

Biosurfactant Production during Saline Oilfield Produced Water Treatment: A Prospect for Coupling Wastewater Management with Enhanced Oil Recovery Applications

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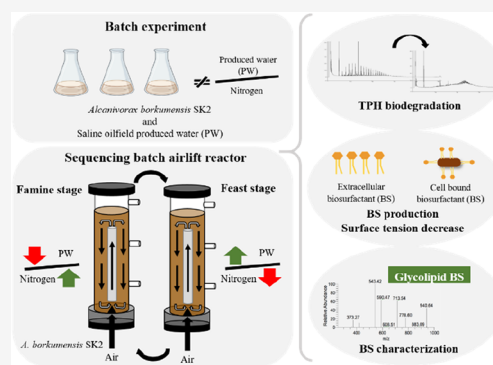
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ABSTRACT: Considerable efforts have been made by the oil industry to develop sustainable strategies for managing produced water (PW) and enhancing oil recovery. This study evaluated biosurfactant (BS) production during PW treatment (0.26 g L⁻¹ of total petroleum hydrocarbon (TPH) and 3.5% salinity) using *Alcanivorax borkumensis* SK2 as an inoculum. In batch experiments (24 and 96 h of incubation), nitrogen concentration was a key factor influencing BS production. A cell-bound and an extracellular BS were produced during the exponential and stationary growth phases, resulting in a surface tension (ST) decrease of up to 27%. BS production scaled up in a sequencing batch airlift reactor (23 days of operation, 3 cycles) showed ST reduction from 72 to 49.6 ± 0.5 mN m⁻¹ (in a 166 h cycle duration), coupled with a TPH removal efficiency of 97%. BS (crude extract) production yield (Y_{P/S}) of 0.059 g g⁻¹ was attained (15 mg L⁻¹ of BS). Chemical characterization identified the BS as a glycine-glycolipid. BS production from PW treatment is a promising strategy for its valorization in applications such as microbial enhanced oil recovery, supporting a circular economy in which the wastewater generated is simultaneously treated, valued, and reused.

KEYWORDS: biosurfactant, glycolipid, sequencing batch airlift reactor, produced water, *Alcanivorax borkumensis* SK2



1. INTRODUCTION

The world's economy is highly dependent on nonrenewable fossil fuels, such as crude oil. Energy demand is projected to rise in the coming years, and despite the growing adoption of renewable energy sources, crude oil is expected to remain the dominant source of energy in the future.¹

In the last decades, the discovery of new oil fields has declined, and consequently, current crude oil exploitation results primarily from mature reservoirs.² As wells age, oil production declines,³ and the conventional recovery methods, such as water injection, are often insufficient to maintain economically viable production.⁴ Thus, the oil and gas industry faces two major problems with mature reservoirs, the generation of large volumes of produced water (PW) containing hazardous organic and inorganic pollutants (e.g., hydrocarbons and salts) that, without proper treatment, can cause serious environmental consequences,⁵ and the decrease of oil recovery efficiencies.²

Oil pollution in the marine environment, as a result of oil discharge and/or oil spills due to offshore installations, has a severe negative impact on marine biodiversity, human and animal health, and, in the long term, leads to a socioeconomic decline of the affected areas.¹ The European Blue Growth has emerged as a long-term strategy designed to support

sustainable growth in marine and maritime sectors, and the marine environmental safety is one of its biggest concerns.¹ As a result, there is a growing need to develop innovative, sustainable technologies, such as bioremediation, to restore marine areas at risk of oil pollution, along with more efficient and eco-friendly solutions for produced water (PW) treatment that deliver maximum degradation in the shortest possible time.

Aerobic biological treatment using hydrocarbonoclastic bacteria (HCB) has proven to be an effective strategy for treating saline oilfield PW.⁶ High treatment efficiency (up to 98% total petroleum hydrocarbon (TPH) removal) has been demonstrated alongside the valorization of PW through lipid production (triacylglycerol (TAG), wax esters (WE), and polyhydroxyalkanoates (PHA)) in a sequencing batch airlift reactor (SBAR), inoculated with *Alcanivorax borkumensis* SK2 and operated under nutrient limiting conditions (e.g., nitrogen

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and oxygen).^{6,7} In biological processes, hydrocarbon uptake by bacteria is difficult due to its low aqueous solubility.^{14,8} To overcome this, several marine HCB have developed strategies, namely, the production of tensioactive molecules as bio-surfactants (BS), to improve hydrocarbon emulsification.^{9,10}

BS are amphiphilic biomolecules and are capable of reducing surface (ST) and interfacial tensions (IFT), stabilizing oil-in-water and water-in-oil emulsions, as well as acting as detergents, solubilizers, and lubricants, thereby improving hydrocarbon bioavailability and biodegradation.¹¹ BSs are classified according to their chemical composition and molecular weight. Generally, BS are classified into glycolipids (e.g., rhamnolipids and sophorolipids), lipopeptides (e.g., surfactin), polymeric BS (e.g., emulsan and alasan), and particulate BS (e.g., vesicles). According to their molecular weight, they are grouped as high-molecular-weight BS (e.g., polysaccharides, proteins, and lipoproteins) or low-molecular-weight BS (e.g., glycolipids, lipopeptides, and phospholipids).¹² The low-molecular-weight BS are generally involved in the decrease of ST and IFT, while the high-molecular-weight BS function as emulsion-stabilizing agents.¹² Both low- and high-molecular-weight BS can be produced as extracellular or as a part of the cell membrane by bacteria.⁹

Glycolipids are among the most common microbial BSs, composed of a carbohydrate linked to a fatty acid molecule.¹³ *A. borkumensis* SK2 produces a low-molecular-weight anionic glycolipid BS in the presence of hydrocarbons, that can be cell-bound (glycine-containing precursor), aiming to increase the cell hydrophobicity and its affinity for oil droplets or extracellular (glycine-lacking form).^{8,14} Microbial BS are considered a promising and sustainable alternative to synthetic BS, with potential applications in remediation technologies as oil spills dispersants and in the microbial enhanced oil recovery process (MEOR).^{2,13} The main advantages of microbial BS are its low toxicity, high selectivity and biodegradability, and the capability to maintain activity at extreme conditions of temperature, pH, and salinity.^{15–17}

The interest in biotechnological approaches within the Oil and Gas industry is growing, with MEOR emerging as a promising economic and environmentally friendly strategy for oil recovery, compared to the commonly used chemical processes.² MEOR consists of a process in which microorganisms (either already present or introduced into the reservoir) and/or their metabolic byproducts (i.e., biopolymers, biosurfactants) are injected into the well to increase oil recovery, thereby extending the reservoir's life.^{2,18} *In situ* BS production at concentrations sufficient to mobilize significant amounts of residual oil has already been demonstrated using specific microorganisms, highlighting their potential for MEOR purposes.^{19,20} HCB, naturally present in the reservoir and with the ability to produce surface-active compounds, can be a suitable option for MEOR. Furthermore, bioremediation has emerged as a sustainable biotechnology strategy that explores the potential of microorganisms and/or their byproducts for the removal of contaminants from the environment. The exploration of the potential of marine microorganisms is vast, as are their applications. BS production by marine microorganisms can be a sustainable strategy for MEOR and for the remediation of oil-polluted marine sites.

In this study, the production of surface-active compounds by *A. borkumensis* SK2 from saline oilfield PW as a C-source was assessed, and the potential to combine PW treatment (in terms of TPH content) with BS production, aiming at its reinjection

into reservoirs for EOR, was successfully demonstrated in a 2 L SBAR. To the best of our knowledge, this integrated approach for treated PW valorization was not demonstrated before. The influence of the total petroleum hydrocarbon-to-nitrogen (TPH/N) ratio (in particular the nitrogen concentration) on BS production was studied, and its chemical characterization was assessed.

2. EXPERIMENTAL SECTION/METHODS

2.1. Strain, Carbon Source, and Seed Culture Preparation. *A. borkumensis* SK2 (DSM number 11573) was grown in saline media (ONR7a) according to DMSZ (Braunschweig, Germany) and used as an inoculum in batch experiments (Erlenmeyer flasks) and in an airlift reactor operated in sequencing batch mode. The ONR7a media²¹ had the following composition: ammonium chloride (NH_4Cl) (0.27 g L^{-1}); boric acid (H_3BO_3) (0.027 g L^{-1}); calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (1.46 g L^{-1}); iron dichloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) (0.002 g L^{-1}); magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (11.18 g L^{-1}); sodium bromide (NaBr) (0.083 g L^{-1}); potassium chloride (KCl) (0.72 g L^{-1}); sodium bicarbonate (NaHCO_3) (0.031 g L^{-1}); sodium chloride (NaCl) (22.79 g L^{-1}); sodium fluoride (NaF) (0.0026 g L^{-1}); sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) (0.089 g L^{-1}); sodium sulfate (Na_2SO_4) (3.98 g L^{-1}); strontium dichloride hexahydrate ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$) (0.024 g L^{-1}); TAPSO ($\text{C}_7\text{H}_{17}\text{NO}_7\text{S}$) (1.30 g L^{-1}).

