a relative germination of 100%. *P. vulgaris* were also affected by exposure to the biosurfactant since relative germination was 50% for 10 mg/L and 83.3% for 50 mg/L. When the seeds were exposed to SDS, the increase in the concentration of SDS led to the decrease in the germination of *B. napus* seeds leading to average relative germination of 83.3% for 10 mg/L, 66.06% for 50 mg/L and 50% for 100 mg/L. *P. sativum* vegetable seeds were also adversely affected by exposure to SDS since the germination was less than 86.3% for all the test solutions of 10 mg/L, 50 mg/L and 100 mg/L. *P. vulgaris* seeds only achieved relative germination of 50% at the highest concentration of 100 mg/L of SDS. Table 2 shows the germination index of *B. napus*, *P. sativum* and *P. vulgaris* at different concentrations of the test solutions. The results show that SDS had substantial adverse effects on seed germination and root length represented by the extremely low values of germination index as compared to the biosurfactant. The results obtained are similar to previous reports were the biosurfactant produced by *Candida lipolytica* allowed seed germination at all biosurfactant concentrations but had a continuously decreasing germination index at every increase in biosurfactant concentration when exposed to *Brassica oleracea* var. *botrytis L.*, *Brassica oleracea* var.*capitata*, and *Lactuca sativa* L. seeds (Santos et al., 2017). Their studies indicated that *Brassica oleracea* var. *botrytis L.* was the most adversely affected vegetable seed (Santos et al., 2017). Contrary to these findings, Sharma et al. (2014) reported that the exposure of *Brassica nigra* and *Triticum aestivum* seeds to the xylolipid biosurfactant produced by a *Lactobacilli* strain led to the continuous increase in root elongation and germination index with an increase in biosurfactant concentration much as SDS had led to an inherent decrease in root elongation and

Table 2
Germination index of all the three seed types after exposure to the biosurfactant and SDS.

	Biosurfactant (mg/L)			SDS (mg/L)		
Seed type	10	50	100	10	50	100
B. napus	81.14	80.00	54.29	22.18	5.46	1.19
P. sativum	34.11	27.13	26.16	74.87	19.56	3.28
P. vulgaris	33.26	29.43	25.22	23.21	16.07	8.93

Table 3
Factor combinations generated by design expert and their respective responses obtained after running the experiments.

Runs	Factors			Responses	
	A: Voltage (V)	B: Biosurfactant (mg/L)	C: Distance between electrodes (mm)	Y_1: Microbial growth (CFU/mL)	Y_2: Hydrocarbon removal (%)
3	20	56	260	9.80	76.80
4	20	56	260	9.60	74.30
6	20	56	260	9.86	77.20
7	20	56	260	9.92	76.56
18	20	56	260	9.92	77.50
19	20	56	260	9.80	76.80
8	20	28	260	9.45	75.53
9	20	84	260	9.90	78.00
10	10	56	260	8.97	75.70
14	20	56	185	9.59	80.00
16	30	56	260	10.96	85.70
17	20	56	335	8.79	74.00
1	10	28	335	7.60	66.50
2	10	84	185	10.69	78.34
5	30	28	185	9.35	74.77
11	30	84	185	11.52	92.00
12	30	84	335	8.85	82.77
13	10	28	185	8.57	70.34
15	10	84	335	9.69	75.50
20	30	28	335	8.75	68.93

germination index at all concentrations of the test solution. In other studies, the exposure of the seeds to the biosurfactant produced by *Candida sphaerica* UCP 0995 led to a decrease in root elongation and germination index for *Brassica oleracea and Cichorium intybus* at concentrations higher than 200 mg/L but the same concentrations led to an increase in elongation and germination index for *Solanum gilo* (Sarubbo et al., 2013). In the present study, *P. sativum* and *P. vulgaris* were the most affected seed type in terms of germination index which means the biosurfactant is more likely to affect distinct seed types and plants differently (Sarubbo et al., 2013; Singh et al., 2020).

