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Biosurfactant Produced by Serrati sp. and its Application in Bioremediation Enhancement of Oil Sludge

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The petroleum industry is one of the most pivotal sectors of the global economy but with it comes the production of atrocious amounts of waste especially during production and refining of the oil. Oil sludge which is an example of such waste has been categorized as hazardous because it is composed of asphaltenes, heavy metals and heavy hydrocarbons such as long-chain paraffins among others. The bioremediation of hydrocarbons in this waste is limited by low availability; biosurfactants were therefore applied to evaluate their efficacy in promoting the bioremediation process. The biosurfactants were produced by a hydrocarbon-degrading bacterium identified by 16S ribosomal RNA as *Serrati* sp. Biosurfactants were applied to the batch reactors containing oil sludge in different concentrations of 7 g/L, 14 g/L, and 28 g/L. The biosurfactants enhanced oil sludge biodegradation by 23.38%, 70.28%, and 55.6% respectively in 14 days. The increase in biosurfactant concentration showed a subsequent increase in microbial growth after every biosurfactant amendment due to the increase in bioavailability of the pollutants to the microbes which is utilized as the carbon and energy source. The biosurfactants were characterized by Fourier-transform infrared spectroscopy and Thin Layer chromatography showing the presence of amino acids. The biosurfactant produced by the hydrocarbon-degrading bacteria of *Serratia* sp. SA1 showed great potential in improving the bioremediation of recalcitrant hydrocarbons in the oil sludge and can, therefore, be used as an alternative to chemical surfactants.

1. Introduction

The most significant wastes generated in the petroleum industry is oil sludge. Oil sludge is a complex emulsion of various petroleum hydrocarbons (PHCs), water, heavy metals, and solid particles (Hu et al., 2013). The disposal of oil sludge into the environment such as on land or in water may have prolonged effects after the contamination event with some ecosystems such as mangrove swamps and salt marshes experiencing the effects for decades after the event (Kingston, 2002). This is because petroleum oil sludge is usually composed of petrochemical pollutants such as PHC's which are composed of alkanes, cycloalkanes, benzene, toluene, xylenes, naphthalene, phenols, and various polycyclic aromatic hydrocarbons (PAH's) (Das and Chandran, 2011). The greatest concern regarding contamination by hydrocarbons lies in the mutagenic, carcinogenic and toxic characteristics of such contaminants (Souza et al., 2014). Basing on the source and chemical composition, oil extracts can be classified as chemical/Inorganic wastes, spent catalysts, hydrocarbon wastes, contaminated soils, and solids, and aqueous waste (Islam, 2015). The combination of oil and soil creates a stable W/O emulsion of solids (colloids), water and metals preventing coalescence of similar molecules of water and oil. These emulsions are a result of the activity of emulsifiers such as asphaltenes, resins, oil-soluble organic acids, fine solids, nitrogen-oxygen and sulphur which are major components of petroleum hydrocarbons (Hu et al., 2013). The degradation of hydrocarbons is inhibited by low bioavailability of substrate (hydrocarbons) to the microorganisms due to their recalcitrant and hydrophobicity properties (Hu et al., 2013). The reduced level of bioavailability of the hydrocarbons to the bacteria is because of the tendency of the petroleum hydrocarbons to bind to the soil compounds making it so difficult to be degraded (Das and Chandran, 2011). Bioavailability is reported to be dependent on the general mass transfer and movement of the pollutant into the aqueous bulk phase (Volkering et al., 1998). Biosurfactants are reported to have the ability to increase the bioavailability of hydrocarbons 5–20-fold hence significantly increasing their rate of biodegradation (Chauhan et al., 2008). Biosurfactants have been reported to be more effective in

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improving the bioavailability of hydrophobic substances as compared to chemical surfactants (Ron and Rosenberg, 2002). When biosurfactants are applied, the mobility of PHCs is enhanced by the hydrophobic tail when it makes the biosurfactant molecule to gather at the interfaces of the emulsion thereby reducing the interfacial or surface tension while the hydrophilic head increases PHCs solubility by allowing the surfactant molecule to dissolve in the water phase (Mulligan, 2009). The hydrophobic moiety is made of hydroxyl fatty acids, long-chain fatty acids, or a-alkyl-b-hydroxyl fatty acids while the hydrophilic moiety can be a phosphate, an alcohol, cyclic peptide, amino acid, carbohydrate, or carboxylic acid (Bezza and Chirwa, 2015). Biosurfactants have been rendered a better substitution for synthetic surfactants because of greater environmental compatibility, higher biodegradability, higher foaming capacity, lower toxicity, higher selectivity, able to function at extreme pH, temperature, salinity (Bezza and Chirwa, 2015). In this research, we investigated the application of biosurfactants produced by *Serratia* sp. as an enhancement for the degradation of hydrocarbon pollutants. The research involved the production, purification, characterization, and application of biosurfactant with amendments of 7 g/L, 14 g/L and 28 g/L. The effect of biosurfactant application on microbial growth and total carbon reduction was then evaluated.