A concentrate of saline oilfield PW (TPH content of $202 \pm 16 \text{ g L}^{-1}$), obtained from a full-scale physical separation process from Shell (The Netherlands), was used as a carbon source (C-source), and its chemical composition has been previously reported in ref 7. *A. borkumensis* SK2 precultures were performed in Erlenmeyer flasks with 50 mL of ONR7a media containing pyruvate (10 g L^{-1}) as C-source, and incubated on a rotary shaker at 150 rpm and 30°C for 48 h. Subsequently, the cells were harvested by centrifugation at $16,654g$ for 10 min at 10°C in a centrifuge Multifuge X3FR, washed with NaCl (0.9%, w/v), and resuspended in fresh ONR7a media to be used as inoculum for the batch experiments and the SBAR operation. In the last case, the inoculum was prepared in 3 growth stages with a sequential increase in the culture volume in order to achieve a high amount of cellular biomass, as described in ref 6. At the start of the operation, the biomass concentration inside the bioreactor was 0.7 g L^{-1} .

2.2. Batch Experiments. Batch experiments were carried out in Erlenmeyer flasks using ONR7a as culture media, with saline oilfield PW and ammonium chloride (NH_4Cl) serving as carbon and nitrogen sources, respectively, at a 3.5% salinity. The NH_4Cl concentration in the ONR7a media was modified according to the desired total petroleum hydrocarbon-to-nitrogen (TPH/N) ratio. Two different ratios, both with nitrogen in limiting concentration to promote the accumulation of storage compounds, were tested, namely, a TPH/N ratio of 4 (70 mg L^{-1} of nitrogen and 0.26 g L^{-1} of TPH) and a TPH/N ratio of 9 (30 mg L^{-1} of nitrogen and 0.26 g L^{-1} of TPH). *A. borkumensis* SK2 was used as an inoculum. The incubations started with an initial optical density of 0.2 at 600 nm ($\text{OD}_{600 \text{ nm}}$) and were conducted at 30°C , pH 7.8, and 150 rpm. The inoculum volume was 10 and 5 mL for Erlenmeyer flasks with 100 and 50 mL of ONR7a media, respectively.

The batch experiments were performed under sterile conditions. However, the C-source (saline oilfield PW) was

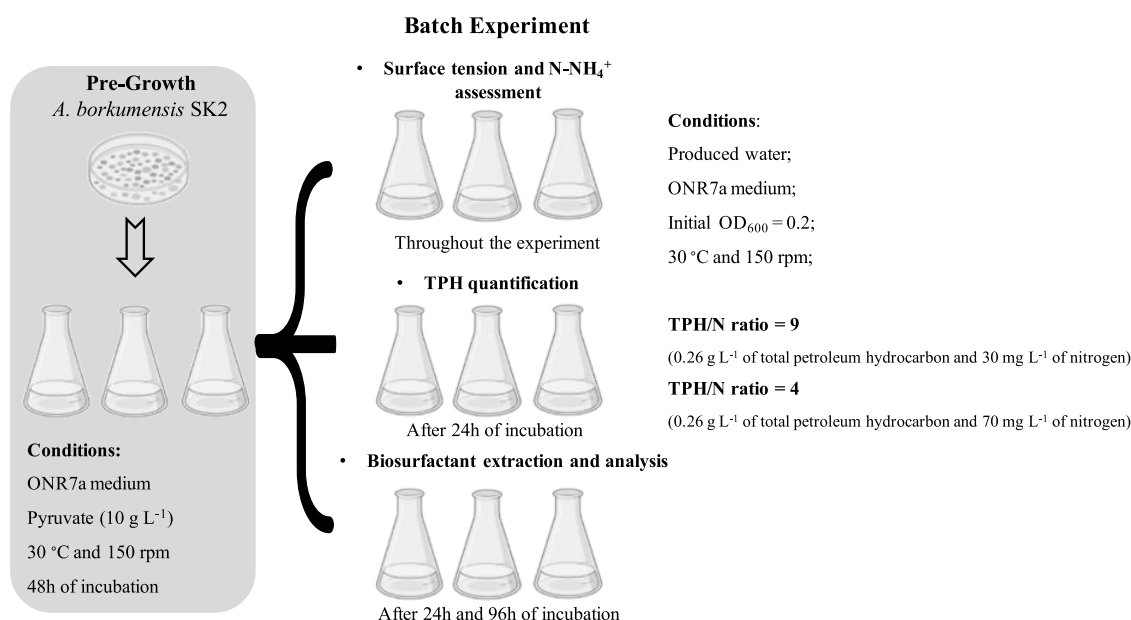


Figure 1. Schematic diagram representing the experimental procedure of batch experiments performed with *A. borkumensis* SK2 and saline oilfield PW as the carbon source.

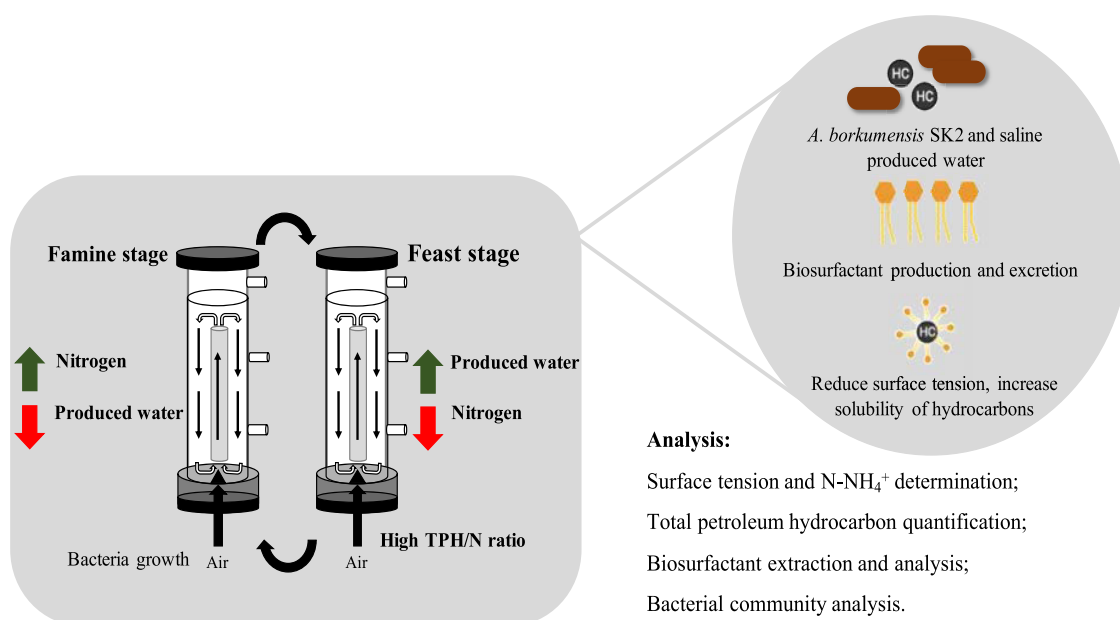


Figure 2. Experiment design of biosurfactant production by *A. borkumensis* SK2 from saline oilfield PW in a SBAR.

added without previous sterilization to avoid compromising its physical and chemical characteristics. BS production and microbial growth were assessed by the determination of ST and ammonium nitrogen (N-NH₄⁺) concentrations, respectively. During the incubation period, 3 mL samples were collected from Erlenmeyer flasks with 100 mL of the ONR7a media (triplicates) for ST measurement and ammonium nitrogen (N-NH₄⁺) quantification. The possible contribution of PW indigenous microbial population was evaluated in a control flask, devoid of inoculum.

TPH removal and the BS chemical structure were also studied during the experiment. At the late exponential (after 24 h of incubation) and stationary phases (after 96 h of incubation), all the contents of 6 extra Erlenmeyer flasks with 100 mL of the ONR7a medium (3 flasks for each time

point) were used for BS extraction and analysis. In parallel, all the contents of 3 Erlenmeyer flasks with 50 mL of ONR7a media (after 24 h of incubation) were extracted for the TPH content determination. The samples collected for TPH quantification were acidified with HCl (5 M) to a pH below 2 and stored at 4 °C until extraction. A scheme of the batch experiments is represented in Figure 1. For each TPH/N ratio tested (4 and 9), triplicates were made for all of the studied parameters, and all data were expressed as the mean ± standard deviation of at least three independent replicates.

2.3. Evaluation of Biosurfactant Production in a Sequencing Batch Airlift Reactor (SBAR). An airlift reactor with a cylindrical acrylic structure was used in the experiment with the following characteristics: 7.5 cm diameter, 60 cm length, and a total and working volume of 2.59 and 2.5 L,

respectively. To ensure constant air flux and the mixture inside the bioreactor, an air diffuser was placed at the bottom of the reactor. Moreover, a draft tube (acrylic material) with 3 cm diameter and 39 cm length was located in the middle of the reactor and approximately 7.5 cm above the bottom (Figure 2).