3.4. Removal of hydrocarbons from soil

Bioremediation, biosurfactants, and electrokinetic remediation were combined to form a hybrid removal system to remove hydrocarbons from the soil contaminated. The experiments were run according to the experimental design generated by the design expert software. A total of 20 experiments were run with different combinations having variations in biosurfactant concentration, the distance between electrodes and voltage (Table 3). Microbial growth and hydrocarbon removal were the two main dependent variables that were monitored and measured. The results obtained show that the combination of a voltage of 30 V, electrode spacing of 185 mm and a biosurfactant concentration of 84 g/L had the highest microbial growth of 11.52 CFU/mL and hydrocarbon removal of 92%. Contrary to this, the combination of a voltage of 10 V, electrode spacing of 335 mm and a biosurfactant concentration of 28 g/L had the lowest microbial growth of 7.6 CFU/mL and hydrocarbon removal of 66.5%.

The removal of hydrocarbons from soil using biosurfactant enhanced bio-electrokinetic remediation uses a combination of different processes to achieve efficient hydrocarbon removal. The liquid phase (oil and water) in the system moves towards the electrode compartments as a result of electroosmosis (Peppicelli et al., 2018), charged ions in the system move as a result of electromigration to their oppositely charged electrodes (Xu et al., 2017), and charged colloidal particles move towards their oppositely charged electrodes as a result of electrophoresis (Peppicelli et al., 2018). Furthermore, the strong oil-water-solid emulsions are broken down by electro-demulsification due to the application of current or the reduction of surface and interfacial tension by the biosurfactant (Batista et al., 2006; Elektorowicz et al., 2006). Combining the highest voltage with the highest biosurfactant concentration and the lowest distance between electrodes led to the highest microbial growth and carbon removal. In contrast, the lowest voltage and biosurfactant concentration combined with the highest distance between electrodes led to the lowest carbon removal. This is because the increase in voltage leads to the rise in electroosmotic flow, electro-demulsification, electrophoresis, electromigration and electrolysis (Gidudu and Chirwa, 2020c; Mena et al., 2016). Electroosmotic flow improves the removal of hydrocarbons by moving the oil away from the soil towards the electrode compartments (Gidudu and Chirwa, 2020b; Yang et al., 2005). Indeed, in all the experiments, the oil was extracted from the soil in the medium compartment and moved towards the anode compartments. In contrast, the water (anolyte) continuously moved from the anode compartment towards the cathode compartment. More oil was transferred towards the anode compartment at the highest voltage of 30 V compared to the lowest voltage of 10 V, similar to reports made in previous studies (Gidudu and Chirwa, 2020c). The process of demulsification resulting from the application of the biosurfactant and current also leads to the increase in the electroosmotic flow of oil from the soil and the bioavailability of the hydrocarbons to the microbes that utilize it as a carbon source. Biosurfactants enhance the bioavailability of the hydrocarbons to the microbes by reducing the interfacial tension and surface tension between the oil-water-solid emulsions (Batista et al., 2006). The increase in voltage led to increased electroosmotic flow and bioavailability, supporting the bacteria's favourable growth. The increase in electroosmotic flow and bioavailability led to improved hydrocarbon removal due to the multiplier effect of these processes (Das and Chandran, 2011).

3.5. Effect of the independent factors and their interactions on microbial growth

The growth of the hydrocarbon-degrading bacteria is very important in the combination of bioremediation, biosurfactants and electrokinetic remediation as a hybrid system but it could be affected by the voltage and its associated electrokinetic processes (Beretta et al., 2019; Lear et al., 2007). It was, therefore, essential to evaluate which of the independent variables had a substantial effect on bacterial growth using the response surface methodology. A hierarchical model with a predicted R^2 of 0.406 and an adjusted R^2 of 0.6216 was suggested. This was modified with the backward elimination tool and the hierarchical model selection tool to remove the insignificant terms and improve the fitting of the model. The model was modified to obtain a significant model with an F-value of 44.48 with only a 0.011% chance that the model with such a high value could occur due to noise. A, C, AB, AC, BC, C^2, ABC, A^2B, AB^2 terms of the model were significant with *p*-values less than 0.05 (Eq. (5)). The rest of the terms such as B, B^2, A^2, were insignificant but were not eliminated from the model to support the hierarchical selection of the model (Eq. (5)). The lack of fit was also insignificant with an F value of 4.3 and *p*-value of 0.082. The model also had an R^2 of 0.9871 and an adjusted R^2 of 0.9649. An adequate signal with an adeq precision of 28.744 meant that the model was good enough to navigate the design space.

