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Production and Application of a New Biosurfactant for Solubilisation and Mobilisation of Residual Oil from Sand and Seawater

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Abstract: Significant research has been conducted to minimise environmental impacts and promote the sustainable use of resources and raw materials. Microbial surfactants are an example of advanced materials obtained from sustainable production processes. In the present study, a biosurfactant was produced by the yeast *Starmerella bombicola* ATCC 22214 grown in a previously selected low-cost mineral medium containing 10% sucrose, 1.2% canola oil, and 0.5% corn steep liquor. The biosurfactant reduced surface tension from 72 ± 0.1 to 32.76 ± 0.3 mN/m. The yield was 23 g/L, and the critical micelle concentration was 0.6 g/L. The biosurfactant emulsified 96.25 ± 0.08% of used motor oil, was characterised as a sophorolipid, and exhibited stability under extreme conditions with no significant loss of its properties. Toxicity was assessed by exposing the microcrustacean *Artemia salina* and the zebrafish (*Danio rerio*) to the biosurfactant. The biosurfactant proved efficient for use in remediation processes, removing 97.8% and 69.2% of the petroleum derivative from sand in kinetic and static tests, respectively, and removed 91.5% of the contaminant from seawater. The results indicate the potential of this new biosurfactant for the mobilisation and solubilisation of hydrocarbons in the marine environment. This green biomolecule is a promising technology for the replacement of chemical dispersants in the remediation of aquatic and soil systems.

Keywords: *Starmerella bombicola*; biosurfactant; bioemulsifier; toxicity; oil derivative; bioremediation

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1. Introduction

Oil and its derivatives have played a central role in driving the economy for more than a century [1]. The primary sources of water and soil contamination by petroleum hydrocarbons are oil spills and the improper disposal of oily waste generated from leaks during the production, drilling, extraction, transportation, and storage of oil. This includes damage to pipelines and leaks from underground storage tanks [2]. When released into the environment, oil hydrocarbons spread horizontally, affecting a large area, and are decanted due to the action of gravity, resulting in the blockage of pores in the soil [3]. These durable, stable contaminants remain in the environment for long periods of time and do not easily undergo degradation [4].

Separation from the soil is difficult for two main reasons: (1) contaminants can be strongly absorbed by organic matter and encapsulated in minerals in the soil, which hampers the remediation process; and (2) soil is a complex, heterogeneous system and mass transfer processes play an important role in separation. Organic and inorganic colloids, flora, fauna, and microorganisms in the soil can also interfere with remediation [3,5].

Different methods are used to treat oily wastewater, such as flotation, sedimentation, coagulation, filtration, ultrafiltration, and reverse osmosis. As water and oil are practically immiscible, they are easy to separate. Water containing suspended, emulsified, or solubilised residual oil is treated and sent to an appropriate destination [6].

As environmental contamination with oil hydrocarbons constitutes a considerable threat to human health, different technologies are employed to remediate contaminated sites. Environmental remediation constitutes a set of methods used to mitigate the harmful effects of these pollutants. However, such methods involve the use of synthetic surfactants [7], which are highly toxic [8]. Thus, replacing synthetic compounds with natural products has attracted growing interest.

Concerns related to the depletion of fossil fuels have shifted the focus toward sustainable alternatives to conventional chemical processes that rely on petroleum [9]. The advancement of biorefinery practices has facilitated the obtainment of fuels, polymers, and chemicals produced from microbial sources [10]. Fermentation techniques that employ microbial strains enable the obtainment of high-value products, such as vitamins, carboxylic acids, biofuels, enzymes, etc. Natural products derived from microbial cells offer ecological compatibility and also require less land and water [11]. An environmental biodiversity analysis revealed that 99% of microbes exist in the form of consortia and have applications as functional ingredients in different industries [9,12].

There are approximately one million types of products of a natural origin. One-quarter of these are biologically active compounds. More than half are derived from plants, and the remainder are obtained from microorganisms, which produce about 23,000 secondary metabolites, with fungi accounting for 42% and filamentous bacteria accounting for 32% [13]. These statistics demonstrate the considerable biodiversity and utility of microorganisms for industrial purposes. Natural products constituted approximately 52% of all novel chemical products that received approval from the US Food and Drug Administration between the years 1981 and 2006 [14]. Numerous strategies have been developed and put into practice to obtain byproducts from microorganisms. However, a small number of microbial-based natural products are employed in the industrial sector [15], which underscores the need to establish a commercial market for bioproducts derived from microbes.

Biosurfactants constitute a valuable by-product obtained from microbial cells and have become increasingly competitive with synthetic surfactants derived from petroleum [16]. The increasing interest in microbial surfactants is due to the fact that such compounds are expected to replace synthetic petroleum surfactants, such as sodium dodecylbenzenesulfonate (SDBS), which have a slow degradation process. For instance, sulfonate surfactants are produced from alkyl aryl hydrocarbons, such as dodecylbenzene, which have a linear hydrocarbon structure without an aromatic component, and degradation takes three to eight days, compared to the 24 days that synthetic petroleum surfactants require [17].

Biosurfactants are biomolecules with hydrophobic and hydrophilic portions. The hydrophobic portion has long-chain fatty acids, whereas the hydrophilic portion may be an amino acid, carbohydrate, phosphate carboxyl acid, cyclic peptide, or alcohol [18]. This amphipathic nature gives biosurfactants surface-active properties that enable the reduction of surface tension and interfacial tension in aqueous solutions and mixtures with hydrocarbons. These metabolites are synthesised by yeasts, bacteria, and filamentous fungi of the genera *Candida*, *Pseudomonas*, *Bacillus*, *Starmerella*, *Rhodococcus*, and others [19–21].

The advantages of biosurfactants over synthetic surfactants include lower toxicity, greater biodegradability, and high stability over a broad range of pH, salinity, and temperature values. Thus, these biomolecules have applications in a variety of fields, including the food, biomedical, and environmental industries [22]. Moreover, the critical micelle concen-

tration (CMC) of biosurfactants is lower. The CMC is the lowest amount of a surfactant needed to achieve the greatest reduction in surface tension, improving economic efficiency in different applications.

The properties of biosurfactants include humectation, emulsification, foaming, phase solubilisation, and dispersion. Depending on the combination of molar mass, mode of action, and physicochemical properties, biosurfactants can have low or high molecular weight. Those with low molecular weight reduce surface tension and interfacial tension, whereas those with high molecular weight are denominated bioemulsifiers and are more effective at ensuring the stabilisation of emulsions [18,23]. Biosurfactants generally have a molar mass ranging from 500 to 1500 Da [24]. With regards to the chemical composition, these biomolecules can be glycolipids (rhamnolipids, mannosylerythritol lipids, trehalose lipids), lipopeptides (surfactins, fengycins, lichenysines), glycolipopeptides, glycoproteins, phospholipids, neutral lipids, polymeric biosurfactants (emulsan, alasan, biodispersan), and particular biosurfactants (protein–sugar–lipid complex molecules) [25].

Biosurfactants can be used to enhance the washing and removal of lyophilic contaminants through displacement and solubilisation. Displacement occurs when the concentration of biosurfactant is below the CMC, whereas solubilisation occurs at higher concentrations. With the displacement mechanism, biosurfactant molecules accumulate at the interface between the soil and pollutant or between water and soil, causing the soil to roll and changing the affinity of the system for water. The adsorption of biosurfactant molecules to the surface of the contaminant results in repulsion forces between the main chemical groups of the contaminant and the soil particles, enhancing the separation of the contaminant. With the solubilisation mechanism, the contaminant is incorporated into the micelles formed by the presence of the biosurfactant, favouring partitioning towards the water phase. Pollutants that partition towards micelles can be recovered and demulsified, electrochemically destroyed, or adsorbed to activated carbon. The washing solution (biosurfactant) can be discarded or recycled to reduce remediation costs. Biosurfactants also increase the bioavailability of organic pollutants in the soil to microbial cells by lowering the surface tension of the aqueous phase, thereby facilitating biodegradation [26].

The compound annual growth rate of the global biosurfactant market is expected to be 0.8%, increasing from USD 1.3754 billion in 2020 to USD 1.4427 billion in 2026, although the cost of producing a biosurfactant is not yet compatible with the market demand. Foaming during processing, limited yields, expensive raw materials, and costs related to purification and downstream processing constitute some of the challenges to face when producing biosurfactants on an industrial scale. To solve this problem, industrial and agricultural byproducts have been used as substrates. Moreover, the use of waste products minimises pollution and reduces waste treatment costs. Statistical approaches and engineering methods have also been successfully used to reduce costs and optimise biosurfactant production operations [18,27].