The airlift was fed with saline oilfield PW (0.26 g L⁻¹ TPH, in ONR7a media) and operated in sequencing batch mode for 23 days at the operational conditions of 25 ± 5 °C, pH 7.8, and 7–8 mg L⁻¹ of dissolved oxygen (DO) concentration. DO concentration was measured with a AD630 DO meter (ADWA, Hungary).

Throughout the operation, the airflow was maintained at approximately 1602 ± 3 mL min⁻¹.

The SBAR was operated during three alternate cycles of the famine and feast stages. The famine stage started with the addition of nitrogen (30 mg L⁻¹) as NH₄Cl to the bioreactor medium in order to stimulate microbial growth. After approximately 48 h of operation, saline oilfield PW (0.26 g L⁻¹ TPH) was added to the bioreactor, starting the feast stage.

The TPH/N ratios applied to the SBAR were determined by dividing the initial TPH concentration added at the beginning of the feast stage (0.26 g L⁻¹) by the N-NH₄⁺ concentration inside the bioreactor at that moment. Thus, in cycles I and II, a TPH/N ratio of 9 was applied to the bioreactor, and a ratio of 11 in cycle III.

Microbial growth was monitored during all of the operations by the decrease in the N-NH₄⁺ concentration inside the bioreactor.

For ST determination, 5 mL samples were collected, and ST was measured in noncentrifuged samples and after centrifugation (10 min, 4 °C, and 17,000g). ST of centrifuged samples was determined in the supernatant and in the biomass resuspended in 1× phosphate-buffered saline (PBS), pH 7.4, and with the following composition: 1.8 mM potassium dihydrogen phosphate (KH₂PO₄), 2.7 mM potassium chloride (KCl), 10 mM disodium hydrogen phosphate (Na₂HPO₄), and 137 mM NaCl.

For BS extraction and analysis, 25 mL of samples were collected during the SBAR operation at the following sampling points: cycle II—56 and 166 h and cycle III—56 and 169 h and stored at 4 °C until further analysis.

For TPH content determination, 30 mL samples were collected from the bioreactor after 54, 78, and 143 h of operation in cycle I, 56, 71, 166 h in cycle II, and 56, 71, 169 h in cycle III. Afterward, the TPH samples were acidified with HCl (5 M) to a pH below 2 and stored at 4 °C.

After the stabilization of N-NH₄⁺ concentration in each cycle (end of the feast stage), approximately 1.5 L of the bioreactor media (supernatant and biomass) was removed. Then, fresh ONR7a media was added to the remaining media inside the bioreactor, up to a total volume of 2.5 L, and a new cycle was started. The process scheme can be found in Figure 2.

Since the bioreactor operation was not conducted under sterile conditions, other bacterial species beyond *A. borkumensis* SK2 (seed culture), initially present in saline oilfield PW, may also have developed during the operation. Therefore, at the end of the feast stage of cycle II and III, 15 mL samples were centrifuged (Multifuge X3FR for 10 min, at 16,654g and 4 °C) and the pellet resuspended in 1× PBS. Subsequently, the DNA was extracted and the bioreactor bacterial community was analyzed by 16S rRNA gene sequencing, as previously

described in ref 7. Sequencing reads were deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB46493 samples SAMEA 8990568 (cycle II) and SAMEA 8990569 (cycle III).

2.4. Analytical Methods. 2 mL samples were taken at different time intervals during batch and SBAR operations and centrifuged at 16,654g for 10 min in a centrifuge Multifuge X3FR. The commercial kits LCK 304 and 338 (Hach Lange, Germany) were used for total soluble (TSN) and total nitrogen (TN) determination, respectively. The N-NH₄⁺ concentration inside the bioreactor was measured in the supernatant using the kit LCK 304. Biomass concentration was estimated from cell organic nitrogen content using the empirical formula C₅H₇O₂N (113.1 g mol⁻¹) for heterotrophic microbial biomass. Accordingly, organic nitrogen was calculated as (TN-N-NH₄⁺), and biomass was estimated using the following equation:

$$\text{biomass concentration (g L}^{-1}\text{)} = (\text{TN-N-NH}_4^+) \times \frac{113.1}{14.0} \quad (1)$$

The bioreactor airflow was monitored using a gas mass flow controller (MC-5 SLPM-D/M, ALICAT scientific), and pH was measured using a benchtop meter (inoLabVR pH 7110, WTW, Germany) and manually controlled by adding HCl (5M) (no sterile) with a syringe to the bioreactor.

TPH extraction and analysis were performed according to the international standard method for determining hydrocarbon oil index (ISO 9377-2) with the modifications mentioned in ref 7. Briefly, the TPH content was analyzed by liquid–liquid extraction with hexane as an organic solvent and quantified by gas chromatography equipped with a flame ionization detector (GC-FID) in a GC Clarus 580 for PerkinElmer. The GC-FID specifications and the method conditions used were previously described and can be found in ref 7.

2.5. Surface-Activity Measurements. ST measurements (in triplicate) were performed using the Du Noüy ring (1.9 cm) method that measures the force of the wetted length acting on a ring as a result of the tension of the withdrawn liquid lamella when moving the ring from one phase to another. The measurements were performed in a KRÜSS K20 Tensiometer at 21 ± 5 °C, as described elsewhere.¹⁵ All of the values of surface tension reported here are the maximum values on a plot of surface tension against ring displacement. Statistical analysis was performed by applying a *t*-test with Microsoft Excel (*p* < 0.05).

2.6. Biosurfactant Extraction and Purification. BS produced by *A. borkumensis* SK2 can be excreted into the culture media (extracellular BS) or kept attached to the cell wall (cell-bound BS).⁸ The production of both BS types was assessed in this study. The samples taken during batch and SBAR experiments were centrifuged at 16,654g for 10 min (centrifuge Multifuge X3FR) for BS analysis. Extracellular BS production was evaluated by analyzing the cell-free supernatants of 100 and 25 mL samples in the batch and SBAR operations, respectively. The cell-bound BS was analyzed by extracting the cell pellet with 1× PBS according to the following procedure: the cell pellet was washed twice with 25 mL of demineralized water, and the cells were resuspended in 30 mL of 1× PBS and incubated for approximately 4 h at 150 rpm at room temperature (21 ± 5 °C). Then, the cellular content in 1× PBS (cell-bound BS), as well as the cell-free

supernatants (extracellular BS) were mixed individually with chloroform: methanol (2:1, v/v) in a separation funnel (1:1 v/v) for 2 min and left to rest for 30 min for the separation of the aqueous (upper phase) and organic phases (bottom phase). Afterward, the organic phases were dried by applying a light nitrogen stream in a TurboVapLV, Biolage (51 °C for 40 min). The obtained dried extracts were resuspended in chloroform (0.5 mL). Subsequently, BS production was studied by thin-layer chromatography (TLC) and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). Once *A. borkumensis* SK2 is able to accumulate and excrete neutral lipids,⁶ the crude BS extracts (extracellular and cell-bound) obtained from batch experiments were further purified to remove lipidic compounds and other impurities. Thus, a 125 mL column was filled with silica gel 60 (resuspended in chloroform) up to two-thirds of its capacity and left to equilibrate. The crude extracts, resuspended in chloroform, were added to the column, which was further washed with the following solutions (at a flow rate of approximately 3 mL min⁻¹): chloroform, chloroform: methanol (180:5, v/v), (180:10, v/v), (180:15, v/v), (180:20, v/v), and methanol. The purification scheme and corresponding elution volumes of each solution used are presented in Table 1. The fractions

Table 1. Elution Scheme for Biosurfactant Purification

fraction Label	elution solution	volume (mL)
fraction 1	chloroform	200
fraction 2	chloroform/methanol (180:5, v/v)	200
fraction 3	chloroform/methanol (180:10, v/v)	200
fraction 4	chloroform/methanol (180:15, v/v)	200
fraction 5	chloroform/methanol (180:20, v/v)	200
fraction 6	methanol	100

recovered with each solution were dried in a TurboVapLV (Biolage) under the conditions mentioned above and resuspended in 0.5 mL of the corresponding elution solution used in the purification process. Each fraction was analyzed by TLC in order to identify those containing BS. Afterward, the BS was quantified gravimetrically, and its chemical characterization was assessed by ESI-MS.

2.7. Biosurfactant Analysis and Chemical Characterization. **2.7.1. Thin-Layer Chromatography (TLC).** The BS crude extracts and the respective fractions obtained after the purification process were analyzed by TLC in silica gel plates (60 F254 20 cm × 20 cm, Merck) using chloroform: methanol: water (65:25:4, v/v/v) as the mobile phase. The visualization was performed by evenly spraying the TLC plate with a solution containing orcinol (0.19%, w/v) in sulfuric acid (53%, w/v) and incubating at 105 °C until the plots became visible. A commercial mixture of rhamnolipids (RLs) (R90, Sigma-Aldrich Co.) containing monorhamnolipids (mono-RLs) and dirhamnolipids (di-RLs) was used as a standard. In order to compare the samples, the retention factor (R_f) value of each visible band was calculated by dividing the distance between the origin (where the sample was placed in the TLC plate) and the point where the spot emerged by the total distance run by the solvent. The purified fractions containing bands that could correspond to BS were analyzed again by TLC with chloroform: methanol: water (65:25:4, v/v/v) as the mobile phase and with primulin as the reveal agent. The identified bands were scraped from the TLC plate and extracted from silica using a dichloromethane and methanol solvent system.