$$Microbial\ growth\ \left(\frac{CFU}{mL}\right) = +4.2 + 0.10995A - 0.126B + 0.04C + 0.0047AB + 0.00046AC + 0.000117BC - 0.0096A^2 + 0.0018B^2 \quad (5)$$

The effect of the independent factors on microbial growth was evaluated using the measured influence of the independent factors and coefficients of each term in the model (Eq. (5)). The results indicated that the increase in voltage does not have a detrimental effect on microbial growth but rather enhances the growth of the microbes in the reactor. In contrast, the decrease in voltage leads to a decline in microbial growth. The exposure of the bacteria to current is reported to

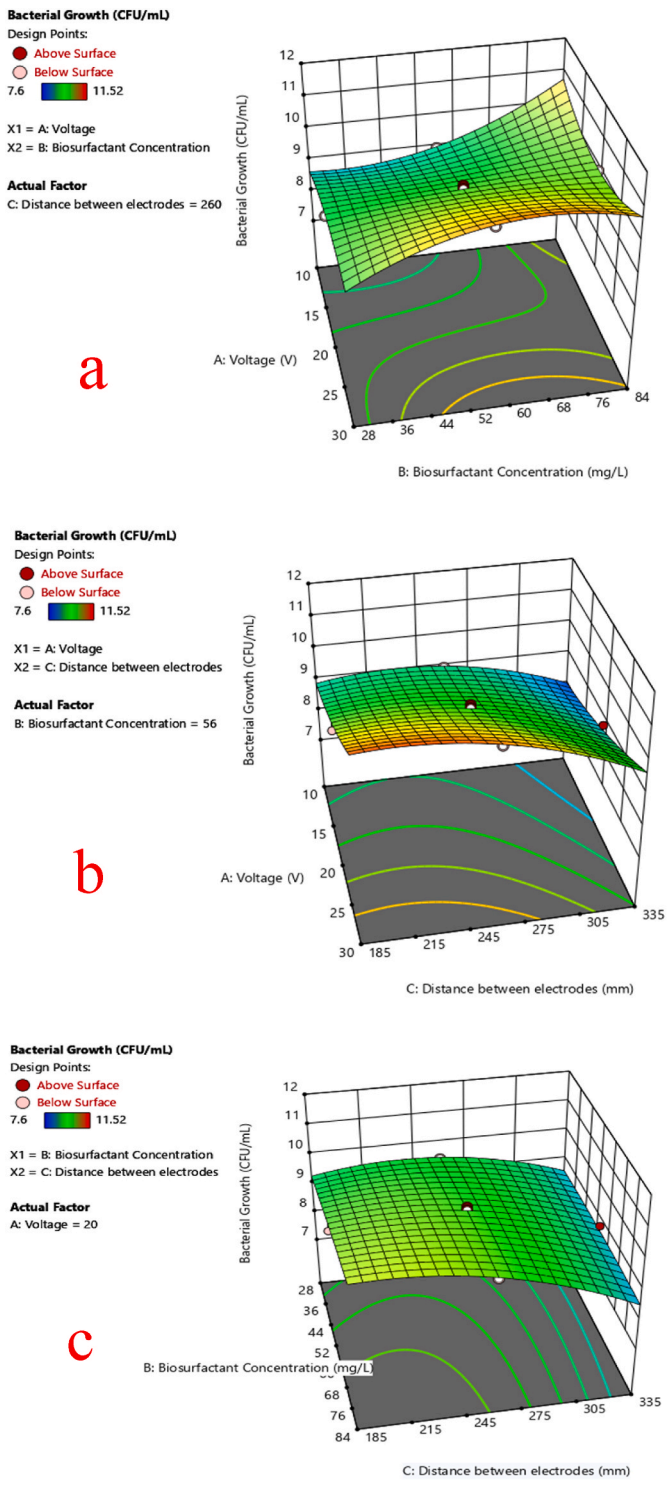

Fig. 6. The effect of the interactions of the factors on microbial growth.