The viability of industrial biosurfactant production depends on the target market. Production can only be performed on a small scale for food, medicinal, and cosmetic products due to the purification steps required. The use of crude fermentation broths, on the other hand, could be a viable solution for environmental applications, which do not require a purification step. Moreover, biosurfactants for such applications can be produced from microorganisms grown in media containing a combination of low-cost carbon sources, ensuring economic and environmental sustainability [28]. Thus, crude biosurfactants are promising for environmental remediation and wastewater treatment, taking advantage of the hydrophobicity of the microbial cell surface, which is an essential aspect of biodegradation [29].

The main challenges for biosurfactants are related to technical and economic issues. Technical problems include low production yields, low product concentration, high mass intensity, and high specific energy demands [30,31]. Economic issues arise from biosurfactant separation and purification processes, which involve technologies such as foam fractionation, membranes, gravity separation (e.g., acid precipitation, crystallization), and

ultrafiltration, all of which have high capital and operating costs [32]. These challenges have hindered the widespread replacement of chemical surfactants with biosurfactants. Various process improvements have been proposed, including metabolic engineering, bioprocess engineering, chemical engineering, and process engineering. For example, genetically modified microorganisms (GMOs) have been developed to increase the yield of biosurfactants [33]. Hybrid production schemes, which integrate product formation and separation, have also been studied to reduce processing steps and production costs, but these methods are still in the research stage [34]. Few studies have focused on the conceptual design of biosurfactant production processes from a techno-economic and environmental perspective to explore potential technologies for upscaling. However, it is important for the development of biosurfactants to expand beyond the academic sector, as the industrial development of biosurfactants is linked to establishing a circular bioeconomy by using renewable sources for production [35]. The increasing development of biosurfactants is driven by current laws and policies regulating the use of environmentally harmful chemicals. Such laws and policies have been announced and implemented throughout the world, further promoting the shift towards the use of sustainable eco-friendly products [22]. For example, BASF SE (Ludwigshafen, Germany) has entered partnership agreements with Allied Carbon Solutions Co. Ltd. (Numazu, Japan) and Holiferm Ltd. (Manchester, United Kingdom) to advance sustainable biosurfactant production for the personal and home care sector. These actions set an example to encourage the production of bio-based products in line with policies, laws, and financial incentives [36].

The research addressed in this paper focuses on technical and environmental information regarding the production of a new biosurfactant. The aim was to produce a biosurfactant from the yeast *Starmerella bombicola* ATCC 22214 and evaluate its properties, toxicity, and safety for use in remediating soil and water contaminated with an oil derivative. In the following sections, we will explain how we were able to achieve high yields in the production of this novel biosurfactant using low-cost substrates. This biomolecule is highly efficient, non-toxic, and environmentally friendly, and can be used without requiring multiple purification steps.

2. Materials and Methods

2.1. Microorganism, Maintenance Medium, and Growth Medium

The yeast *S. bombicola* ATCC 22214 purchased from the American Type Culture Collection was used for biosurfactant production and was maintained in yeast mould agar (YMA) medium at 5 °C. The medium was composed of distilled water (100 mL) with peptone (0.5%), agar (2%), D-glucose (1%), and yeast extract (0.3%), pH 7.0. To maintain viability, transfers to fresh agar slants were performed every month. The growth medium was yeast mould broth (YMB), with the same composition as the YMA medium, but without the agar.

2.2. Growth of Inoculum

To standardise the inoculum, the culture was transferred to a tube containing the YMA medium at 28 °C, obtaining a young culture, and the sample was placed in a 250 mL Erlenmeyer flask with YMB medium (50 mL). Incubation was performed under aerobic conditions, with constant agitation (150 rpm) for 48 h at 28 °C. The mixture was diluted until reaching 10^4 cells/mL. A Neubauer chamber was used for the cell count. The inoculum was used at a concentration of 5% (v/v).

2.3. Production Media and Culture Conditions

Different media (500 mL) were used for biosurfactant production based on descriptions in the literature (Table 1). Erlenmeyer flasks with a capacity of 1000 mL were used for fermentation to produce biosurfactants. The pH was adjusted to 6.0. An autoclave operating at 121 °C was used to sterilise the media (15 min), which were then incubated under aerobic conditions with 5% of the pre-inoculum and kept under orbital agitation at 200 rpm for

192 h at a temperature of 28 °C. The pH of the media was not adjusted during cultivation. All analyses were performed in triplicate and did not vary more than 5%.

Table 1. Composition of biosurfactant production media.

Medium	Components of Production Medium (g/L)
Medium 1	Sunflower oil: 50; Glucose: 25; Yeast extract: 1; KH_2PO_4: 0.5; $MgSO_4 \cdot 7H_2O$: 0.5; $NaNO_3$: 3
Medium 2	Sunflower oil: 50; Sucrose: 25; Yeast extract: 1; KH_2PO_4: 0.5; $MgSO_4 \cdot 7H_2O$: 0.5; $NaNO_3$: 3
Medium 3	Canola oil: 12; Glucose: 100; Corn steep liquor: 5; K_2HPO_4: 1; $(NH_4)_2SO_4$: 4; $MgSO_4 \cdot 7H_2O$: 0.5
Medium 4	Canola oil: 12; Sucrose: 100; Corn steep liquor: 5; K_2HPO_4: 1; $(NH_4)_2SO_4$: 4; $MgSO_4 \cdot 7H_2O$: 0.5
Medium 5	Canola oil: 12; Glucose: 100; Corn steep liquor: 5
Medium 6	Canola oil: 12; Sucrose: 100; Corn steep liquor: 5
Medium 7	Crude cotton seed oil: 100; Glucose: 100; Urea: 1.5; K_2HPO_4: 1; Corn steep liquor: 4; NaCl: 0.1
Medium 8	Refined cotton seed oil: 100; Glucose: 100; Urea: 1.5; K_2HPO_4: 1; Corn steep liquor: 4; NaCl: 0.1
Medium 9	Crude cotton seed oil: 100; Sucrose: 100; Urea: 1.5; K_2HPO_4: 1; Corn steep liquor: 4; NaCl: 0.1
Medium 10	Refined cotton seed oil: 100; Sucrose: 100; Urea: 1.5; K_2HPO_4: 1; Corn steep liquor: 4; NaCl: 0.1

2.4. Isolation of Biosurfactant

Liquid–liquid extraction was used to isolate the biosurfactant. For such, ethyl acetate (proportion of 1:4 (*v*/*v*) with the non-centrifuged medium) was used twice. After centrifugation of the organic phase at 2600× *g* for 20 min, filtration was performed, and the filtrate was placed in a separatory funnel with a solution of saturated sodium chloride (NaCl) for separation of the remaining aqueous phase. Next, the organic phase was placed into an Erlenmeyer flask, followed by the addition of anhydrous magnesium sulphate ($MgSO_4$) until the formation of granules. Filtration was then performed using a qualitative paper filter (Whatman No. 1), followed by drying at a temperature of 50 °C [37].

2.5. Biosurfactant Characterisation

The ionic charge of the biosurfactant was determined using the agar double diffusion method [38]. Two rows of wells were prepared in 1% agar. The wells of one row received the isolated biosurfactant solution, and the wells of the other row received a pure compound, the ionic charge of which was known. The anionic substance was sodium dodecyl sulphate (SDS) (20 mM), and the cationic substance was barium chloride (50 mM). The ionic nature of the biosurfactant was indicated by precipitation lines between the wells after monitoring for 48 h at room temperature.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses were performed in a 500 MHz spectrometer (Bruker INOVA, Varian, Palo Alto, CA, USA). For such, 20 mg of the isolated biosurfactant was dissolved in deuterated chloroform (CDCl3; Sigma-Aldrich, Taufkirchen, Germany) (500 µL) at 300 MHz and 298.1 K. The ppm scale relative to normal tetramethylsilane (TMS) was considered for the analysis of chemical shifts (δ). Fourier transform infrared (FTIR) spectroscopy (400 Perkin Elmer) was also used to characterise the isolated biosurfactant in the wavenumber range of 4000 to 400 (resolution: 4 cm^{-1}).

2.6. Surface Tension

A Sigma 700 Tensiometer (KSV Instruments Ltd., Helsinki, Finland) with a du Noüy ring was used at room temperature for the automatic measurement of surface tension in samples of the cell-free broth (crude biosurfactant) centrifuged at 10,000× *g* for 15 min.

2.7. Emulsification Index (E_{24})

Two mL of the crude biosurfactant solution (cell-free broth) were placed in a screw-top tube (100 mm × 13 mm) to which 2 mL of used motor oil was added for the determination of emulsification activity following the method proposed by Cooper and Goldenberg [39]. Motor oil used as a contaminant was obtained from a local automotive manufacturer in the city of Recife, Brazil. This oil is commercially available for use in flex engines (gasoline, CNG, and alcohol) and is labelled SAE 20W-50 with synthetic blend (PETROBRAS, Rio de Janeiro, Brazil). It is composed of a paraffinic base lubricating oil (a complex mixture of hydrocarbons) and includes performance-enhancing additives. The viscosity of the oil is 98.0 cSt (at 40 °C) and its density is 0.9420 g/mL (at 20 °C). The contents of the tube were mixed in a vortex at 50 Hz for two minutes. The emulsification index (E_{24}) was determined after 24 h using Equation:

$$E_{24} = (he/ht) \times 100$$

in which: he = height of emulsion layer; ht = total height of mixture measured with a ruler in mm. The samples were stored at 27 °C [40].