Then, the samples were filtered and dried under a nitrogen stream. Finally, the samples, as well as the commercial mixture of rhamnolipids (RLs), were analyzed by ESI-MS.

2.7.2. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and Electrospray Ionization-Mass Spectrometry (ESI-MS). The functional groups of the BS crude extracts (cell-bound and extracellular) resuspended in 0.5 mL of chloroform were identified by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) as described in ref 7. Chemical characterization of compounds present in the different bands extracted from TLC plates (Section 2.7.1) was carried out by mass spectrometry (MS) using electrospray ionization (ESI) obtained in a Q-ToF 2 (Micromass, Manchester, U.K.). For ESI-MS analysis, the samples were resuspended in several volumes of chloroform (100–500 μ L) and acquired in positive mode. ESI conditions in the electrospray Q-ToF mass spectrometer were as follows: the electrospray voltage was 3 kV in the positive mode with a cone voltage of 30 V. The source and desolvation temperatures were 80 and 150 °C, respectively. MS/MS spectra were recorded using argon as collision gas and energy between 30 and 40 V. Data was acquired using a Mass Lynx data system (V4.0).

2.8. Statistical Analysis. Significant differences between the data obtained were evaluated by applying a unique factor analysis of variance (ANOVA) using Microsoft Excel. Statistical significance threshold was set at $p < 0.05$ level.

3. RESULTS AND DISCUSSION

3.1. Effect of Nitrogen Concentration on Biosurfactant Production by *A. borkumensis* SK2 from Saline Oilfield PW. The ability of *A. borkumensis* SK2 to produce BS in the presence of saline oilfield PW was studied in batch mode (Erlenmeyer flasks). The capability of this HCB to accumulate storage compounds in response to nutrient limitations in the environment was previously demonstrated.^{22,23} Nitrogen availability was identified as the most relevant factor promoting neutral lipid accumulation (TAG and WE) by *A. borkumensis* SK2 during saline oilfield PW treatment.⁶ Nevertheless, the influence of the TPH/N ratio, specifically the nitrogen concentration, on BS production by this bacterium is poorly explored. Therefore, two different TPH/N ratios of 4 (0.26 g L⁻¹ of TPH and 70 mg L⁻¹ of nitrogen) and 9 (0.26 g L⁻¹ of TPH and 30 mg L⁻¹ of nitrogen) were applied to evaluate the effect of nitrogen concentration on the production of BS by *A. borkumensis* SK2.

Microbial growth and BS production were studied by monitoring the N-NH₄⁺ consumption (Figure 3A) and the ST decrease (Figure 3B,C) throughout the experiment, respectively. It was observed that the N-NH₄⁺ concentration decreased over the first 24 h of incubation for both TPH/N ratios tested. After this period, the N-NH₄⁺ concentration stabilized at approximately 51.3 ± 0.5 and 6.9 ± 0.7 mg L⁻¹ for the TPH/N ratios of 4 and 9, respectively (Figure 3A). This consumption profile indicates that hydrocarbon biodegradation occurred within the first 24 h (Figure 3A), since at this time point, TPH removal efficiencies of 89 ± 0.1 (TPH/N = 4) and 86 ± 1% (TPH/N = 9) were achieved (Table 2) (remaining TPH concentration of 29–37 mg L⁻¹).

The TPH/N ratio of 4 resulted in the highest biomass concentration (0.66 ± 0.03 g L⁻¹) and nitrogen consumption rate (1.09 ± 0.08 mg L⁻¹ h⁻¹), which was expected given the higher nitrogen concentration (70 mg L⁻¹) added to stimulate

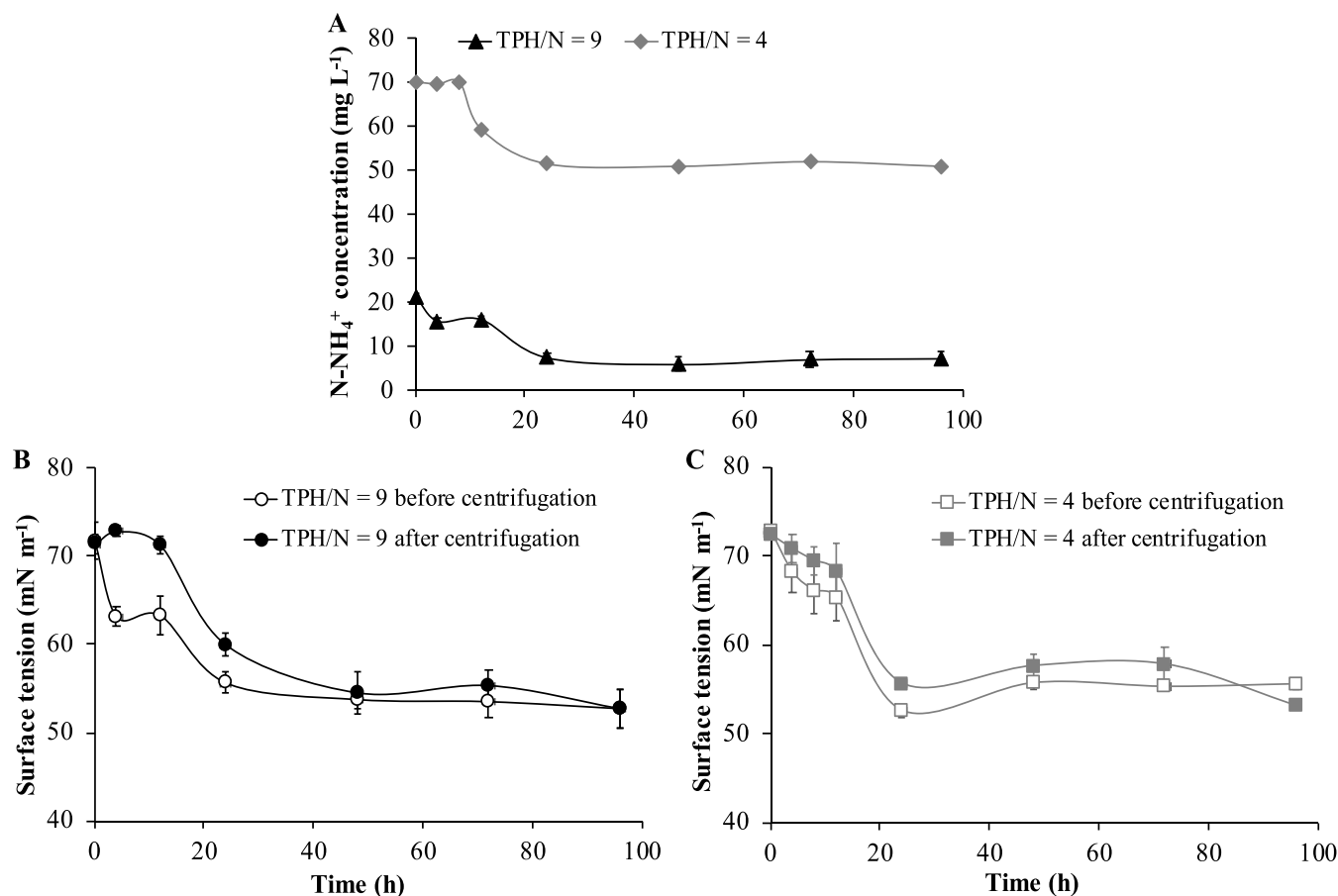


Figure 3. (A) Ammonium nitrogen (N-NH_4^+) consumption profile (mg L^{-1}). (B, C) Surface tension (ST) values (mN m^{-1}) obtained during batch experiments with *A. borkumensis* SK2 and saline oilfield PW for TPH/N ratios of 9 and 4, respectively. The error bars correspond to the standard deviation of three independent measurements.

Table 2. Nitrogen Consumption Rate, Biomass Concentration, Surface Tension Values (before and after Centrifugation), and TPH Removal Efficiency and Rate Achieved for TPH/N Ratios of 4 and 9 in the Batch Assays Performed with *A. borkumensis* SK2 and Saline Oilfield PW

TPH/N ratio	nitrogen (mg L^{-1})	TPH (g L^{-1})	nitrogen consumption rate ($\text{mg L}^{-1} \text{h}^{-1}$)	biomass ^a (g L^{-1})	surface tension ^b (mN m^{-1})		TPH removal efficiency ^c (%)	TPH removal rate ^c ($\text{mg L}^{-1} \text{h}^{-1}$)
					before centrifugation	after centrifugation		
4	70	0.26	1.09 ± 0.08	0.66 ± 0.03	52.6 ± 0.7	55.7 ± 0.5	89 ± 0.1	4.8 ± 0
9	30	0.26	0.73 ± 0.09	0.55 ± 0.06	55.6 ± 1.2	59.9 ± 1.3	86 ± 1	3.5 ± 0

^aBiomass concentration obtained in batch assays (TPH/N = 4 and 9) after 24 h of incubation. ^bMinimum ST values obtained in batch assays (TPH/N = 4 and 9) after 24 h of incubation. The initial ST value was $72.3 \pm 1.4 \text{ mN m}^{-1}$. ^cTPH removal efficiency and rate obtained after 24 h of incubation in batch assays for a TPH/N ratio of 4 and 9.

bacterial growth (Table 2). In contrast, the TPH/N ratio of 9, with a limiting nitrogen concentration (30 mg L^{-1}), yielded a lower biomass concentration and a reduced nitrogen consumption rate after 24 h of incubation ($0.55 \pm 0.06 \text{ g L}^{-1}$ and $0.73 \pm 0.09 \text{ mg L}^{-1} \text{h}^{-1}$, respectively) (Table 2).