have detrimental effects on bacteria by affecting their cell membrane, cell metabolism and mobility (Beretta et al., 2019; Lear et al., 2007), but this was not the case even at the highest voltage of 30 V as seen in Table 3. The increase in voltage leads to increased microbial growth by stimulating bacterial growth through oxidation processes resulting from the electrolysis of water (Shu et al., 2015). The electrolysis of water due to the application of current at the electrodes leads to the production of OH^- ions and H_2 gas at the cathode and the production of H^+ ions and O_2 gas at the anode (Jeon et al., 2015; Shen et al., 2007). The production of oxygen in the system favours the growth of bacteria and the ultimate degradation of the contaminants by the aerobic *P. aeruginosa* strain (Beretta et al., 2019). The model revealed that the increase in biosurfactant concentration led to a substantial increase in microbial growth compared to the increase in voltage. Comparing the experiments operated at the same voltage and the distance between electrodes but with variation in biosurfactant concentration, the experiments with the highest biosurfactant concentration of 84 g/L had the highest microbial growth. Biosurfactants enhance the bioavailability of hydrocarbons to the microbes by reducing interfacial tension and surface tension between oil-water-solid emulsions (Batista et al., 2006).

It was also revealed that the increase in the distance between electrodes had a significant adverse effect on the growth of the bacteria. In contrast, the decrease in the spacing of electrodes enhanced the growth of the bacteria. This is attributed to the fact that the increase in distance between electrodes leads to the prolonged exposure of bacteria to a surrounding in the medium compartment that generally has low pH while the low distance between electrodes provides a neutral pH environment (Gidudu and Chirwa, 2020c). This is as a result of the production of H^+ ions at the anode leading to the formation of an acidic front moving towards the cathode and OH^- ions at the cathode leading to the formation of an alkaline front moving towards the anode with H^+ ions as twice as mobile as OH^- ions (Cameselle et al., 2013; Shu et al., 2015). With a lower distance between electrodes, the ions reach their destination faster than when the distance between the electrodes is more extensive, resulting in the early intersection of the acidic front and alkaline front (Gidudu and Chirwa, 2020c). The intersection of the pH fronts leads to the formation of a neutral pH environment in the medium (soil compartment) compartment where the microbes survive and grow. The neutral pH generated by the low electrode spacing provided amiable conditions for the growth of *P. aeruginosa* which requires an optimum pH of 7 (Das and Mukherjee, 2007; Gidudu and Chirwa, 2020c). Of the three factors, the model revealed that biosurfactant concentration had the most outstanding effect on microbial growth (Fig. 6 a).

Fig. 6 shows the effect of the multifactor interactions on the growth of the microbes. The model disclosed that the variation of voltage between 10 V and 30 V at a constant biosurfactant concentration of 84 g/L leads to microbial growth higher than 9.5 CFU/mL at any given voltage with 10.72 CFU/mL at 10 V, 9.94 CFU/mL at 20 V and 10.74 CFU/mL at 30 V. In contrast, the same voltage variation at a constant biosurfactant concentration of 28 g/L only achieves the highest microbial growth of 9.61 CFU/mL at 30 V, with 8.63 V at 10 V and 9.49 CFU/mL at 20 V (Fig. 6 a). With 84 g/L the increase in voltage did not have any significant effect on microbial growth but the variation in voltage under a constant biosurfactant concentration led to a direct increment in microbial growth on every increase in voltage.

At constant biosurfactant concentration and electrode distance, the increase in voltage led to increased microbial growth. For instance, at 335 mm and 56 g/L the highest microbial growth was obtained at 30 V with 9.65 CFU/mL. Furthermore, a microbial growth of 8.62 CFU/mL was obtained at 20 V and 8.0 CFU/mL at 10 V. On the other hand, an electrode distance of 185 mm and 56 g/L of the biosurfactant leads to microbial growth of 8.9 CFU/mL at 10 V, 9.83 CFU/mL at 20 V and 11.19 CFU/mL at 30 V (Fig. 6 b).