2.8. Effect of Environmental Factors

The effects of environmental factors on surface tension and emulsification activity in solutions of the crude biosurfactant (cell-free broth) were investigated as described in Sections 2.6 and 2.7, considering different temperatures (0, 5, 28, 70, 100, and 120 °C) for 60 min, pH (2.0, 4.0, 6.0, 8.0, 10.0, and 12.0) after adjustments with HCl or NaOH 6.0 M, and concentrations of NaCl (2, 4, 6, 8, 10, and 12%) at 28 °C [41].

2.9. Determination of Critical Micelle Concentration

The critical micelle concentration (CMC) was determined by diluting sodium hydroxide (NaOH) in a small fraction of distilled water, to which the crude extract of the biosurfactant was added at a proportion of 1:7 (*v*/*v*) ratio. The product was washed in acetone, followed by filtration through a sintered glass filter and drying for evaporation of the solvent. The product (0.1 g) was successively diluted with distilled water, followed by the quantification of surface tension with the aid of the KSV Sigma 700 tensiometer and du Noüy ring up to a constant value (standard deviation less than 0.4 mN/m during 10 successive measurements) to obtain the CMC, which was expressed as g/L of biosurfactant. The ring method involves raising the liquid until it touches the surface. The sample is then lowered again to stretch the film formed beneath the liquid to determine maximum force, which is then used to calculate surface tension. The instrument was calibrated using Mill-Q-4 ultrapure distilled water from Millipore (Burlington, MA, USA). Before use, the platinum plate and all glassware were washed with chromic acid, deionized water, and acetone in sequence and then flamed with a Bunsen burner. Samples were read three times for accuracy [37].

2.10. Toxicity Test with Artemia salina as Indicator

Brine shrimp (*Artemia salina*) larvae were used as the bioindicator in the toxicity test of the biosurfactant. Eggs of the microcrustacean were purchased from a local store, and larvae were obtained after 24 h of incubation. Ten larvae were placed in 10 mL flasks with seawater (5.0 mL), together with 5.0 mL of the crude biosurfactant (cell-free broth) or different concentrations of the isolated biosurfactant (½ CMC, CMC, 2 × CMC, 3 × CMC, and 5 × CMC). The control treatment was seawater alone (without biosurfactant). Mortality was determined after 24 h [42].

2.11. Ecotoxicity Tests with Danio rerio as Indicator

Adult zebrafish (*Danio rerio*) were fed nauplii of *Artemia* sp. four times per day. A 50 L aquarium was used, into which eight females and four males were placed for reproduction. Fertilised eggs were examined using an inverted microscope at a magnification of 40 times. Fertilisation was greater than 90%. Eggs with opacity or coagulation were discarded. The environmental variables of interest were pH (7.5 to 7.9), temperature (27 ± 0.5 °C), and dissolved oxygen (5 to 6.5 mg/L).

The fish embryo test (FET) was used, in which fertilised eggs were exposed to the biosurfactant [43]. The signs of embryo death were egg coagulation, absence of somites after 24 h, as well as the absence of a heartbeat or movement. The number of deaths after 96 h of exposure was compared to the total exposed to each sample (20 individuals) for the calculation of the mortality rate [44].

The general morphology score (sum partial scores for each embryo during morphological development in 96 h of exposure) was used to determine sublethal effects. Developmental markers were investigated at 24 h intervals, with the determination of abnormalities indicating sublethal effects. The effect of the different concentrations of the biosurfactant on the embryos was determined by comparisons with the control. The following characteristics and respective time intervals were considered: detachment of the tail during the initial development of the embryo (24 h); formation of somites (24 and 48 h); development and pigmentation of eyes (24 to 96 h); movement of the embryo (24 to 96 h); the presence of blood circulation (24 to 96 h); the presence of heartbeats (48 to 96 h); pigmentation of head and body (48 to 96 h); pigmentation of the tail (48 to 96 h); the presence of an extension of a nearly empty vitelline sac related to a nearly completely resorbed vitelline sac (96 h), enabling a space where the swim bladder will be; presence of pectoral fins (72 to 96 h); presence of a salient mouth (72 to 96 h); hatching (72 to 96 h). The maximum GMS is 18 at the end of 96 h, indicating a perfectly developed larva [44–46].

Statistical analysis: A logistic curve was used to calculate the lethal effects of $LC_{50/96h}$ with the aid of R software 4.0.2 (R Development Core Team, 2020, Auckland, New Zealand) [47]. One-way analysis of variance (ANOVA) was used to determine mean GMSs obtained from embryos exposed to different concentrations of the biosurfactant, considering a 5% significance level. Dunnett's post hoc test was used to detect significant differences from the control. Statistical analysis was performed with the aid of the SigmaPlot software, version 12 (Jandel Scientific, Erkrath, Germany).

2.12. Remediation Experiment with Oil Derivative Adsorbed to Sand—Kinetic Test

Sand (10 g) contaminated with a 10% motor oil solution was placed in 100 mL of drinking water, to which 1 mL of sugarcane molasses donated by a local processing plant was added. The mixture was sterilised under fluent vapor and constituted the control condition. Next, 2% solutions of different concentrations of the isolated biosurfactant (CMC and 2 × CMC) were added, followed by 15% of the inoculum containing 10^7 colony-forming units/mL cultivated in the YMB medium. The mixtures were incubated at 150 rpm and 28 °C for 75 days. The experiments were performed in triplicate using 250 mL Erlenmeyer flasks. The following conditions were analysed: Control—contaminated sand + molasses; Condition 1: contaminated sand + molasses + *S. bombicola*; Condition 2: contaminated sand + molasses + biosurfactant (CMC) + *S. bombicola*; Condition 3: contaminated sand + molasses + *S. bombicola* + biosurfactant (2 × CMC). Molasses (1%) was added at regular intervals (Days 15, 30, 45, and 60). Aliquots (5 mL) were withdrawn every 15 days for analysis, totalling five samplings (Days 15, 30, 45, 60, and 75) [48]. The oil removal rate was determined by gravimetry, as described in Section 2.14.

2.13. Removal of Oil Derivative Adsorbed to Sand in Packed Columns—Static Test

Sand (10 g) contaminated with a 10% motor oil solution (*w*/*w*) was placed in glass columns (55 × 6 cm). The surface was then inundated with 200 mL of the biosurfactant solution at the CMC (Condition 1) and 2 × CMC (Condition 2) under the action of gravity.

The crude biosurfactant (cell-free broth) was tested with the same quantity (Condition 3). A column containing the sand and water (200 mL) was the control. After 24 h, no further percolation of the biosurfactant solutions was observed [48,49]. The oil removal rate was determined by gravimetry, as described in Section 2.14.

2.14. Analysis of Oil Derivative Removed from Sand

One hundred mL of n-hexane was added to the liquid phase in a decantation funnel and agitated for 10 min. The mixture was either placed into a rotary evaporator to evaporate the hexane or hexane was evaporated in a laboratory oven at 68–70 °C. The beaker with the residual oil was weighed.

The role of the aging factor in pollution phenomena of soil and sand particles, which is an important element in the effective dynamics of contaminant removal, was considered using motor oil samples that were previously exposed to room temperature (30–35 °C) for a period of one year prior to use.

2.15. Remediation Experiment of Oil Derivative in Seawater

Bioremediation tests were performed based on the methods described in the Standard Methods for the Examination of Water and Wastewater [50]. Motor oil removal experiments were conducted in 250 mL Erlenmeyer flasks with 50 mL of seawater collected from Port Suape and 1% motor oil. The medium was sterilised, followed by inoculation with 5% of the inoculum of the biosurfactant-producing microorganism (10^7 colony-forming units/mL with an O.D. of 0.7 to 600 nm). The flasks were agitated at 150 rpm in a rotary shaker for 30 days, with samples withdrawn for analysis every 10 days (totalling three samples). The experiments involved the following different conditions: Control − seawater + motor oil; Condition 1: seawater + motor oil + *S. bombicola*; Condition 2: seawater + motor oil + *S. bombicola* + biosurfactant (CMC); Condition 3: seawater + motor oil + *S. bombicola* + biosurfactant (2 × CMC). The oil degradation efficiency was determined as described in Section 2.16.

2.16. Calculation of Degradation Efficiency of Oil Derivative from Seawater

The oil degraded from the samples and control medium was quantified following extraction with n-hexane. The residual oil was separated using a separatory funnel with the same volume of hexane in a beaker that had previously been weighed.