In the control flask, used to evaluate the possible contribution of the PW indigenous microbial population, no significant growth (as indicated by the minimal change in N-NH_4^+ concentration from 70 to 68 mg L^{-1}) or surface tension variation ($72\text{--}70 \text{ mN m}^{-1}$) was detected.

Throughout the experiment, the ST profile followed the N-NH_4^+ consumption for both TPH/N ratios tested (Figure 3). The ST values were significantly reduced by 27 and 23% (after 24 h of incubation), decreasing from $72.3 \pm 1.4 \text{ mN m}^{-1}$ (ONR7a media ST, control) to 52.6 ± 0.7 (TPH/N = 4) and

$55.6 \pm 1.2 \text{ mN m}^{-1}$ (TPH/N = 9) ($p < 0.05$) (Figure 3B,C and Table 2). After 24 h of incubation, hydrocarbon dispersion in the aqueous phase became evident, with ST values stabilizing at the minimum, along with the N-NH_4^+ concentration, which also reached its lowest level during the same period (Figure 3). Olivera et al.¹⁰ reported a ST reduction up to 50 mN m^{-1} after 48 h of *A. borkumensis* SK2 growth was observed in the presence of hexadecane. In this work, the ST decreased to $54.1 \pm 2.1 \text{ mN m}^{-1}$ after 24 h of incubation in the presence of real wastewater from the Oil and Gas industry.

After sample centrifugation, higher ST values were recorded for both TPH/N ratios tested (Table 2). Specifically, ST values (after 24 h) increased from 52.6 ± 0.7 to $55.7 \pm 0.5 \text{ mN m}^{-1}$ and from 55.6 ± 1.2 to $59.9 \pm 1.3 \text{ mN m}^{-1}$ for TPH/N ratios

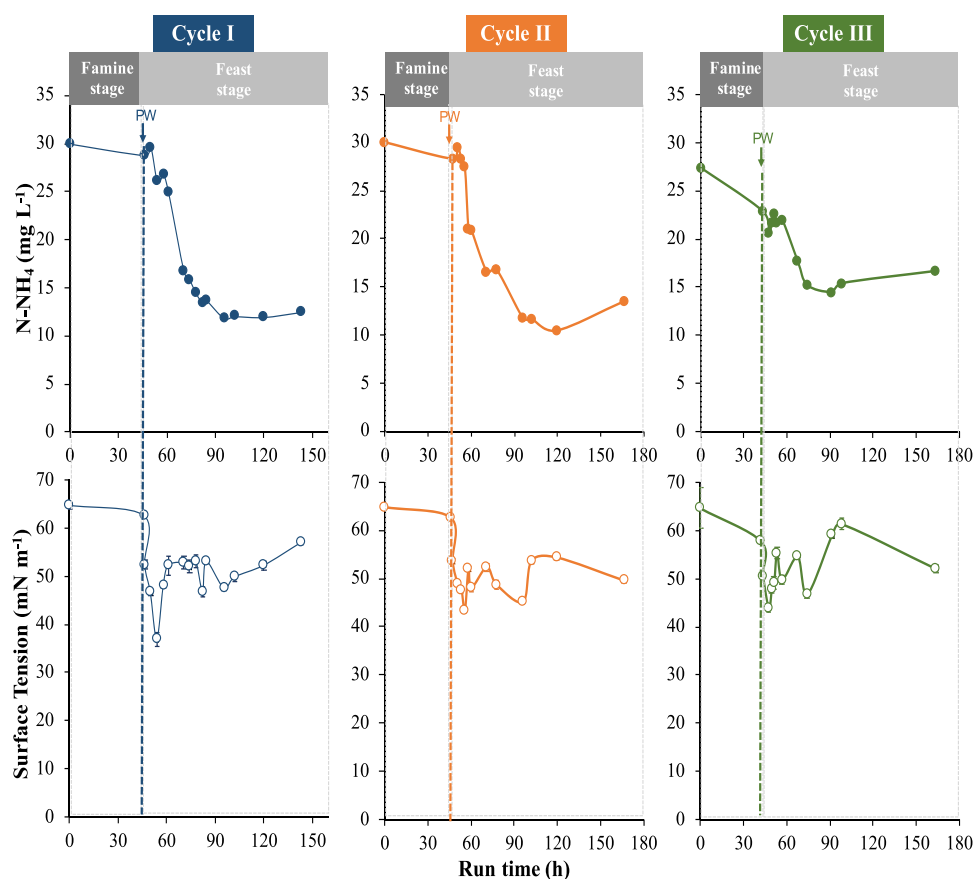


Figure 4. Ammonium nitrogen (N-NH_4^+) (mg L^{-1}) and surface tension (mN m^{-1}) (noncentrifuge samples) profile throughout the SBAR operation with *A. borkumensis* SK2 and saline oilfield PW. Three independent cycles (feast and famine stage) were made. A TPH/N ratio of 9 was applied to cycles I and II and a TPN/N ratio of 11 to cycle III. The surface tension values correspond to the average of three independent measurements $\pm$ standard deviation.

of 4 and 9, respectively ($p < 0.05$; Table 2). The lowest ST values observed with a TPH/N ratio of 4 ($p < 0.05$), compared to a ratio of 9, indicate that nitrogen concentration in the medium plays a critical role in the production of surface-active compounds by *A. borkumensis* SK2 under batch conditions. A lower C-to-N ratio resulted in lower ST values ($52.6 \pm 0.7 \text{ mN m}^{-1}$) and, consequently, higher TPH removal efficiency ($89 \pm 0.1\%$) after an incubation period of 24 h (Table 2). High C/N ratios are known to promote the formation of storage compounds (i.e., triacylglycerides and wax esters) in *A. borkumensis* SK2.⁶ These byproducts have the same precursor molecules from *de novo* fatty acid synthesis as BS, directly competing with BS production.²⁴ Nevertheless, the obtained results offer a new possibility for the use of *A. borkumensis* SK2 in biotechnological applications, particularly for the bioremediation of oil-contaminated sites, since the nitrogen addition can increase BS production (lower ST values) and hydrocarbon dispersion, thereby improving bioremediation rates. Nutrient stimulation (e.g., nitrogen and phosphorus) has been used in petroleum bioremediation strategies, resulting in higher biodegradation rates.²⁵ For instance, Wei et al.²⁶ reported an improvement of approximately 76% on the TPH removal with the application of an integrated biochar, biosurfactant (i.e., rhamnolipids), and nitrogen treatment strategy for the bioremediation of oil-contaminated coastal wetland soils.

Furthermore, the lower ST values observed prior to sample centrifugation (for both TPH/N ratios of 4 and 9) suggest the production of a cell-bound BS that contributes actively to ST

reduction. The TLC profile of the crude BS extracts (both extracellular and cell-bound BS) from experiments performed with a TPH/N ratio of 4 (after 24 and 96 h of incubation), provided further insights into the BS structure (see the Supporting Information). A commercial mono-RL and di-RL mixture served as a standard, and a single spot with a R_f value of approximately 0.47 was observed in each sample of the crude BS extracts (see the Supporting Information). The ability of *A. borkumensis* SK2 to produce a glucose-lipid BS in the presence of *n*-alkanes is well documented in the literature. Abraham et al.²⁷ reported that *A. borkumensis* produces two forms of a glycolipid: a glycine-containing precursor, linked to the cell surface (cell-bound BS), and a glycine-lacking form that is released from the cell to the surrounding media (extracellular BS).

In the present work, both cell-bound (BS-PBS-24h and BS-PBS-96h) and extracellular (BS-24h and BS-96h) BS were detected during the late exponential growth and stationary phases. The cell-bound BS extracted in PBS (BS-PBS-24h and BS-PBS-96h) reduced the ST of PBS by 34% and 23%, decreasing from 72.4 ± 0.3 (PBS ST value) to 48.1 ± 0.2 and $56.1 \pm 0.3 \text{ mN m}^{-1}$, respectively ($p < 0.05$).