2. Methodology

2.1 Microbial culture, media and growth conditions

Strain SA1 used in the experiments was obtained from API (Atmospheric tank) tank sludge in South Africa by selective enrichment to obtain efficient hydrocarbon degraders according to Trummler et al. (2003). 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. (2003) by dissolving in 1 L of distilled water: 6.0 g $(NH_4)_2SO_4$; 0.4 g $MgSO_4 \cdot 7H_2O$; 0.4 g $CaCl_2 \cdot 2H_2O$; 7.59 g $Na_2HPO_4 \cdot 2H_2O$; 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 pure microbial culture of *Serratia* species used in this study was sourced from a sample of disposed petrochemical oil waste in South Africa and identified using the16S ribosomal RNA (rRNA) sequencing.

2.2 Biosurfactant production

To produce biosurfactant, 30 g of a pure strain of *Serratia* sp. 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 min. 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. The biosurfactant supernatant was then obtained by centrifugation at 10,000 rpm at 4 °C for 10 min. The biosurfactants were then recovered by acid precipitation as described by (Bezza and Chirwa, 2015). The inoculum was screened using the drop collapse method and the oil spreading test to conform biosurfactant production. In the drop collapse method, 2 mL 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 (Bodour and Miller-Maier, 1998). 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. (2000) 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.

2.3 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 (TLC) 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 ^{o}C for 5 min until the appearance of the respective colors (Noparat et al., 2014).

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 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) (Bezza and Chirwa, 2015).

2.4 Degradation studies

Biodegradation studies were carried out in 250 mL Erlenmeyer flasks as batch reactors. The Erlenmeyer flasks containing 500 g of oil sludge were inoculated with 30 g of cells of *Serratia* sp*.* The main experimental setups were as follows: (A). oil sludge + cells + distilled water (No biosurfactants), (B). oil sludge + cells + 7 g/L of biosurfactants, (C). oil sludge + cells + 14 g/L of biosurfactants, (D). Oil sludge + cells + 28 g/L of biosurfactants. All the experiments were incubated in an orbital shaker at pH 7, 180 rpm and 35 ^{o}C for 14 days. Samples were drawn from the flasks every after 2 days and aliquots of appropriate dilutions were plated in triplicates on to agar plates for viable cell counting. The samples were also taken for total carbon analysis using a Schimadzu Total Organic Carbon Analyzer.

3. Results and Discussions

3.1 Screening, isolation, and identification of the strain for hydrocarbon degradation and biosurfactant production

The hydrocarbon-degrading and highest biosurfactant producing strain SA1 obtained after isolation and passing the biosurfactant screening test using the drop collapse method and the oil spreading test was identified using the 16S rRNA sequence analysis. The 16S rRNA sequence of SA1 showed the highest similarity to genus *Serratia* related to *nematodiphila and marcescen* species with query cover of 100 % as shown in the phylogenetic tree presented in Figure 1 below.

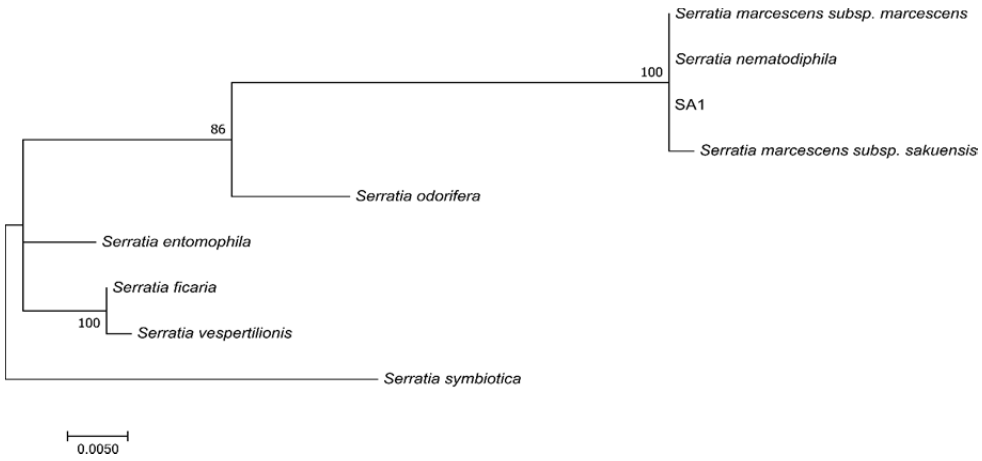

Figure 1. Phylogenetic tree based on the 16S rRNA genotype fingerprinting method with a scale bar corresponding to 0.0050 estimated nucleotide distance per sequence position.