2.17. Statistical Analysis

Statistical analysis was performed with the aid of Statistica® (version 7.0) using the one-way procedure, followed by linear one-way analysis of variance (ANOVA). The results of triplicate experiments were expressed as mean and standard deviation. Tukey's post hoc test was used with a 95% significance level for the determination of significant differences.

3. Results and Discussion

3.1. Biosurfactant Production, Isolation, and Emulsifying Capacity

Biosurfactants were produced by *S. bombicola* ATCC 22214 using different media, followed by the determination of the surface tension of the crude biosurfactants, yield (through the isolation of the organic solvent), and emulsifying capacity. The results are displayed in Table 2.

Surface tension is one of the most important properties for the determination of the effectiveness of a biosurfactant [51]. According to Akbari et al. [52], biosurfactants with the capacity to reduce the surface tension of water from 72 to 35 mN/m are effective. Thus, all biosurfactants produced in the present study achieved satisfactory results (Table 2). The similar surface tension of the different biosurfactants demonstrates that *S. bombicola* ATCC 22214 can produce biosurfactants using a variety of substrates. Previous studies have also reported the versatility of this yeast in producing biosurfactants in different media [53–55]. Jiménez-Peñalver et al. [56] produced a biosurfactant from *S. bombicola*

ATCC 22214 in a low-cost medium that lowered surface tension to 33.8 mN/m. Jadhav et al. [57] cultivated *S. bombicola* MTCC 1910 in a medium in which the carbon source was 10% residue from a sunflower oil refinery and obtained a biosurfactant that lowered the surface tension to 35.50 mN/m. Shah et al. [58] cultivated *S. bombicola* ATCC 22214 in a medium with 10% palm oil and produced a biosurfactant that lowered the surface tension to 35.35 mN/m. Gaur et al. [59] produced a biosurfactant from the yeast *Candida albicans* SC5314 that reduced the surface tension to 42 mN/m. These results are similar to those found in the present investigation.

Table 2. Surface tension results, yield, and motor oil emulsification index of biosurfactants produced by *S. bombicola* ATCC 22214 in different production media (data expressed as mean ± SD of triplicate determinations).

Biosurfactant	Surface Tension (mN/m)	Yield (g/L)	Emulsification Index (%)
Medium 1	32.4 ± 0.2	8.1	83.3 ± 0.1
Medium 2	32.8 ± 0.1	7.3	84.4 ± 0.4
Medium 3	38.0 ± 0.1	11.4	77.0 ± 0.1
Medium 4	32.7 ± 0.3	23.0	96.2 ± 0.1
Medium 5	32.8 ± 0.5	7.7	90.2 ± 0.1
Medium 6	32.9 ± 0.2	5.3	90.5 ± 0.1
Medium 7	33.6 ± 0.6	5.4	72.9 ± 0.1
Medium 8	33.1 ± 0.1	8.7	91.0 ± 0.1
Medium 9	33.4 ± 0.4	10.1	89.7 ± 0.1
Medium 10	31.3 ± 0.1	19.5	92.4 ± 0.5

The simplified isolation method carried out in the laboratory was used to determine biosurfactant yields. The advantage of this method is the elimination of the initial centrifugation and filtration steps involved with other methods and the use of a smaller quantity of solvent. The present results are in agreement with data described in previous studies involving the isolation of biosurfactants produced by microorganisms of the genus *Candida*. Marcelino et al. [60] cultivated *S. bombicola* in a mineral medium supplemented with soybean oil and corn industry residue, obtaining a biosurfactant yield of 15.6 g/L. Dierickx et al. [61] cultivated *S. bombicola* in a 7.5 L bioreactor and obtained a biosurfactant yield of 87.12 g/L, indicating that it is possible to scale up the production of the biosurfactant produced by this species.

The emulsification index was calculated to determine the capacity of the biosurfactants produced in the different media to emulsify residual motor oil. The results were above 90% for all tests, indicating the high emulsifying activity of the biomolecules. Using a biosurfactant produced by *C. albicans* IMRU 3669, El-Sheshtawy et al. [62] obtained 65% emulsification of a petroleum derivative. Lira et al. [63] produced a biosurfactant from *C. guilliermondii* UCP 0992 in a medium supplemented with 5% corn steep liquor, 5% soybean waste frying oil, and 5% sugarcane molasses and obtained 71.4% emulsification of motor oil. Santos et al. [64] produced a biosurfactant from *C. lipolytica* UCP 0988 in a medium supplemented with industrial waste products and achieved 60% emulsification of motor oil.

Emulsion systems containing hydrocarbons and water have low stability. Therefore, surfactants are used to stabilise these systems. The stability provided by surfactants demonstrates the surface activity of the compound. However, it is important to note that surfactants and emulsifiers serve different purposes. Surfactants primarily function to reduce interfacial tension, while emulsifiers adsorb more slowly to the droplet surface, providing long-term stability [65]. The biosurfactant produced by *S. bombicola* in medium 4 (1.2% canola oil, 10% sucrose, 0.5% corn steep liquor, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$) was selected for further experiments, as the best yield and the highest emulsification index were achieved with this medium. The replacement of glucose with sucrose is an important point, as it reduces the final production cost.

3.2. Biosurfactant Characterisation

The double diffusion method in agar showed that the biosurfactant is anionic. Using the same method, other biosurfactants produced by yeasts were also described as anionic [66,67].

A ^{1}H NMR spectrum was obtained from the purified biosurfactant (Figure 1). The peaks between 0 and 3 ppm suggest aliphatic groups, with the presence of hydrogens of methyl groups (0–1.2 ppm), aliphatic carbons (1.3–1.8 ppm, 1.8–2.0 ppm), and the carbonyl group (2.2–2.8 ppm). The peaks between 4 and 4.4 ppm suggest the presence of hydroxyl (-OH). Moreover, a double bond was found at 5.3 ppm. Gaur et al. [59] found similar peaks in the characterisation of a sophorolipid. Similar peaks were also found in the analysis of the biosurfactant produced by *S. bombicola* NRRL Y-17069 [68].

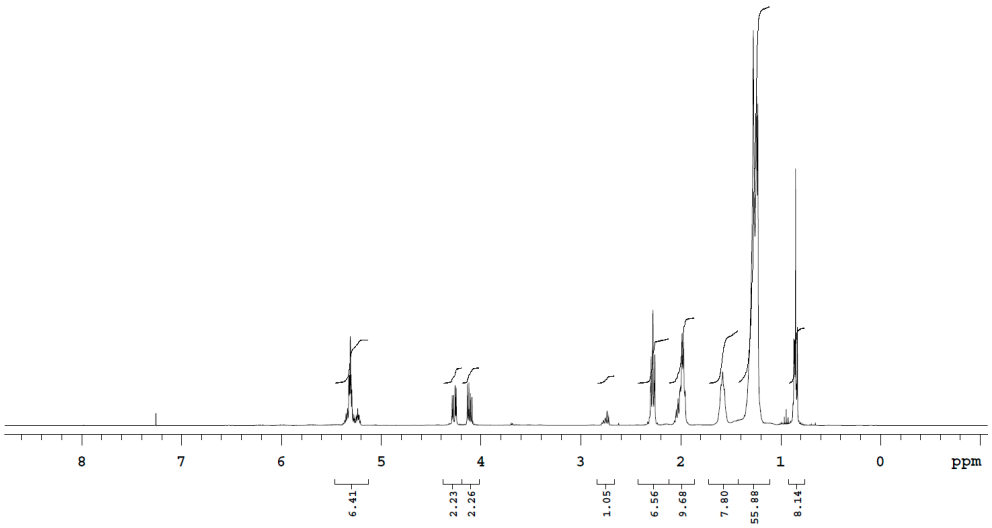

Figure 1. ^{1}H NMR spectrum (CD3OH, 300 MHz) of biosurfactant isolated from *S. bombicola* ATCC 22214 cultivated in 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

In the ^{13}C NMR spectrum, the peak at 173 ppm was attributed to the C=O of carboxyl acid or ester. Double-bond peaks were found between 120 and 140 ppm. Hydroxyl (-OH) was detected between 60 and 70 ppm. Signals were also detected between 10 and 40 ppm, which are characteristic of aliphatic carbons (Figure 2).