The interaction between biosurfactant concentration and the distance between electrodes reveals that the variation of biosurfactant concentration between 28 g/L and 84 g/L at a constant voltage of 20 V and 335 mm electrode distance does not significantly affect microbial growth since viable cell microbial counts for different biosurfactant concentrations do not change substantially as it remains at an average of 8.6 CFU/mL (Fig. 6 c). In contrast, the variation of biosurfactant concentration at a constant voltage of 20 V and 185 mm electrode distance leads to an increase in microbial growth with an increase in biosurfactant concentration. This is why 9.27 CFU/mL was obtained at 28 g/L, 9.83 CFU/mL at 56 g/L and 10.2 CFU/mL at 30 V.

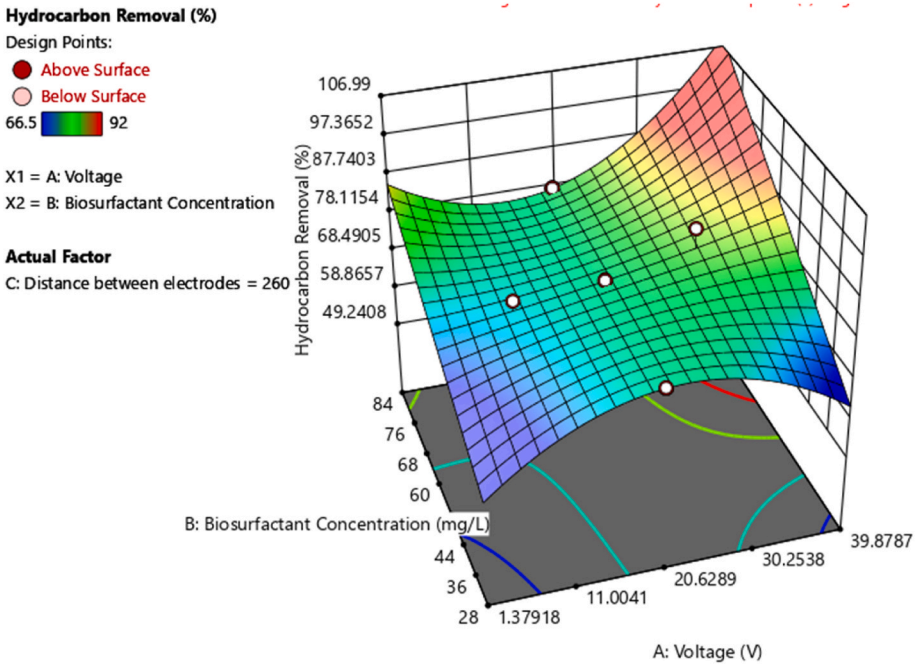

Fig. 7. The effect of the interaction between voltage and biosurfactant concentration on hydrocarbon removal.

3.6. Effect of the independent factors and their interactions on hydrocarbon removal

A linear model with an adjusted R^2 of 0.7641 and a predicted R^2 of 0.6474 was suggested by the software for the hydrocarbon removal response. To obtain a better fitting of the model, the model was modified using the backward elimination method combined with the hierarchical model selection to remove insignificant terms, one at a time. The model obtained has an F-value of 20.84 and *p*-value <0.0001, implying that the model is significant since there is only a 0.01% chance that an F-value this large could occur due to noise. In this case, A, C, AB, A^2B were the significant model terms. B and A^2 terms are not significant since their values were greater than 0.1 (Eq. (6)). These were however left in the model to support hierarchical model selection. The lack of fit was insignificant with a *p*-value of 0.0517 which means the lack of fit did not necessarily occur due to noise. The predicted R^2 of the model was 0.7463 while the adjusted R^2 was 0.8623. The difference between the predicted R^2 and the adjusted R^2 was less than 0.2 which means the two values of the model are in reasonable agreement. The adeq precision obtained for the model was 20.488 way higher than the desirable of 4. The model could therefore be used to navigate the design space with an adequate signal in terms of signal to noise ratio. The actual equation that can be used to make predictions of the response for given levels of each factor was obtained (Eq. (6)).

