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Thermophilic Hydrocarbon-Utilizing Bacilli from Marine Shallow Hydrothermal Vents as Producers of Biosurfactants

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Abstract: The exploitation of thermophilic hydrocarbon-utilizing bacilli could provide novel environmentally friendly surfactants. In this work, 80 thermophilic bacilli isolated from shallow hydrothermal vents of the Eolian Islands (Italy) were screened for their ability to utilize hydrocarbons and produce biosurfactants (BSs). Among them, 15 strains grew with kerosene or gasoline (2% v/v) as the only carbon and energy source, and most of them were positive to the methylene blue agar as prescreening assay for BSs production and displayed emulsifying activity. The cell-free supernatants (CFSs) from two selected strains, *Bacillus licheniformis* B3-15 and *Bacillus horneckiae* SBP3, were both surface active and able to emulsify different hydrocarbons and vegetable oils. BSs from B3-15 (910 mg L$^{-1}$) and SBP3 (950 mg L$^{-1}$) were chemically different surfactin-like lipopeptides, with specific mineral-, castor- and crude oil removal ability from the cotton matrix. CFSs from the 15 thermophilic strains, which harbor both lipolytic and surfactant abilities, could be suitable for industrial-based applications and environmental issues, such as oil recovery and removal from polluted areas or surfaces, (e.g., oil pipelines, bilge tankers, or industrial silos), whereas the crude BSs, as high-value compounds, may be used in different fields of application, as detergent, cosmeceutical, and pharmaceutical industries.

Keywords: Bacillus; biosurfactant; hydrocarbon; shallow-hydrothermal vent; thermophile

1. Introduction

Hydrophobic compounds, such as hydrocarbons, possess low water solubility, thus, microorganisms are able to increase the bioavailability of these compounds, as potential carbon and energy source, by producing surface-active molecules (SAMs) [1]. The role of bacterial SAMs for growth on hydrocarbons as a carbon source has been previously described by Rosenberg [2]. Compared with industrially synthesized surfactants, bacterial SAMs possess better emulsification, detergency, and dispersion properties, and arise a great interest in different fields of application, including bioremediation, biodegradation, and oil recovery, and pharmaceutical, food, cosmeceutical, and cleaner industries [3–6]. Based on their molecular weight, bacterial SAMs can be distinguished into two classes. Compounds that have low molecular weights and efficiently reduce surface and interfacial tension are called biosurfactants (BSs) and include lipopeptides, (e.g., surfactin, iturin, and fengycin), glycolipids, (e.g., rhamnolipids, trehalose lipids, and sophorolipids) and phospholipids, (e.g., phosphatidylethanolamine) [7,8]. On the other hand, bioemulsifiers are higher in

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molecular weight than BSs, as they are complex mixtures of polysaccharides, lipopolysaccharides, lipoproteins and proteins, and complexes of biopolymers, (e.g., emulsan and alasan) [9–11], that can efficiently emulsify hydrophobic substrates and a wide range of hydrocarbons, (i.e., n-tetradecane, n-hexadecane, n-octane, xylene mineral light and heavy oils, petrol, and crude oil) in water.

The distribution of marine hydrocarbon-utilizing bacteria, mostly Gram-negative, from temperate and tropical zones, is widespread [12,13]. Among the Gram-positive bacteria, many Bacillus strains were reported to be able to utilize petroleum compounds, mainly related to mesophilic species, such as Bacillus subtilis and B. pumilus, and to the thermotolerant B. licheniformis [13]. Among thermophilic species, Geobacillus stearothermophilus, G. thermoleovorans, G. subterraneus and G. uzenensis were reported to utilize or degrade hydrocarbons [14–16].

Marine extreme habitats, such as deep and shallow hydrothermal vents, are inhabited by microorganisms, or extremophiles, well adapted to physico-chemical conditions (high temperatures, low pH values, high content in CO₂, H₂S, heavy metals, hydrocarbons, etc.), that are restrictive for most of the organisms [17]. The common occurrence of hydrocarbons in emitted fluids [18,19] may suggest the development of special microbial adaptive strategies in shallow hydrothermal vents, and therefore they can offer a rich source of unexplored hydrocarbon-utilizing microorganisms able to produce valuable unexploited SAMs.