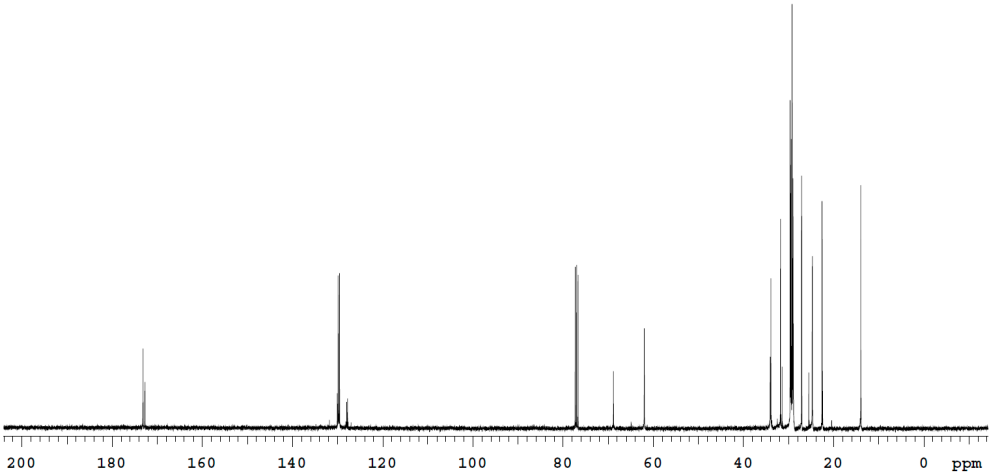

Figure 2. ^{13}C NMR spectrum (CD3OD, 300 MHz) of biosurfactant isolated from *S. bombicola* ATCC 22214 cultivated in 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

FTIR spectra (Figure 3) revealed a characteristic stretching of O–H near 3500 cm^{-1} in the structure of the biosurfactant. The peak at 3006 cm^{-1} was attributed to =C–H stretching. The peaks at 2924 cm^{-1} and 2854 cm^{-1} were attributed to –C–H stretching. An absorbance band was found at 1747 cm^{-1} and attributed to the vibration of the stretching of –C=O. The bands at approximately 1463 cm^{-1} and 1377 cm^{-1} are associated with the asymmetrical and symmetrical bending of CH_3, respectively. Stretching characteristic of C (=O)–O–C was found at 1163 cm^{-1}. C–O–C stretches were obtained at 1120 cm^{-1} and 1096 cm^{-1}, suggesting a glycosidic bond. The structural details of the biosurfactant produced by *S. bombicola* ATCC 22214 were similar to those described for sophorolipid biosurfactants characterised in previous studies [58,59,69,70].

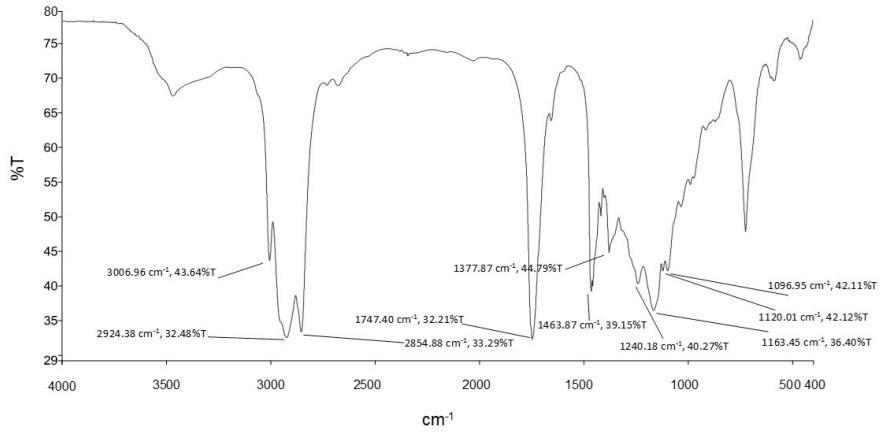

Figure 3. FTIR absorption spectra of biosurfactant produced by *S. bombicola* ATCC 22214 cultivated in 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

3.3. Critical Micelle Concentration

The effectiveness of a biosurfactant in reducing surface tension is determined by its critical micelle concentration (CMC), which is the concentration at which the surfactant starts forming micelles. Therefore, a lower CMC indicates a more effective surfactant, making it more desirable for the industries by improving its cost–performance profile, which determines its commercial viability [71,72]. Figure 4 shows the change in surface tension as a function of the concentration of the biosurfactant produced in medium 4. The CMC of the biosurfactant produced by *S. bombicola* ATCC 22214 was reached at a concentration of 0.6 g/L, indicating satisfactory efficiency. Studying a biosurfactant produced by *C. bombicola* URM 3718, Silva et al. [37] determined a CMC of 0.5 g/L. Ashish and Debnath [73] produced a biosurfactant from *C. tropicalis* MTCC230 and determined a CMC of 0.0325 g/L.

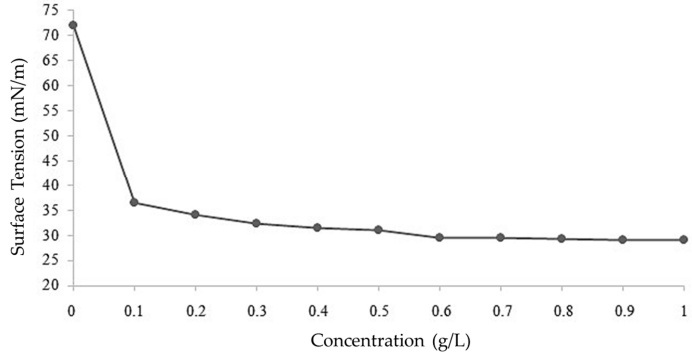

Figure 4. CMC of biosurfactant produced by *S. bombicola* ATCC 22214 in medium composed of 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

3.4. Stability of Biosurfactant

The stability study of a biosurfactant is required to determine its applicability, as applications in specific environments can pose a challenge. For a biosurfactant to have industrial use, it must maintain its surface-active characteristics irrespective of the environmental conditions to which it is subjected [74].

Biosurfactant yields obtained in the fermentation process are essential for their use in the bioremediation of large areas contaminated with hydrocarbons. Thus, using crude biosurfactants (cell-free broth) is recommended in view of economic considerations. Therefore, the stability of the biosurfactant produced in medium 4 was investigated in its crude form considering the effect on surface tension and emulsification capacity in the presence of residual motor oil (Table 3).

Table 3. Influence of saline (NaCl) concentration, temperature, and pH on reduction in surface tension and emulsifying activity of residual motor oil in cell-free broth containing biosurfactant produced by *S. bombicola* ATCC 22214 cultivated in 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$ (data expressed as mean ± SD of triplicate determinations).

NaCl (*v*/*v*)	Surface Tension (%)	Emulsification Index (%)
2	33.3 ± 0.02	95.5 ± 0.3
4	33.7 ± 0.03	95.4 ± 0.3
6	32.2 ± 0.01	96.2 ± 0.2
8	33.5 ± 0.02	96.2 ± 0.1
10	31.8 ± 0.02	96.2 ± 0.2
12	32.4 ± 0.01	96.9 ± 0.1
Temperature (°C)	Surface Tension (mN/m)	Emulsification Index (%)
0	33.7 ± 0.02	96.3 ± 0.2
5	33.9 ± 0.01	96.4 ± 0.2
28	32.7 ± 0.01	96.3 ± 0.4
70	32.8 ± 0.00	92.7 ± 0.2
100	32.8 ± 0.00	92.6 ± 0.4
120	33.5 ± 0.18	90.4 ± 0.3
pH	Surface Tension (mN/m)	Emulsification Index (%)
2	35.0 ± 0.02	94.4 ± 0.1
4	34.9 ± 0.03	94.5 ± 0.3
6	32.7 ± 0.01	95.5 ± 0.2
8	31.8 ± 0.01	95.5 ± 0.2
10	34.2 ± 0.01	96.4 ± 0.2
12	33.9 ± 0.01	97.6 ± 0.1

The biosurfactant maintained the ability to reduce surface tension in the presence of all concentrations of NaCl added to the cell-free broth (2–12%), indicating good tolerance to salinity. The concentrations of NaCl also did not exert a substantial influence on the emulsification index. As the salinity of the ocean is around 3%, the biosurfactant produced by *S. bombicola* ATCC 22214 can be applied in environments with a high saline concentration.

Surface tension remained around 33 mN/m, with little variation in the cell-free broth at temperatures ranging from 0 to 120 °C. This indicates that the biosurfactant can be applied in environments that undergo a significant change in temperature, such as industries in which sterility is achieved by heat. The emulsification indices of motor oil also demonstrated thermal stability.

The surface tension of the cell-free broth fluctuated somewhat with the change in pH, remaining relatively stable (around 35 mN/m) at more acidic pH (2 and 4) and more basic pH (around 34 mN/m) and dropping to 32 mN/m at pH 6 and 8. The emulsification of the motor oil increased with the increase in pH. Ashish and Debnath [74] found similar behaviour.

Based on the results, the crude biosurfactant can be applied in environments with extreme salinity, temperature, and pH, as in oil recovery activities and the bioremediation of polluted marine environments, with no significant change in its properties. This factor is important, as the purification step can correspond to as much as 60% of the total production cost of a biosurfactant. The elimination of this step lowers the application cost of the biosurfactant produced by *S. bombicola* ATCC 22214, constituting another advantage of this novel biomolecule in the petroleum market.