3.2. Evaluation of Biosurfactant Production during SBAR Operation Treating Saline Oilfield PW. BS production was also assessed during the operation of a SBAR treating the saline oilfield PW. N-NH_4^+ consumption and ST profile (noncentrifuged samples) are presented in Figure 4, while overall bioreactor performance is summarized in Table 3.

Table 3. TPH/N Ratio, Feast and Famine Stage Intervals, Nitrogen Consumption Rate, Biomass Concentration, Surface Tension Measurements, and TPH Removal Efficiency and Rate throughout the SBAR Operation

cycle	TPH/N ratio ^a	feast stage duration (h)	famine stage duration (h)	nitrogen consumption rate ^b (mg L ⁻¹ h ⁻¹)	time ^c (h)	biomass (g L ⁻¹)	surface tension ^d (mN m ⁻¹)	TPH removal efficiency (%)	TPH removal rate ^e (mg L ⁻¹ h ⁻¹)
I	9	97	47	0.39	54	n.d. ^f	36.9 ± 1.4	n.d.	n.d.
					78	n.d.	53.2 ± 1.3	n.d.	n.d.
					143	0.65	57.0 ± 0.5	n.d.	n.d.
II	9	119	47	0.28	56	0.53	43.4 ± 0.5	29 ± 19	9.0 ± 5.8
					71	0.58	52.3 ± 0.8	62 ± 17	6.8 ± 1.8
					166	0.65	49.6 ± 0.5	97 ± 0.03	2.1 ± 0
III	11	120	44	0.17	56	0.55	43.9 ± 0.8	49 ± 9	16.7 ± 3.1
					79	0.57	46.8 ± 0.7	59 ± 8	5.0 ± 0.6
					169	0.61	52.0 ± 0.9	97 ± 0.9	2.1 ± 0

^aTPH/N ratio in each cycle. The ratio was calculated by dividing the TPH concentration added (0.26 g L⁻¹) to the bioreactor by the nitrogen concentration measured at the beginning of the feast stage. ^bCorresponds to the nitrogen concentration consumed since the beginning of the feast stage (PW addition) until the N-NH₄⁺ stabilization. ^cCycles duration in hours since the beginning of the famine stage (nitrogen addition to the bioreactor). ^dSurface tension values of noncentrifuged samples. ^eThis rate was calculated by dividing the TPH concentration consumed at each sampling point by the duration in hours of the feast stage. ^fn.d.—not determined.

An adaptation period of the bacterial community to the SBAR conditions (cycle I) was performed; thus, TPH biodegradation (TPH removal rate and efficiency) and BS analysis were only evaluated in cycles II and III.

N-NH₄⁺ consumption started after the addition of saline oilfield PW to the bioreactor (feast stage beginning) (Figure 4). N-NH₄⁺ consumption rates were higher in cycles I (0.39 mg L⁻¹ h⁻¹) and II (0.28 mg L⁻¹ h⁻¹) compared to cycle III (0.17 mg L⁻¹ h⁻¹), probably due to the higher TPH/N ratio of 11 (lower nitrogen concentration) applied in the last cycle (Table 3). Biomass concentration increased along the operation, reaching a maximum of 0.65 g L⁻¹ (cycle I and II) and 0.61 g L⁻¹ (cycle III) at the end of the feast stage (Table 3).

In contrast to batch experiments, the ST profile during the SBAR operation did not follow the N-NH₄⁺ consumption trend. Instead, ST dropped immediately upon C-source addition to the bioreactor (beginning of feast stage), decreasing by approximately 19%, from 64.3 ± 3.3 mN m⁻¹ (ST value during the famine stage) to 52.2 ± 1.4 mN m⁻¹ (ST value after the addition of C-source) ($p < 0.05$) (Figure 4). This initial reduction likely resulted from the hydrophobic hydrocarbon molecules present in the saline oilfield PW. Afterward, ST continued to decrease, reaching the minimum value of 36.9 ± 1.4, 43.4 ± 0.5, and 43.9 ± 0.8 mN m⁻¹ after 54 h in cycle I and 56 h in cycles II and III, respectively (Table 3), followed by a dynamic fluctuation in the ST values within the ranges of 46.8 ± 1.0–57.0 ± 0.5 mN m⁻¹ (cycle I), 45.1 ± 0.4–54.3 ± 0.5 mN m⁻¹ (cycle II), and 46.8 ± 0.7–61.4 ± 1.2 mN m⁻¹ (cycle III) (Figure 4). With the addition of saline oilfield PW, there was an excess of hydrocarbons inside the bioreactor, which probably triggered the production of tensioactive compounds and, consequently, the ST reduction.

The genetic annotation of biosurfactant biosynthesis in *A. borkumensis* SK2 remains unclear; however, some genes are reported as potentially involved in its synthesis and transportation to the cell membrane, being upregulated in the presence of hydrocarbons and downregulated in their absence,²⁸ which can explain the observed results. The maximum TPH removal rates were attained after 56 h of operation (9.0 ± 5.8 and 16.7 ± 3.1 mg L⁻¹ h⁻¹ in cycles II and III, respectively), which corresponded to the sampling points where the minimum ST values were obtained, decreasing subsequently (Table 3). Moreover, the maximum

TPH removal efficiency obtained was approximately 97% at the end of the feast stage of cycles II and III (Table 3) (TPH in the treated effluent in the range of 8–9 mg L⁻¹).

The cell-bound (BS-PBS) and extracellular (BS) crude extracts collected during cycles II (56 and 166 h) and III (56 and 169 h) were further analyzed by TLC (see the Supporting Information). A single spot was detected in the crude extracts of both cycles, namely, II-BS-56h, II-BS-PBS-166h, and II-BS-166h in cycle II and III-BS-56h, III-BS-PBS-169h, and III-BS-169h in cycle III, with the same R_f value (0.47) as the one detected in the TLC of batch experiments, suggesting that a similar BS was produced in both operation mode (see the Supporting Information). A BS (total crude extract, cycle II) production of approximately 15 mg L⁻¹ was obtained, corresponding to a production yield ($Y_{P/S}$) of 0.059 g g⁻¹.

Summarizing, the TLC results demonstrated the presence of the extracellular BS at 56 and 166 h of operation in cycle II and after 56 and 169 h in cycle III. Despite the fact that the lowest ST values were attained after 56 h of operation (Figure 4), the TLC profile showed the presence of the cell-bound BS only at the end of the feast stages (II-BS-PBS-166h and III-BS-PBS-169h) (see the Supporting Information). Furthermore, in cycle II, the ST value of the extracellular BS sample (supernatant) (II-BS-56h) was 34% lower (44.1 ± 2.7 mN m⁻¹) than the obtained for the cell-bound BS after centrifugation and extraction with PBS (II-BS-PBS-56h) (66.4 ± 1.3 mN m⁻¹) ($p < 0.05$), hence suggesting that the extracellular BS was the main responsible for the decrease of the media ST until 56 h of operation. It is reported in the literature that low-molecular-weight BS can significantly reduce ST from 72 to 35 mN m⁻¹ and the IFT of *n*-hexadecane from 40 to 1 mN m⁻¹, while high-molecular-weight BS, such as bioemulsifiers (BE), are less efficient in reducing ST (72 to 40–50 mN m⁻¹) but play an important role in the formation and stabilization of emulsions.^{29–31} Although the ST values achieved in this study are similar to the ones reported for BE (72 to 40–50 mN m⁻¹), the emulsifying activity with hexadecane as a hydrophobic substrate in samples collected in cycles II (II-BS-56h and II-BS-166h) and III (III-BS-56h and III-BS-169h) did not exhibit the formation of oil-in-water (O/W) emulsions (data not shown).

Bacterial access to hydrophobic substrates, such as hydrocarbons, depends on the cell surface hydrophobicity. Many bacteria can regulate this property through the production of