Thermophiles (with optimal growth temperature from 45 °C to 70 °C) are fast growing and their biomolecules may have a great advantage, including thermostability, over those from mesophilic or psychrophilic microorganisms, as they can be produced in large quantities, using a relatively simple purification process [17]. Thermotolerant and thermophilic Bacillus and Geobacillus spp., isolated from the shallow hydro-thermal vents of the Eolian Islands (Italy), are able to produce different kinds of enzymes and molecules, as exopolysaccharides (EPSs) involved in the cell protection and bacterial adhesion to solid surfaces, cell-to-cell interactions and microbial competitions [17,20,21]. EPSs from Eolian thermophilic bacilli, such as those from B. licheniformis strains B3-15 and T14 [13,22], and G. thermodenitrificans B3-72 [23], have been reported to possess unique properties, mainly thermostability and non-cytotoxicity, and biological activities, including antimicrobial, antiviral, antibiofilm, and immunostimulant effects, that make them potentially useful in different biotechnological applications [13,17,22–27]. The isolation of thermotolerant, hydrocarbon-utilizing bacilli and thermophilic Geobacillus strains (5-2, 10-1, and 1bw) from different Eolian vents was previously reported [13,28]. However, the production and characterization of BSs from Eolian, thermophilic hydrocarbon-utilizing bacilli have not been reported up to date, and their exploitation could provide novel environmentally friendly surfactants.

The aim of the present work was to evaluate the ability of thermophilic bacilli, isolated from shallow hydrothermal vents of the Eolian Islands, to utilize hydrocarbons and produce BSs for their possible exploitations in different fields of applications, including bioremediation and oil recovery, and detergent, pharmaceutical and cosmeceutical industries. For these purposes, a set of thermophilic and thermotolerant strains were firstly screened for their ability to utilize kerosene or gasoline as only carbon sources. BSs production in their cell-free supernatants (CFSs) was tested with preliminary screening assays for both surface-active properties (oil drop-collapse test and surface tension reduction) and emulsification activity. BSs from selected strains were produced under optimized conditions, by providing different carbon and nitrogen sources, and characterized by ATR-FTIR. Finally, their oil-removal capabilities from a cotton matrix were investigated.

2. Materials and Methods
2.1. Bacterial Strains

Thermophilic bacterial strains (80) used in this work are part of the collection of the “Research Centre for Extreme Environments and Extremophiles” at the Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences of the University of
Messina, Italy. All strains were previously isolated from hydrothermal fluid and sediment samples collected from the vents of Eolian Islands (Italy) [17,20] (Table 1). All strains are routinely grown on Marine Agar 2216 (MA, Difco Laboratories, Detroit, MI, USA) and frozen at −80 °C in 40% (v/v) glycerol for long-term storage.

Table 1. Characteristics of fluid (F) and sediment (S) samples collected from the hydrothermal vents of Eolian Islands (Italy), and designation of the 80 isolates.

| Island   | Site          | Station and Sample Type (F,S) | Depth (m) | Temp (°C) | pH  | Conductivity (mS cm⁻¹) | Isolate                        |
|----------|---------------|-------------------------------|-----------|-----------|-----|------------------------|--------------------------------|
| Vulcano  | Levante Harbor| A (F)                          | 0.3       | 25        | 5.2 |                        | A1-3, A2-4, A3, A2-3a S1, S1-1 |
|          |               | S1 (S)                         | sl        | 93        |     |                        | S3 B1-2                         |
|          |               | S3 (S)                         | sl        | 85        |     |                        | B2-3, B2-4, B2-5, B2-70-1,T1-3, T2-3, T2-4, T2-5, T2-6 |
|          |               | B1 (F)                         | 6.0       | 24        | 6.4 |                        | B3-3, B3-4, B3-6, B3-11, B3-12, B3-13, B3-15, B3-16, B3-18, B3-24, B3-27, B3-28, B3-S, B3-S2, B3-S3, B3-S4, B3-S5, B3-71, B3-72, B3-73, B3-74, B3-75, B3-76, T3-1, T3-2, T3-3 |
|          |               | B2, T2 (F,S)                   | 6.0       | 43        | 6.4 |                        | B3-2, B2-5, B2-70-1,T1-3, T2-3, T2-4, T2-5, T2-6 |
|          |               | B3, T3 (F,S)                   | 0.7       | 65        | 5.2 | nd                     | B3-2, B2-4, B2-5, B2-70-1,T1-3, T2-3, T2-4, T2-5, T2-6 |
|          |               |                               |           |           |     |                        | B3-3, B3-4, B3-6, B3-11, B3-12, B3-13, B3-15, B3-16, B3-18, B3-24, B3-27, B3-28, B3-S, B3-S2, B3-S3, B3-S4, B3-S5, B3-71, B3-72, B3-73, B3-74, B3-75, B3-76, T3-1, T3-2, T3-3 |
| La Roya  |               |                               | 1 (F)     | 6.3       | 48  | 5.6                    | 47.9                           |
|          |               |                               |           |           |     |                        | s1a-1, s1a-2, s1a-2-1, 1as-1, slb-1a, slb-3 |
|          |               |                               |           |           |     |                        | 3s-1, 3s-2, md3s-1, md3s-2 |
|          |               |                               |           |           |     |                        | 4-1, 4s-1, md4-1-1, md4-1-2, su4-4, s4-4, s4-5, s4-6 |
| Pta      | Conigliara    |                               |           |           |     |                        | 5-1, 5-2, 5s-1, 5s-2, 5s5s-1, 5s5s-2 |
| Lipari   | Inzolfata     | 5 (F,S)                       | 3.1       | 30        | 5.9 |                        | 5-1, 5-2, 5s-1, 5s-2, 5s5s-1, 5s5s-2 |
| Panarea  | La Calcara    | 7 (S)                         | 19.8      | 95        | 5.1 |                        | s7s-1, s7s-3g, s7s-5 SBP3 |
|          | Black point   |                               | 23.0      | 130       | 3.3 | 46.2                    | APA, APB |
|          | Campo 7       | AP (W)                        | 21.3      | 60        | 4.9 | 49.2                    | APA, APB |
| Stromboli| Zurro         | 10 (F)                        | sl        | 36        | 6.7 |                        | 10-2, 10-1-65, 10-1-55, md10-1, g10 |
|          | Ginostra      | 11 (F)                        | sl        | 39        | 6.7 |                        | 11-1, g11-2 |