$$Hydrocarbon\ removal\ (\%) = +46.65 + 3.69A + 0.6B - 0.037C - 0.06AB - 0.09A^2 + 0.0017A^2B \quad (6)$$

The effects of the variation of the independent variables were evaluated using the perturbation tool of the software. The results showed that the decrease in voltage and biosurfactant concentration both led to the reduction in hydrocarbon removal, while the decrease in the distance between electrodes led to a substantial increase in hydrocarbon removal and vice versa. This occurs when the biosurfactant facilitated bio-electrokinetic removal of hydrocarbons from the soil is driven by a combination of electro-demulsification and biosurfactant supported demulsification of oil-solid-water stable emulsions which enhance the electroosmotic flow of oil from the soil (Batista et al., 2006; Beretta et al., 2019; Gidudu and Chirwa, 2020c). In addition, the introduction of oxygen in the system by electrolysis improves the bioavailability of the hydrocarbons to the microbes leading to enhanced hydrocarbon removal (Batista et al., 2006; Beretta et al., 2019; Gidudu and Chirwa, 2020c). Electro-demulsification and electrolysis are all dependent on the intensity of the voltage applied so the increase in voltage increases the rate of electrolysis and electro-demulsification (Boulakradeche et al., 2015; Elektorowicz et al., 2006; Mena Ramirez et al., 2015). The comparisons of the three independent factors indicate that both voltage and biosurfactants are the most significant factors since voltage has a coefficient of 3.69 and biosurfactant concentration has a coefficient of 0.6.

Fig. 7 shows the effect of the interactions of the factors on hydrocarbon removal. The model reveals that if the voltage was to be reduced to a constant voltage of 1.37 V under the application of 28 g/L, only 50% hydrocarbon removal would be achieved. The further increase of the biosurfactant concentration to 60 g/L at a constant voltage of 1.37 increases the hydrocarbon removal to 68%. If the biosurfactant concentration is increased further to 84 g/L, the hydrocarbon removal also increases to 82%. This was also reported by Gidudu and Chirwa (2020b), where the lowest biosurfactant concentration of 28 g/L led to an average hydrocarbon removal of 74.54%, followed by a concentration of 54 g/L with 78.81%. The highest carbon removal of 92.47% was obtained when the highest biosurfactant concentration of 84 g/L was used.

On the other hand, if a constant biosurfactant concentration of 28 g/L was to be applied at 1.37 V, a hydrocarbon removal of 50% would be achieved. A further increase of voltage to 25 V with a biosurfactant concentration of 28 g/L increases the hydrocarbon removal to 72%. If the voltage increases further to 39 V with the same biosurfactant concentration of 28 g/L, the hydrocarbon removal decreases to 62%. At a constant biosurfactant concentration of 84 g/L, every increase in voltage leads to a significant increase in hydrocarbon removal. For instance, 77.5% levels of hydrocarbon removal are achieved with 10 V, 77.9% with 20 V and 88.65% with 30 V.

This means that voltage has a threshold value at which the remediation process is most efficient; however, hyper increases in voltage may not improve the remediation process when combined with low biosurfactant concentration. But the simultaneous increase in voltage and biosurfactant concentration leads to a significant increase in hydrocarbon removal (Fig. 7). Furthermore, Fig. 7 shows that the increase in