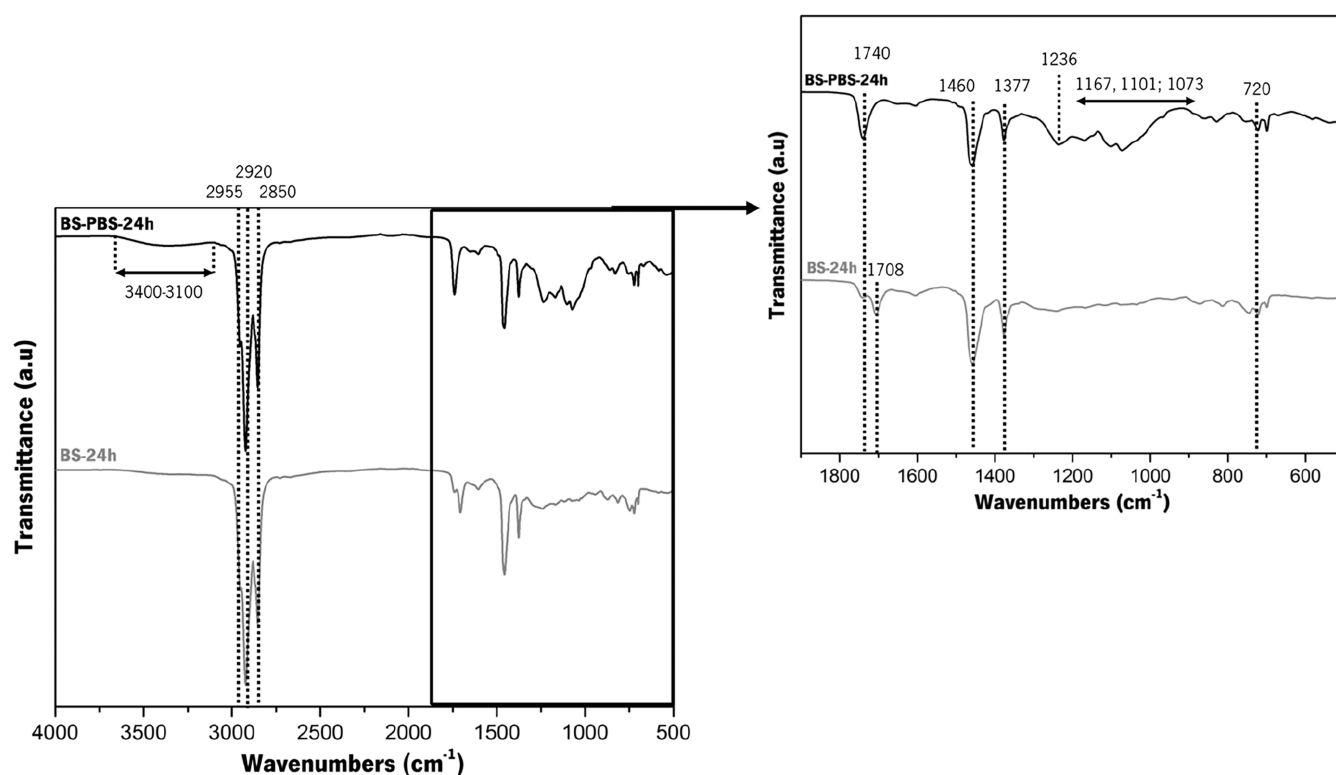


Figure 5. Spectrum of the cell-bound (BS-PBS-24h) and extracellular (BS-24h) crude extracts after 24 h of incubation in batch assays (TPH/N = 9).

cell-bound BS, which can locate its hydrophobic moieties outwardly or inwardly, leading to changes in the hydrocarbon access mode during the different growth stages.³² For example, in *Gordonia* sp. BS29, the cell surface is hydrophobic during the early exponential growth phase in *n*-hexadecane due to the presence of a cell-bound BS.³³ However, during the late exponential growth phase, the cell surface hydrophobicity is reduced, and at the same time, *Gordonia* sp. BS29 releases an extracellular BE allowing the hydrophilic cells to attach to the hydrophilic outer layer of the emulsified oil droplets.³³ Similarly, Abraham et al.²⁷ reported that the cell-bound BS produced by *A. borkumensis* SK2 likely serves as precursors to extracellular BS, enhancing cell hydrophobicity and, consequently, its affinity to oil-suspended droplets. In the case of the extracellular BS, its main function is to promote oil–water emulsions, thereby improving hydrocarbon bioavailability.⁸

Indeed, cell-bound BS have been linked to ST decrease by increasing cell hydrophobicity and its adherence to the oil/water interface.³⁴ Higher cell surface hydrophobicity allows cells to adhere more effectively to alkane droplets, enhancing uptake and promoting the degradation of water-insoluble substances.³⁵ This aligns with the results from the batch experiments conducted in this study, where both cell-bound and extracellular biosurfactants (BS) were detected in crude extracts at 24 and 96 h (see the [Supporting Information](#)). However, this pattern was not observed in the SBAR system, as cell-bound BS was not detected at the sampling point corresponding to the lowest surface tension (ST) value, recorded at 56 h of operation (see the [Supporting Information](#)). In this case, the continuous flux of air, promoting mixing inside the bioreactor, may hinder the attachment of *A. borkumensis* SK2 to the oil-suspended droplets. As a result, BS produced is likely excreted into the

extracellular medium to enhance hydrocarbon availability, which likely explains the observed decrease in surface tension at 56 h of operation. During the feast stage, the hydrocarbons present in the C-source were consumed, reducing the need for BS production and excretion. As a result, the cell-bound form remained associated with the cell surface, explaining its detection only at the end of the feast stage. Moreover, while BS enhances the bacterium's affinity for suspended oil droplets and promotes hydrocarbon emulsification, its presence on the cell surface may also contribute to outer membrane functionality,²⁷ which may explain its detection during the late exponential phase.

The obtained results suggest that the bioreactor's hydrodynamic conditions can influence both the production and excretion of BS by *A. borkumensis* SK2, as well as the bacterium's interaction with hydrocarbons in its environment.

Besides the main band observed in the TLC analysis (R_f 0.47), other bands with R_f values of 0.5 and 0.8 were also detected at the end of the feast stages of cycles II (II-BS-PBS-166h and II-BS-166h) and III (III-BS-PBS-169h and III-BS-169h), indicating the possible presence of structurally distinct BS compounds (see the [Supporting Information](#)). Lipphardt et al.³⁵ reported the production of several glycine-glucolipid congeners by *A. borkumensis* when grown in pyruvate and hexadecane as C-sources. Several congeners differing in chain length (C6–C12) and degree of unsaturation (0–1 double bonds) of the bound 3-hydroxy-alkanoic acids were identified.³⁵

Alcanivorax was the dominant genus in the SBAR bacterial community, with relative abundances of 89% (cycle II) and 84% (cycle III). *Mesonnia* was the second most abundant genus detected, with 5–9%, followed by *Thalassospira* with 1.4%. Other taxa detected (up to 41) showed relative abundances

significantly lower than 1%. BS production was, therefore, attributed to *Alcanivorax*. The ability of *A. borkumensis* SK2 to produce BS likely explains its dominance in bacterial communities, particularly during oil spill events. A detailed table with all the identified bacterial taxa is available in the [Supporting Information](#).

3.3. Biosurfactant Chemical Characterization. ATR-FTIR analysis of the cell-bound and extracellular crude extracts produced during both batch (TPH/N ratios of 4 and 9) and SBAR operations revealed similar spectra, indicating that neither the operational mode nor the TPH/N ratio significantly influenced the type of compounds produced. Thus, only the spectra of the cell-bound (BS-PBS-24h) and extracellular (BS-24h) BS of batch incubation (TPH/N = 9) (lower ST values) are shown in [Figure 5](#).

Both spectra displayed characteristic peaks associated with fatty acids, including bands at 2955 cm^{-1} ($\nu_{\text{as}}(\text{CH}_3)$), 2920 cm^{-1} ($\nu_{\text{as}}(\text{CH}_2)$) and 2850 cm^{-1} ($\nu_{\text{s}}(\text{CH}_3)$), as well as acyl chains peaks at 1460 cm^{-1} ($\delta_{\text{as}}(\text{CH}_3)$) and 720 cm^{-1} ($\rho(\text{CH}_2)$) (Silva et al.³⁸) ([Figure 5](#)). The BS-PBS-24h spectrum shows a highly intense peak of the ester group $\nu(\text{C}=\text{O})$ in conjugated fatty acids at 1740 cm^{-1} .^{36,7} The same absorption peak was detected in the BS-24h spectrum but with lower intensity. Moreover, both spectra showed absorption peaks at 1377 cm^{-1} attributed to the C–O stretching vibration of carbohydrates³⁷ ([Figure 5](#)). Additionally, the BS-24h spectrum exhibited a distinct band at 1708 cm^{-1} , characteristic of the absorption frequency of the carbonyl group in aliphatic esters ($\nu(\text{C}=\text{O})$), indicating the presence of other lipophilic compounds in the sample^{6,38} ([Figure 5](#)). Previous studies have demonstrated the ability of *A. borkumensis* SK2 to produce and release neutral lipids (TAGs and WEs) to the extracellular media when grown in saline oilfield PW.⁶ The reason why neutral lipids are discharged is not well understood; however, it has been reported that the release of TAGs and WEs creates a hydrophobic microenvironment around the cells, which improves the bioavailability and uptake of hydrophobic compounds, such as hydrocarbons.³⁹

In the BS-PBS-24h spectrum, it is possible to observe a peak at 1236 cm^{-1} assigned to the stretching vibration in phosphorus compounds ($\nu_{\text{as}}(\text{P}=\text{O})$), suggesting the presence of phospholipids³⁶ ([Figure 5](#)). Additionally, defined absorption peaks in the carbohydrate region ($1200\text{--}900\text{ cm}^{-1}$), corresponding to the C–O–C asymmetric stretching vibration of polysaccharides, were observed, as well as the O–H stretching peak in the range of $3400\text{--}3100\text{ cm}^{-1}$; these functional groups are characteristics of carbohydrates.^{40,41}

Together, the ATR-FTIR spectral features indicate the presence of a glycolipid biosurfactant composed of a carbohydrate conjugated with a fatty acid, which aligns with the TLC results ([Supporting Information](#)). A summary of the most relevant peaks herein described and the corresponding molecular assignments is provided in the [Supporting Information](#).