3.2 Characterization of the biosurfactants

FTIR Characterization
The FTIR was used to study the chemical functional groups of the biosurfactants produced by the SA1 strain with fingerprint areas between 4000 and 500 cm^{-1}. The results showed a high similarity with a typical spectrum of rhamnolipids with the vibrations showing the presence of peptides and aliphatic hydrocarbons (Fadhile Almansoory et al., 2017). The infrared spectrum in Figure 2 below shows the FTIR analysis. The low absorption bands at 3295 represents the N-H functional group which is typical of a secondary amine, deformations at 2950 and 2861 cm^{-1} are for C-H (sp^{3}) stretching's, while the C=O functional groups are

represented by the peak of medium absorbance at 1726 cm^{-1} which represents the presence of the carboxylate group (Fadhile Almansoory et al., 2017).

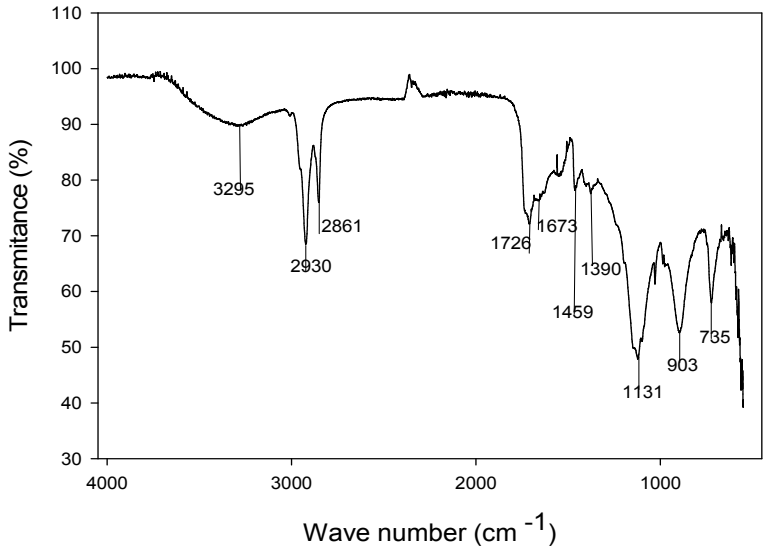

Figure 2. Fourier-transform infrared spectra of the biosurfactant that was produced by the strain PA1

Thin Layer chromatography (TLC)

The TLC results of the biosurfactant extracted from the acid precipitate revealed a pink spot on plates with an Rf value of 0.753 when sprayed with ninhydrin as shown in Figure 3 signifying the presence of amino acids in the biosurfactants as previously reported (Sriram et al., 2011).

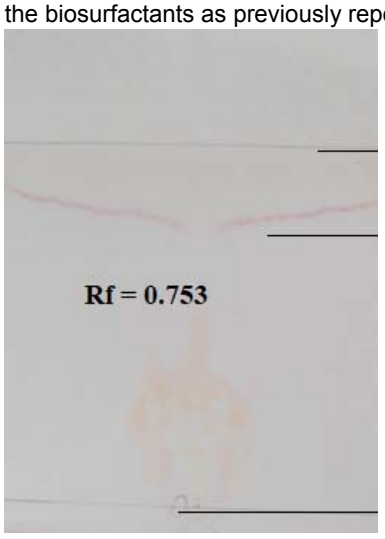

Figure 3. Analysis of Thin Layer Chromatography of the biosurfactant produced by the strain SA1 revealing a pink pigment after spraying with ninhydrin to produce an Rf value of 0.753

3.3 Microbial Growth

The experiments that were supplemented with biosurfactants had high bacterial growth as compared to those without as seen in Figure 4. 28 g/L amendment had the highest bacterial growth of 9.906029 log CFU/mL as compared to 7 g/L with 9.665 and 28 g/L with 9.545. The application of biosurfactants enhanced the growth of bacteria with an increase in every amendment especially from 7 g/L to 14 g/L. But the further increase in biosurfactant concentration from 14 g/L to 28 g/L led to a comparative reduction in growth. The increase in bacterial growth as a result of biosurfactant application and amendments are because biosurfactants improved

the bioavailability of the hydrocarbon pollutants to the bacteria that is used utilized as a carbon and energy source (Ron and Rosenberg, 2002). By reducing surface tension and interfacial tensions biosurfactants increase the surface area of the hydrocarbons which makes it accessible to the available microorganisms (Das and Chandran, 2011). At high biosurfactant concentration of 28 g/L, the bacteria growth was low because the microbes must have been inhibited by the high concentration of the biosurfactants at the beginning of the experiment since such concentrations have been reported to affect the cellular membrane of the bacteria (Bezza and Chirwa, 2017).