3.5. Toxicity of Biosurfactants to Artemia salina

The literature suggests that biosurfactants are less harmful than synthetic surfactants and dispersants. However, the impact of these biomolecules on the environment has not been studied enough [75]. Indeed, recent research emphasises the importance of conducting thorough investigations to understand the properties of new biosurfactants before release into the environment. According to Silva et al. [76], biosurfactants are commonly non-toxic to microorganisms at concentrations close to the CMC.

A bioassay was conducted involving larvae of the microcrustacean *Artemia salina* to determine the toxicity of the biosurfactant produced by *S. bombicola* ATCC 22214 (Table 4). No mortality occurred after exposure to the crude biosurfactant and different concentrations of the isolated biosurfactant (½ CMC, CMC, and 2 × CMC) for 24 h, indicating the absence of toxicity. In contrast, mortality was 10 and 20% at concentrations of 3 × CMC and 5 × CMC, respectively. Acute toxicity tests of a biosurfactant produced by *C. bombicola* to *A. salina* larvae also demonstrated low toxicity in a previous study [77].

Table 4. Toxicity of biosurfactant produced by *S. bombicola* ATCC 22214 cultivated in 10% sucrose, 0.5% corn steep liquor, 1.2% canola oil, 0.1% K_2HPO_4, 0.4% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$.

Biosurfactant Concentration in Saline Water	Mortality of Brine Shrimp Larvae (%)
Cell-free broth	no mortality
½ × CMC	no mortality
CMC	no mortality
2 × CMC	no mortality
3 × CMC	10.000 ± 0.000
5 × CMC	20.000 ± 0.000

3.6. Ecotoxicity of Biosurfactant to Danio rerio (Zebrafish)

Surfactants stand out among synthetic materials due to their unique properties, which enable their application in diverse fields compared to conventional solvents. However, approximately 60% of chemical surfactants enter aquatic environments [78], and the continual emissions negatively alter the health of such environments. Thus, biosurfactants constitute a viable option to diminish the harmful effects on natural ecosystems due to their low toxicity while maintaining the same functions as synthetic products [79]. To assess the toxicity level of these compounds, it is necessary to carry out controlled, standardised ecotoxicology tests that use established organisms as adequate biological models for such analyses. Among the test organisms used for this purpose, the teleost fish *D. rerio* (zebrafish) is currently one of the main biological models of vertebrates used in ecotoxicological tests for environmental monitoring and the laboratory assessment of toxicity at lethal and sublethal levels of isolated substances or mixtures, including morphological and behavioural biomarkers [44,80]. Toxicity tests were performed on samples of the biosurfactant produced by *S. bombicola* ATCC 22214, with the analysis of lethal and sublethal effects [43,45].

One hundred ninety-two embryos were used in the tests performed in culture plates: 24 in the culture water (control) and 7 in plates with different concentrations of the biosurfactant. The mortality of the embryos is displayed in Table 5.

Table 5. Quantity of dead individuals exposed to different concentrations of biosurfactant with percentage of deaths 96 h post-exposure (hpe). Twenty individuals used in each treatment.

Concentration (mg/L)	Deaths 24 hpe	Deaths 48 hpe	Deaths 96 hpe	Mortality Rate (%) at End of Test (96 h)
600	20	-	-	100
300	20	-	-	100
150	9	8	3	100
75	0	4	16	100
37.50	0	4	16	100
18.750	0	3	14	85
9.375	0	0	5	25

The data showed the absence of toxicity with less than 24 h of exposure for all concentrations less than 75 mg/L, unlike what was found for exposure to three synthetic surfactants (dodecyl dimethyl benzyl ammonium chloride, sodium dodecyl sulphate, and fatty alcohol polyoxyethylene ether), for which the mortality rate was 53% at concentrations of 1 mg/L in the first 10 h of exposure, demonstrating high toxicity [81]. A study using a biosurfactant for the control of aquatic pathogens (SPH6) reported 10% toxicity at a concentration of 20 mg/L in 24 h, by which the surfactant was considered a product with low toxicity [82]. In the present study, 15% mortality was found beginning with 48 h of exposure at a concentration of 18.75 mg/L, which demonstrates lower toxicity of the biosurfactant produced by *S. bombicola* ATCC 22214.

The lethal concentration that kills fifty percent of the organisms (LC_{50}) was 134.4, 100.8, and 15 mg/L after 24, 48, and 96 h of exposure, respectively, which, based on data from the U.S. Fish and Wildlife Service, denotes low toxicity [83]. Previous studies using commercial surfactants reported lower LC_{50} than the values found in the present investigation, such as the 18.3 µg/L described by Al-asmakh et al. [84] using AEO-7. Yi et al. [79] performed tests involving sodium dodecylbenzenesulfonate, nonylphenol exthoxylate (NPE), and stearyl trimethylammonium bromide and found LC_{50} values of 5.77 mg/L, 17.24 mg/L, and 0.73 mg/L, respectively, which are in agreement with the low toxicity found in the present study. In a study involving a biosurfactant produced by *Pseudomonas putida*, the LC_{50} was 60 mg/L when zebrafish embryos were exposed for 48 h, which is considered low toxicity. Johann et al. [85] reported an LC_{50} of 100.8 mg/L in the same period used in the present investigation.

With regards to sublethal effects, embryo–larval development was only analysed at the lowest concentrations (9.37 and 18.75 mg/L) due to the low mortality found after exposure for 96 h. No significant differences were found when these concentrations were compared to the control (Figure 5).

Despite the absence of toxicity in the GMS analysis at the lowest concentrations, some embryos exhibited pericardial oedema and haemorrhage when exposed to other concentrations of the biosurfactant (Figure 6), with a frequency of 63% at 150 mg/L, 18.75% at 75 mg/L, and 12.5% at 37.5 mg/L. Such pathologies were not found at the other concentrations. In previous studies, pericardial oedema was also found in zebrafish embryos and larvae exposed to 12.8 µg/L of AEO-7 [84], 10 mg/L of NPE, and 0.5 mg/L of STAB [79], which are much lower concentrations than the lowest concentration at which this effect was found in the present study (37.5 mg/L).

The tests carried out with the biosurfactant produced by *S. bombicola* ATCC 22214 demonstrated low toxicity to zebrafish embryos and larvae at environmentally relevant concentrations and the absence of toxicity at the lowest concentrations. Thus, the biosurfactant is a safe product for use in natural aquatic environments, offering low toxicity to non-target organisms.

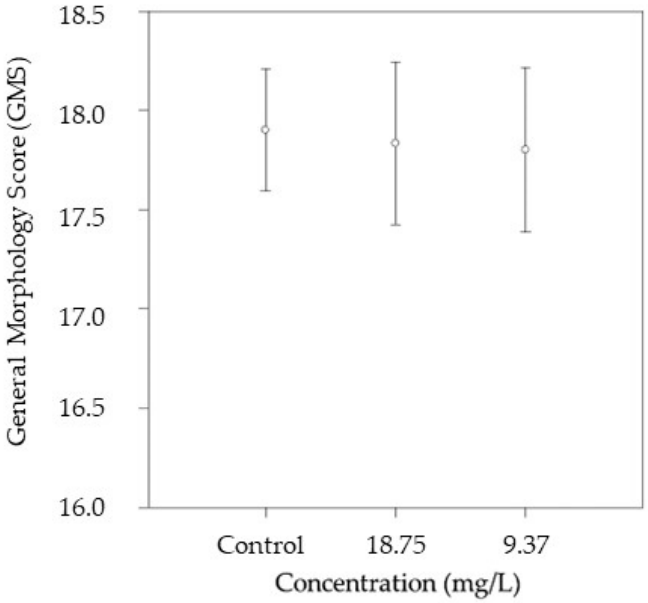

Figure 5. General morphology score for *D. rerio* embryos and larvae following exposure to different concentrations of biosurfactant produced by *S. bombicola* ATCC 22214.

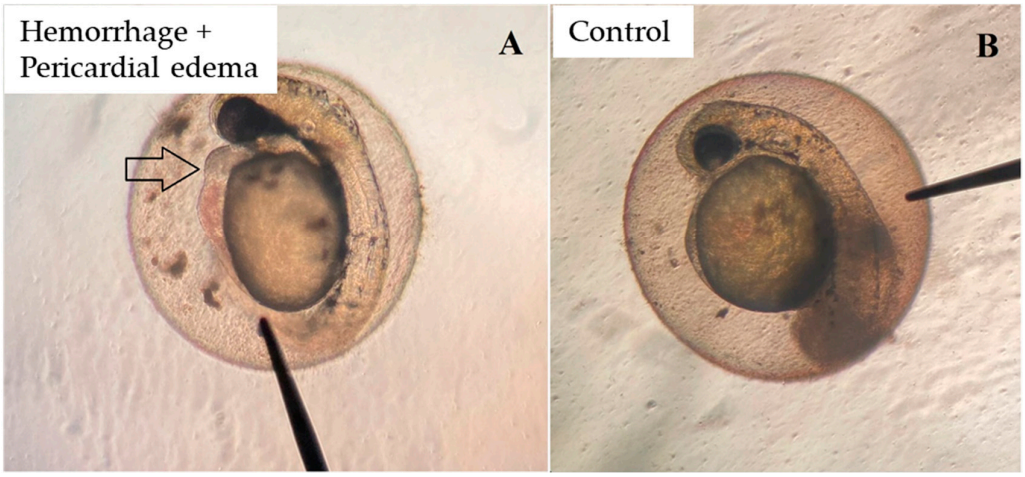

Figure 6. Larva with haemorrhage and pericardial oedema (**A**) exposed to 75 mg/L of biosurfactant produced by *S. bombicola* and control larva without pathologies (**B**).