sl: sea level

2.2. Screening for Lipases

Hydrolysis of Tween 20 (0.5% v/v) and Tween 80 (0.5% v/v) was tested on Sierra agar modified [29]. Activity of esterase (butyrate, C4), esterase lipase (caprylate, C8), and lipase (myristate, C14) was evaluated using the miniaturized API-ZYM test system (bioMérieux, Marcy l’Etoile, France) in triplicate. From selected strains, the 16S rRNA genes were amplified by polymerase chain reaction, using universal bacterial primers: 27F and 1525R. The PCR products were purified using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol and sequenced by BIOFAB (Roma, Italy). A nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi. accessed on 1 February 2022) was performed to obtain sequences with the most significant alignment.
2.3. Petroleum Hydrocarbons Utilization

To screen the ability of the strains to grow in the presence of a hydrocarbon as a unique source of carbon and energy, a basic mineral natural seawater medium (NSW), consisting of (g L$^{-1}$) NH$_4$NO$_3$ 1.0, K$_2$HPO$_4$ 0.2, Fe-citrate 0.02 dissolved in 800 mL of seawater and 200 mL of distilled water, was utilized. The pH was adjusted to 8 with 1N NaOH and the medium was sterilized by autoclaving at 121 °C for 20 min. Kerosene (Petroleum Ether 190–250 °C, Panreac) or gasoline (from Q8 petrol station) maintained at 60 °C in a thermal bath, were sterilized separately by filtration through membrane 0.45 µm and added to the medium (2% v/v) [28]. Strains were inoculated (OD$_{600}$ = 0.1 equivalent to 1.5 × 10$^8$ bacteria mL$^{-1}$) at each optimal temperature for at least 7 days. The bacterial growth was spectrophotometrically evaluated (Ultraspec 3000; Amersham Pharmacia Biotech, Freiburg, Germany). Based on the correspondence between absorbance and bacteria density, absorbance values (OD$_{600}$ ≤ 0.5 were indicative for “high growth”, those ranging from 0.2 to 0.5 for “low growth”, and ≤ 0.2 for “no growth. Each experiment was performed in triplicate.

2.4. Screening for Biosurfactant/Bioemulsifier Production

The methylene blue agar (BM) assay, as a semi-quantitative assay for the detection of extracellular anionic surfactants, was used according to Siegmund and Wagner [30]. Methylene blue was added to agarized NSW at final concentration of 0.2 mg mL$^{-1}$. Strains were inoculated in three replicates and after incubation for 48 h at 45 °C, the formation of a dark blue halo around each colony was considered positive for surfactant production.