To confirm the BS chemical structure, the BS crude extracts (BS-PBS-24h and BS-24h) were purified through silica column chromatography, and the different fractions (as described in [Table 1](#)) were recovered and analyzed through TLC. TLC analysis showed bands in fractions 4 and 6 of the cell-bound BS and fractions 3 and 6 of the extracellular BS, with R_f values different from those of the rhamnolipid standards (see the [Supporting Information](#)), indicating that a structurally different BS was produced by *A. borkumensis* SK2. In fraction 6 (more

hydrophilic fraction), two bands (Fr 6A and B) with high intensity were detected in both cell-bound and extracellular BS ([Supporting Information](#)). The bands observed in all fractions were scraped and analyzed by ESI-MS. All spectra showed abundant ions at m/z 381.4, 710.8, and 738.9, characteristic of phthalates (see the [Supporting Information](#)). These compounds are a class of lipophilic chemicals widely used as plasticizers, and they can be found in laboratory products (e.g., reagents, solvents, and consumables), but they can also be synthesized naturally by bacteria.⁴² Phthalates produced by microorganisms have been reported to function as biologically active compounds, enhancing microbial competitiveness within their communities.⁴² The obtained results did not allow us to determine whether the detected phthalates were synthesized by *A. borkumensis* SK2 or originated from laboratory materials used during the extraction procedure. The spectrum of Fr 6A from the extracellular BS showed a molecular ion with high intensity at m/z 940.7 (see the [Supporting Information](#)). The same ion was also detected in Fr 6B from the cell-bound BS (see the [Supporting Information](#)) but with a lower intensity. The spectrum of the main ion (m/z 940) showed several series of fragments that can be found in [Figure 6](#). A similar

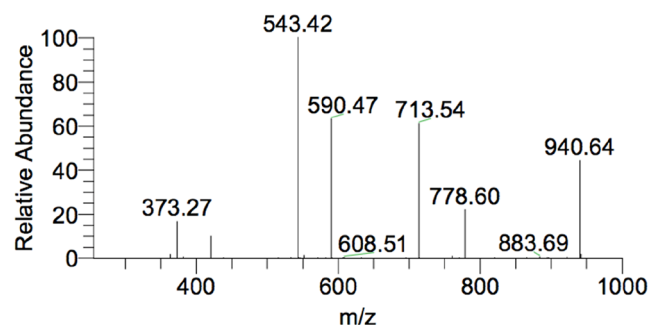


Figure 6. ESI-MS/MS spectrum of ion at m/z 940.6.

fragmentation pattern of m/z 940 was obtained for both Fr 6A extracellular and Fr 6B cell-bound BS, indicating that both BS species have a common structure. The molecular ion species detected, shown in [Figure 6](#), differ from those observed in the rhamnolipid standards spectrum (data not shown), which confirms the TLC results that a structurally different compound was produced. From the $[\text{M} - \text{Na}]^+$ ion at m/z 940, the main sequence of fragments started with a loss of m/z 227 (m/z 713.5) followed by a repeated loss of 170 amu (m/z 543.4 and 373.3) ([Figure 6](#)). Moreover, a loss of 162 amu (m/z 778.6) was also observed, which could correspond to a glycosyl moiety such as a dehydrohexose, which corroborates the ATR-FTIR results of glycolipid production. The fragmentation pattern is similar to the one reported by Abraham et al.²⁷ More recently, Lipphardt et al.³⁵ described the production of several congeners with alterations at the amide linkage in the pyruvate culture extract of *A. borkumensis*. The ionization and fragmentation patterns in that study closely resemble those observed during growth in oilfield produced water.

The main glycine-glycolipid produced by this bacterium has the following chemical structure: 18-(1- β -glucopyranosyl)-6,10,14-triheptyl-4,8,12,16-tetroxy-3-aza-7,11,15-trioxa-pentaeicosanoic acid.²⁷ In addition, the fragmentation pattern of the $[\text{M} + \text{Na}]^+$ ion at m/z 940 showed a neutral loss of 57 amu to yield m/z 883 ([Figure 6](#)). Abraham et al.²⁷ reported the

same fragment (m/z 882), which corresponded to the loss of $C_2H_2O_2$ from the terminal glycine in the BS molecule. The glycolipid extracted from the cell wall had the same structure as the extracellular BS with the exception of having the N-terminal esterified with glycine.²⁷

The purified glycolipid BS corresponding to fractions 6 (cell-bound and extracellular BS) was quantified. *A. borkumensis* SK2 was able to produce approximately 3.7 and 6.4 mg L⁻¹ of cell-bound and extracellular BS, respectively, within 24 h of batch incubation (TPH/N ratio = 9).

Over the past decades, BS have been applied in the bioremediation of oil-contaminated marine environments since it can be a more sustainable and environmentally friendly option compared with chemical surfactants. The use of hydrocarbon-degrading and BS-producing bacteria has emerged as a green and promising strategy for bioremediation.⁴³ Biostimulation and bioaugmentation of petroleum-contaminated marine environments are two of the most common bioremediation techniques applied.²⁵ The addition of BS to contaminated sites can lead to biodegradation efficiencies ranging from 50 to 97%.²⁵ Additionally, introducing biosurfactant-producing microorganisms into petroleum-contaminated environments can increase hydrocarbon solubilization and bioavailability, leading to biodegradation efficiencies between 32.67 and 87.54%.²⁵ In this study, TPH removal up to 89 and 97% was achieved during batch and SBAR operation, respectively, coupled with BS production, demonstrating *A. borkumensis* SK2 has potential for bioremediation applications. Furthermore, both BS and its bacterial producers have been reported as suitable candidates for MEOR applications. Recent work by Deng et al.⁴⁴ demonstrated the emulsifying EOR potential of *A. borkumensis* glycolipid BS in a core flooding experiment, achieving a 20% increase in oil recovery rate.

Declining oil recovery rates and the generation of large volumes of oilfield PW, are some of the biggest problems currently faced by the oil and gas industries. The production of glycolipid BS by *A. borkumensis* SK2 during oilfield PW treatment presents a valuable opportunity for process integration and resource valorization. Reusing treated water and leveraging the biosurfactant-rich effluent for reinjection into reservoirs can contribute to both enhanced oil recovery and more sustainable wastewater.

The combined treatment of saline oilfield produced water and biosurfactant production presents a promising approach to integrating green and blue economy principles, aligning with several United Nations Sustainable Development Goals (SDGs). Biosurfactants, produced from renewable sources such as microorganisms and waste products, offer a sustainable alternative to synthetic, fossil-fuel-derived surfactants, supporting SDG 12 (responsible consumption and production). This shift reduces carbon emissions and encourages environmentally friendly industrial practices, contributing to SDG 9 (industry, innovation, and infrastructure). Moreover, the process addresses the challenge of managing saline produced water, a major byproduct of oil extraction. By treatment of this wastewater, making it safer for reuse or discharge, it supports SDG 6 (clean water and sanitation). Simultaneously, the recovery of valuable biosurfactants enhances resource efficiency and promotes a sustainable blue economy, contributing to SDG 14 (life below water) by protecting aquatic ecosystems and marine biodiversity.

One of the primary limitations in the broader application of BS is the typically low product yield and the cost associated with recovery and purification. With the proposed recycling strategy, the need for BS recovery and purification is eliminated, which significantly reduces the application costs. Moreover, this approach supports a circular economy model, where wastewater from the Oil & Gas industry operations is not only treated but also repurposed, maximizing resource efficiency and environmental benefits.

4. CONCLUSIONS

This study demonstrated the ability of *A. borkumensis* SK2 to produce a glycolipid biosurfactant (BS) that actively reduces surface tension in saline oilfield produced water. Batch experiments showed that the nitrogen concentration influenced BS production, with lower TPH/N ratios (higher nitrogen concentrations) resulting in greater ST reduction and higher TPH biodegradation rates after approximately 24 h of incubation. Treatment of saline oilfield PW, in terms of TPH content, in a sequencing batch airlift reactor coupled with BS production was successfully accomplished (TPH removal efficiencies up to 97%). The extracellular BS was identified as the primary agent responsible for ST reduction to 43.4 ± 6.5 mN m⁻¹ after 56 h of operation. Reactor hydrodynamics were found to influence both BS production and excretion by *A. borkumensis* SK2, and how this bacterium interacts with hydrocarbons in the surrounding environment.

The simultaneous treatment of saline oilfield PW and production of glycolipid BS offer a promising route for resource recovery and wastewater reuse. Reinjection of the BS-containing bioreactor effluent into reservoirs may serve as a cost-effective and eco-friendly strategy for enhancing oil recovery while supporting sustainable water management in the Oil and Gas sector. Field-simulated experiments should be conducted to further validate and optimize this approach.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsestwater.5c00466>.

Additional results: TLC analysis of the biosurfactant extracts and purified fractions; ESI-MS spectra of the purified fractions; functional groups assignment to the bands found in ATR-FTIR spectra; and 16S rRNA gene sequencing analysis of the SBAR bacterial community. (PDF)

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Notes

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