In the context of environmental impact and regulations on the use of biosurfactants, it is important to emphasize that chemical dispersants have been used in the USA since 1969. During the oil spill from Deepwater Horizon in 2010, an unprecedented amount of Corexit dispersants, which contain the anionic surfactant sodium dioctyl sulfosuccinate (DOSS), was used. This raised concerns with regards to the potential toxicity to organisms in the water column. Corexit 9527 and 9500 were extensively on the oil spill. The use of Corexit 9580 was discontinued due to its toxic surfactants and a component that was found to be carcinogenic (2-butoxyethanol) [76,86]. In Brazil, only two chemical dispersants (Corexit EC9500—Nalco Holding Company, Watchung, NJ, USA, and Ultrasperse II®—Ingredion, Westchester, IL, USA) are authorized by the National Environment Council [87] for the treatment of oil spills in the marine environment. Ultrasperse II® is a blend of alcohol, alcohol sulphate, and fatty ester ethoxylate, and few studies have reported its effects on marine life. Although it is allowed in some countries and used by some oil companies with intellectual property protection, its toxicity to marine fish has been demonstrated [86].

Current dispersants are less toxic and more effective than the products used in past decades. However, several challenges to understanding toxicity have been pointed out,

such as the limitations of lab tests and differences in exposure conditions in the field [87]. Laboratory experiments are good for maintaining consistent test material concentrations during exposure, which is necessary for the accurate measurement of toxicological responses. However, these methods fail to fully replicate real exposure scenarios and can affect the availability of the compounds being tested. Despite these challenges, chemical toxicity distribution (CTD) is a risk assessment method that assists in assessing the environmental risks of dispersants. A study by Berninger et al. [88] suggested that dispersants are less toxic than oil alone but more toxic than oil when mixed with this hydrocarbon. Assessing the toxicity of untreated and dispersant-treated oil is complex due to the various toxic compounds to which aquatic organisms are exposed. The presence of polycyclic aromatic hydrocarbons (PAHs) in chemically dispersed oils is also linked to their toxicity. The toxicity of dispersants can be measured using LC_{50} values, which range from 200 to 500 mg/L, with higher concentrations indicating lower toxicity. The US Environmental Protection Agency uses a five-step scale to classify pesticides based on their acute toxicity to aquatic organisms. Microbial biosurfactants are considered eco-friendly alternatives for dispersant formulations due to their low toxicity. Despite the availability of different toxicity assessment methods, the low toxicity of eco-friendly dispersants is likely attributed to the absence of organic solvents and other toxic chemicals, which also enhances the biodegradability of these products [76].

3.7. Remediation of Oil Derivative Adsorbed to Sand by Biosurfactant—Kinetic Test

Oil and its derivatives are among the main environmental problems throughout the world. Bioremediation is considered a promising ecological option for cleaning up oil-contaminated environments with the use of microorganisms or microbial processes to diminish the concentration and/or toxicity of these pollutants. However, the response time can be long, which makes the addition of agents that accelerate this process necessary, such as biosurfactants. The addition of biosurfactants increases the solubility of hydrophobic organic compounds, enhancing their desorption from soil [29,89,90].

Figure 7 displays the results of the bioremediation of soil contaminated with motor oil in the presence of the yeast *S. bombicola* and the biosurfactant produced by the *S. bombicola* ATCC 22214 strain in a kinetic test.

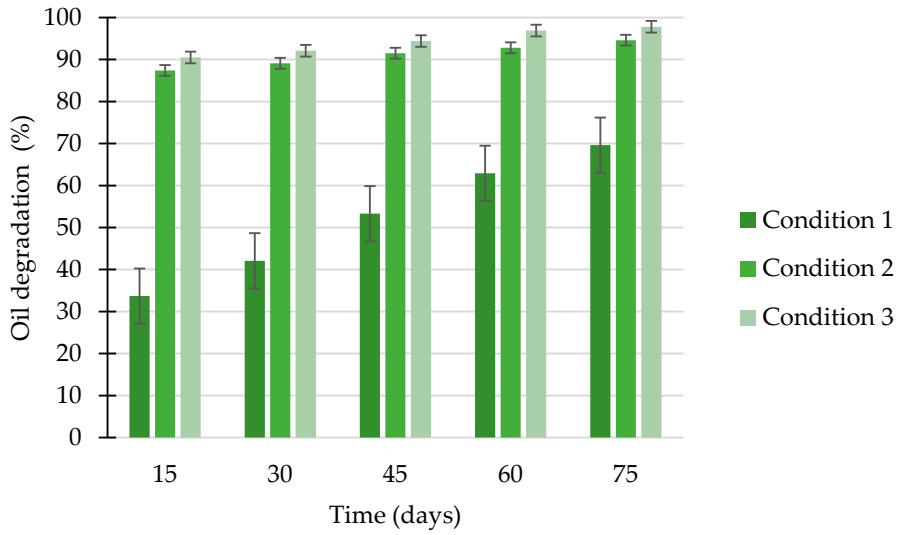

Figure 7. Degradation of oil adsorbed to sand by bioremediation process using biosurfactant produced by *S. bombicola* ATCC 22214. Condition 1: contaminated sand + molasses + *S. bombicola*; Condition 2: contaminated sand + molasses + biosurfactant (CMC) + *S. bombicola*; Condition 3: contaminated sand + molasses + *S. bombicola* + biosurfactant (2 × CMC). Error bars illustrate standard deviations calculated from three independent experiments.

The motor oil degradation rate was increased by the addition of the biosurfactant compared to the control condition (without biosurfactant). The highest removal rate (97.8%) was achieved with the biosurfactant at the 75-day assessment, demonstrating the advantage of this new biosurfactant in remediation processes. The concentration of the isolated biosurfactant exerted an influence on the degradation rate due to the enhancement of solubilisation of the oil in the aqueous phase. According to Oluwaseun et al. [91] and Zhao et al. [92], mobilisation and solubilisation are the main mechanisms of oil removal from soil, which are related to the CMC. Mobilisation occurs when the biosurfactant is used at a concentration lower than the CMC, and solubilisation occurs when the biosurfactant is used at a concentration above the CMC, as stated in Section 1. The reduction in surface and interfacial tensions is associated with the mobilisation mechanism. Surfactants increase the interaction angle between the hydrophobic contaminant and soil, facilitating separation from soil particles. With the mobilisation mechanism, the contaminant is divided in the centre of the micelles of the surfactant.

In this research, the biosurfactant was used at and above its CMC. This means that at higher concentrations, the surfactant molecules replaced water molecules, reducing the polarity of the water phase and surface tension. Consequently, the process of solubilisation of pollutants was accelerated. Micelles were formed, which significantly increased the solubility of the hydrophobic contaminant in the aqueous phase, thus helping to detach it from the soil. Mobilisation is more efficient when pollutants are solubilised in the water phase, enabling removal by either plants and microorganisms (biotic removal) or washing and recovery (abiotic removal) [93].

In a previous study, a biosurfactant produced by *Candida antarctica* was able to remove about half of the oil adsorbed to sand [94]. In a study carried out by Santos et al. [95], a biosurfactant produced from *C. sphaerica* UCP 0995 achieved a 90% motor oil removal rate at a concentration of 2 × the CMC in 90 days.

3.8. Removal of Oil Derivative Adsorbed to Sand by Biosurfactant in Packed Columns—Static Test

Biosurfactants decrease interfacial tension between oil and soil or oil and water, reducing capillary forces that resist the movement of oil through soil. Packed columns were used instead of agitation processes to test the effectiveness of the biosurfactant produced by *S. bombicola* ATCC 22214 under static conditions. Figure 8 displays the results of the static test. The cell-free broth (crude biosurfactant) achieved the best removal rate (69.2%) after 24 h of percolation.