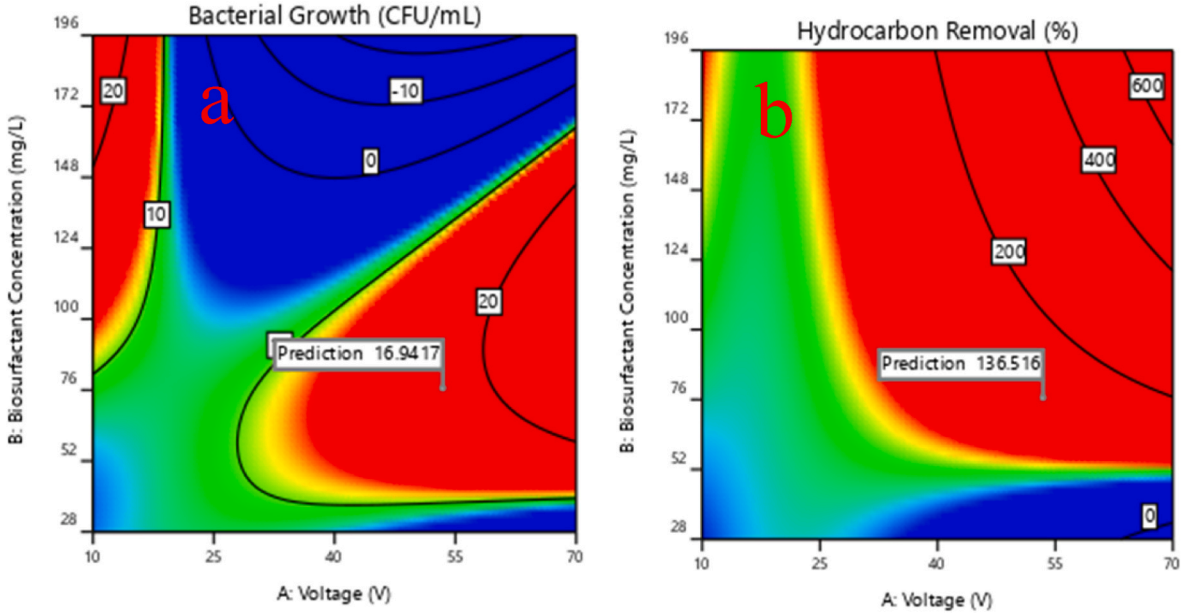

Fig. 8. Optimized predictions for bacterial growth (a) and hydrocarbon removal (b).

biosurfactant concentration improves the remediation process in terms of hydrocarbon removal irrespective of the variation in voltage. Therefore, combining the highest voltage with the highest biosurfactant concentration leads to the highest carbon removal much as the cost of remediation is also most likely to increase due to energy consumption and the cost of biosurfactants.

3.7. Optimization of microbial growth and hydrocarbon removal

Optimization of microbial growth and hydrocarbon removal was based on minimizing voltage to reduce the energy expenditure of the process, minimizing the concentration of the biosurfactant, and maximizing the distance between electrodes. These were all done with the primary goal of maximizing bacterial growth and the removal of hydrocarbons. The optimization generated 12 different solutions. The best solution had the highest desirability of 0.620. To obtain this desirability, the combined voltage of 53.426 V, a biosurfactant concentration of 76.706 mg/L and the distance between electrodes of 308.662 mm were suggested. The combination of these optimized factors was predicted to produce a bacterial growth of 16.942 CFU/mL and a hydrocarbon removal of 136.516% meaning the experimental time has to be reduced to less than 14 days (Fig. 8). The validation test using this configuration obtained a bacterial growth of 10.14 ± 2.15 CFU/mL and total carbon removal of 96 ± 3.72% in 160 h.

4. Conclusion

The rhamnolipid biosurfactant produced by a Pseudomonad strain does not inhibit the growth of hydrocarbon-degrading bacteria. The biosurfactant has lower toxicity to bacteria as compared to SDS. The biosurfactant and SDS both affected the growth of the *Brassica napus* var. *napus, Pisum sativum, Phaseolus vulgaris* vegetable seeds. Each of the seeds was affected differently, but the biosurfactant mostly affected Pisum sativum while SDS mostly affected *Brassica napus* var. *napus*. The interactions between the factors show that biosurfactants are very significant in the remediation process. The increase in voltage must be accompanied by an increment in biosurfactant concentration to have an efficient remediation process. The optimum conditions for removing total hydrocarbons at a lower operational cost were a voltage of 53.426 V, a biosurfactant concentration of 76.706 mg/L, and the distance between electrodes of 308.662 mm.

Declaration of competing interest

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

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