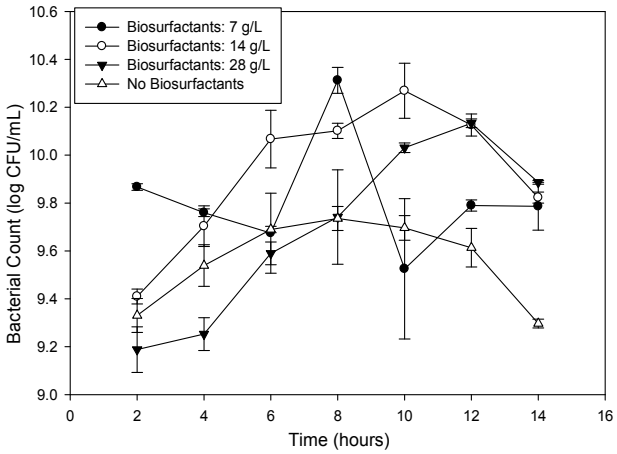

Figure 4: Relationship between biosurfactant supplements and bacterial growth

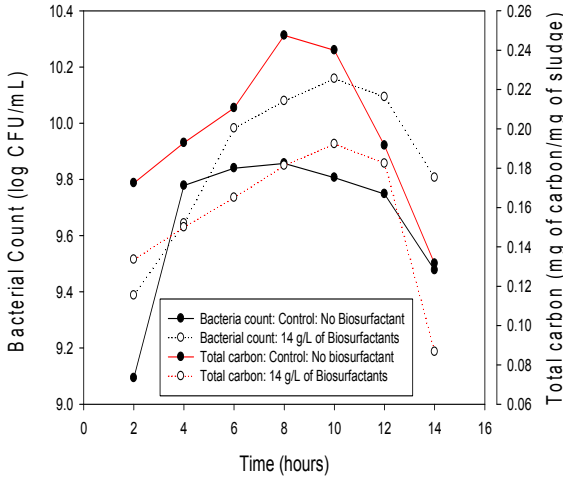

Figure 5: Variation of total carbon with bacteria growth for 14 g/L biosurfactant amendment for a single experiment

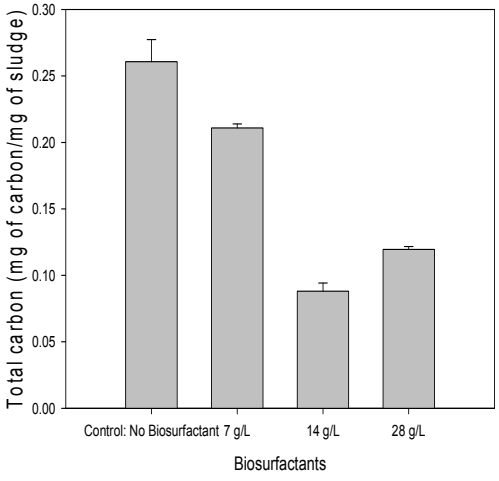

Figure 6: Total carbon left after the experiment for each of the experiments

3.4 Hydrocarbon Degradation

The strain *Serratia* sp. showed great ability in degrading the hydrocarbon pollutants with biosurfactants showing great biodegradation enhancement capabilities. The experiment with 14 g/L of biosurfactants had the highest bacterial growth but also had the lowest carbon content. This is because the bacteria utilized the pollutants as a carbon source which is symbolized by the high microbial growth rates as shown in Figure 5. Much as the experiment supplemented with 28 g/L had growth of the bacteria inhibited at the beginning the subsequent increase in microbial counts towards the end led to a lower reduction of the hydrocarbons as compared to the experiments supplemented with 7 g/L (Figure 6). Comparing experiments supplemented with biosurfactants and those without, it was clearly observed that biosurfactants were able to enhance the

degradation process in general. The biosurfactants enhanced oil sludge biodegradation by 23.38%, 70.28%, and 55.6% for 7 g/L, 14 g/L and 28 g/L respectively in 14 days.

4. Conclusion

The strain SAI identified as *Serratia* sp. has great capabilities of degrading hydrocarbon pollutants. The strain also produces biosurfactants that enhance microbial growth and degradation by increasing mass transfer. It is however important that an optimum dosage of biosurfactants is found since the 14 g/L biosurfactant amendment had the highest degradation as compared to the 28 g/L amendment.

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