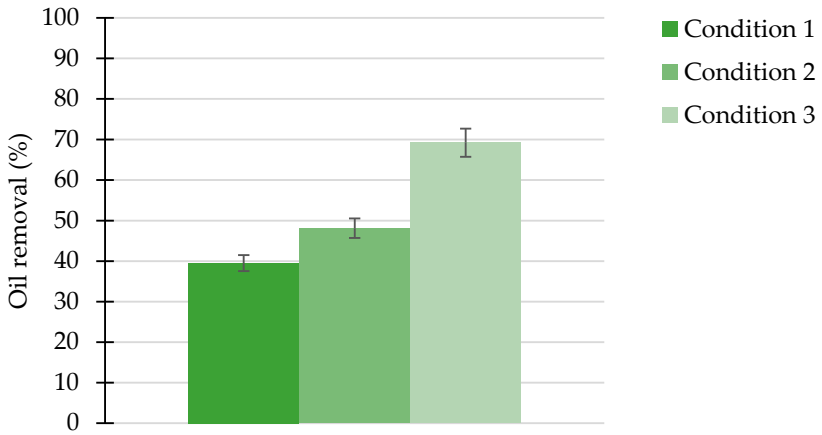

Figure 8. Removal of oil adsorbed to sand by bioremediation process using biosurfactant produced by *S. bombicola* ATCC 22214 in packed columns through static test. Condition 1: biosurfactant at CMC; Condition 2: biosurfactant at 2 × CMC; Condition 3: cell-free broth (crude biosurfactant); Control: distilled water. Error bars illustrate standard deviations calculated from three independent experiments.

This removal capacity is promising, as the biosurfactant did not undergo the extraction process and removed more than half of the oil adsorbed to the sand. The isolated biosurfactant achieved removal rates of 39.5% and 48.1% under Condition 1 (CMC) and Condition 2 (2 × CMC), respectively, whereas water alone (control) was able to remove 19.2% of the contaminant. It is noteworthy that isolation constitutes a large part of the production costs, and high removal rates with the crude biosurfactant are of extreme industrial interest.

Several biosurfactants have shown potential for cleaning soil contaminated with hydrophobic substances in glass columns. Ibrahim et al. [96] reported a 76% oil recovery rate using a biosurfactant in a column in a period of only two hours. Fernandes et al. [97] produced a biosurfactant from *Bacillus subtilis* RI 4114 and reported a 69% recovery rate of residual oil using 600 mg/L in a column. Kavitha et al. [98] produced a biosurfactant from *Bacillus* sp. MTCC 5514 and found a greater than 70% oil removal rate from standard sand and sandy soil. Jain et al. [99] investigated oil removal in glass columns comparing two biosurfactants to synthetic surfactants, reporting greater than 90% recovery rates with the biosurfactants, whereas the synthetic surfactants did not achieve a 70% rate of contaminant removal.

3.9. Remediation of Seawater Contaminated with Spilled Oil Derivative

The bioremediation of seawater requires a surfactant with dispersant capacity. As a phenomenon associated with both interfacial tension and surfactant concentration, dispersion facilitates the access of autochthonous microorganisms to the contaminant, thus promoting bioremediation.

The reduction in surface and interfacial tensions promoted by biosurfactants increases the solubility, mobility, and bioavailability of petroleum hydrocarbons. This facilitates biodegradation by microorganisms, which can break down large oil slicks and make the contaminants more easily accessible for natural processes. As a result, microorganisms can metabolise and reduce or eliminate the contaminants to safe levels [100]. There are two mechanisms by which biosurfactants facilitate hydrocarbon biodegradation: (1) The reduction in surface and interfacial tensions increases the bioavailability of the contaminant to the microorganism; (2) The interaction between the cell surface of the microorganism and biosurfactant alter the cell membrane, facilitating adhesion to the hydrocarbon through the increase in hydrophobicity without causing harm to the membrane. Due to the blocking of hydrogen bonds, biosurfactants enable hydrophobic–hydrophilic interactions that rearrange molecules and reduce the surface tension of the medium, leading to an increase in surface area. This favours bioavailability and consequent biodegradability of the contaminant [101,102].

Time (in days) and the increase in biosurfactant concentration enhanced the degradation rate of the motor oil by the yeast, as shown in Figure 9. The best result was found at 30 days, with the degradation of 91.5% of the oil when the biosurfactant was used at twice its CMC. Good results were also achieved under Conditions 1 and 2 (lower biosurfactant concentrations), with an increase in the degradation rate over time.

Saeki et al. [103] produced a biosurfactant from *Gordonia* sp. JE-1058 that enhanced the degradation polycyclic aromatic hydrocarbons and total saturated hydrocarbons in seawater.

The viscosity of liquid oil plays a crucial role in the performance of cleaning agents [104]. High viscosity slows the dispersion of oil and delays leaching after the collection of the contaminant and the thickness of floating oil exerts a negative impact on the effectiveness and efficiency of treatment [105]. As the residual engine lubricating oil used in the experiments of this work had already undergone physical and chemical changes caused by prolonged storage, the results obtained demonstrate the potential of the biosurfactant in removing very viscous hydrocarbons.

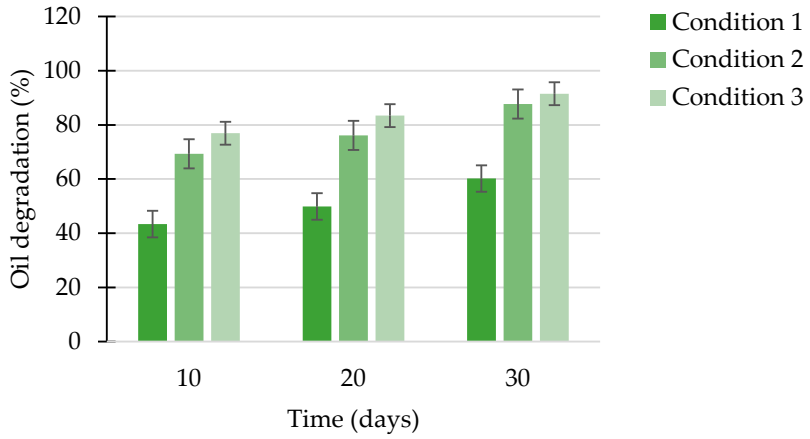

Figure 9. Degradation of oil from seawater by bioremediation process using biosurfactant produced by *S. bombicola* ATCC 22214. Condition 1: seawater + motor oil + *S. bombicola*; Condition 2: seawater + motor oil + *S. bombicola* + biosurfactant (CMC); Condition 3: seawater + motor oil + *S. bombicola* + biosurfactant (2 × CMC).

Several studies have compared biosurfactants with chemical surfactants/dispersants. Couto et al. [106] examined the effects of the chemical surfactant Ultrasperse II® and the biosurfactant surfactin produced by *Bacillus* sp. H2O-1 on marine bacterial communities. The authors found that surfactin stimulated the growth of oil-degrading bacteria more effectively than the chemical surfactant, although the biodegradation of the hydrocarbon was not affected. Additionally, rhamnolipids stimulated hydrocarbon biodegradation in the marine environment more effectively than the chemical surfactants DOSS and GM-2 [107]. Binary systems combining biosurfactants and chemical surfactants have been developed to combat oil spills with less toxicity. For instance, a binary system consisting of the cationic surfactant ethanediyl-1,3-bis(dodecyl dimethyl ammonium bromide) and surfactin produced by *Bacillus subtilis* was successful at reducing the interfacial tension of crude oil [108]. Shah et al. [109] also developed a dispersant based on a binary mixture of an ionic surfactant, choline laurate, and a lactonic sophorolipid produced by *S. bombicola*, which exhibited no toxicity in experiments with fish and had dispersion rates higher than 80%.

4. Conclusions

Despite the advances in biotechnology, few microbial products have reached the industrial scale to replace petroleum-based materials. While biotechnology is important to many industries, the production of microbial surfactants remains too costly to compete with chemical surfactants. Therefore, strategies are needed to improve the affordability of the production of these natural compounds on the industrial level. The microbial strain must be able to use substrates efficiently, and careful selection of the microorganism is required when creating a particular product. The existence of a market for microbial products is another concern, which requires both efficiency and a competitive price. The present study fulfilled some of these requirements. The new biosurfactant produced by *S. bombicola* ATCC 22214 in a low-cost medium demonstrated efficiency and effectiveness and was characterised as a sophorolipid. Moreover, the biosurfactant exhibited stability in the presence of extreme environmental conditions, low toxicity, and the capacity to perform satisfactorily in the removal and degradation of a petroleum derivative in soil and seawater. Thus, the production of this biosurfactant on a large scale for applications in industries as an agent for the mitigation of environmental pollution related to hydrophobic compounds is promising and can significantly contribute to the reduction of environmental impacts on ecosystems. Although the production of surfactants has been a widely explored process for decades, the combination of substrates and cultivation conditions associated with different microorganisms remains a valid strategy for obtaining optimal yields and efficient

biomolecules. After the initial establishment of satisfactory conditions to produce this new biomolecule, as described in this study, the next steps will be scale-up in bioreactors and the optimisation of the extraction steps to use crude or semi-purified formulations of the new agent to meet the demands of the oil industry. Joining efforts to improve the entire process of obtaining each biomolecule is essential for these green surfactants to reach the market.

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