The emulsifying activity of CFSs from aerobically grown bacteria in NSW plus kerosene (2% v/v) (NSW + KER), was evaluated as reported by Cooper and Goldenberg [31], with some modifications. To obtain the CFSs, three replicates of each culture were centrifuged at 3800 $\times$ g for 20 min at 4 °C. An aliquot of each CFS (2 mL) was mixed with an equal volume of kerosene in a glass tube (10 cm high and 1 cm in diameter) and vortexed at high speed for 2 min. The uncultured medium was used as negative control and Triton X-100 (Sigma-Aldrich, Milan, Italy) was used as positive control. The emulsifying index (E$_{24}$) was calculated after 24 h of mixing with kerosene as the ratio between the height of the emulsion layer and the total height, multiplied by 100, and normalized by dividing the E$_{24}$ values by the respective absorbances (OD$_{600}$).

2.5. BSs Optimization and Activity

2.5.1. BSs Optimization

To optimize the BSs production, selected strains with the highest E$_{24}$ were grown in media containing different nitrogen (organic or inorganic), and carbon sources (saccharose and glycerol). The following media were prepared: (i) NSW + KER, (ii) seawater plus yeast extract (0.1%) and saccharose (3%) (Sigma-Aldrich, Milan, Italy) (SWY + SAC), (iii) seawater plus yeast extract (0.1%) and glycerol (3%) (Sigma-Aldrich, Milan, Italy), (iv) Marine Broth 2216 (MB), (v) Marine Broth plus glycerol (3%) (MB + GLY), and (vi) Marine Broth plus saccharose (3%) (MB + SAC). Strains were inoculated (OD$_{600}$ = 0.1) in triplicate and incubated at each optimal temperature of growth, with shaking at 120 rpm for 48 h. Bacterial growth was spectrophotometrically evaluated (OD$_{600}$) after 8, 20, 24, 30, and 48 h.

2.5.2. CFSs Surface-Active Properties

To test the surface-active properties, the oil drop-collapse assay and the reduction of the surface tension (ST) were evaluated at 25 °C. The oil drop-collapse assay was carried out on a polystyrene lid of a 96-microwell plate (Biolog, Harward, CA, USA). Briefly, 100 µL of each CFS were spotted on the lid, then 5 µL of crude oil were added to the surface of each CFS. Biosurfactant-producing culture gave flat drops.

The reduction of the ST was measured (in triplicate) using the digital Wilhelmy plate-type tensiometer K10T (Krüss, Hamburg, Germany) [32]. The ST (STi and STf as initial
and final value, at the beginning and at the end of the incubation time, respectively, was evaluated.

2.5.3. CFSs Emulsifying Properties

To evaluate the ability to emulsify different hydrocarbons and vegetable oils, 2 mL of each CFS were mixed with an equal volume of chloroform, ethyl acetate, decane, gasoline, hexadecane, castor oil, olive oil, kerosene, or mineral oil. Each mixture was vortexed vigorously and left to stand for 24 h, and E_{24} was determined as described above. Uninoculated sterile media were used as negative controls.

2.6. BSs Extraction and Characterization by ATR-FTIR

To extract the BSs from each CFS, acid precipitation method was utilized [33]. Each CFS was acidified at pH 2.0 using 2N HCl and kept overnight. BSs were dissolved into a chloroform–methanol mixture (2:1 v/v), and the organic layer, containing BSs, was separated using a rotary evaporation process (Rotavapor® R-300, BUCHI Italia S.r.l, Cornaredo, Italy), and finally weighed. The dried BSs were analyzed in triplicate by the attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. ATR-FTIR Vertex 70 V spectrometer (Bruker Optics GmbH & Co. KG, Ettlingen, Germany) using Platinum diamond ATR was employed to collect spectra in the 4000 to 1000 cm⁻¹ wavenumber range. The analysis of IR spectra was carried out by using the OMNIC software (Origin Lab Co., Northampton, MA, USA).

2.7. Oil Removal Test

The ability of BSs to remove different oils (mineral, crude, and castor) from a cotton cloth matrix was investigated according to Tripathi et al. [34]. Each piece of dry cotton cloth (2 cm × 2 cm) was soaked in one of the selected oils (300 µL). Each cloth was dried overnight at 60 °C and then weighed. Then, each oil-loaded cloth was washed with an aqueous solution of the selected BSs (1 g L⁻¹, w/v) or in tap water as negative control, followed by stirring at 30 °C for 1 h. The cotton cloths were rinsed in water (100 mL each) and dried at 60 °C overnight. The weight difference before and after the treatment with each biosurfactant was measured, and the oil-removal percentage was calculated as reported by Chen et al. [35].

2.8. Statistical Analysis

The experiments were carried out in triplicate and data are expressed as averages and standard deviations or relative errors (where specified). To compare different experimental groups, data were analyzed by two-way ANOVA and the Tukey’s test was used for post hoc analysis (GraphPad Software Inc., La Jolla, CA, USA). All statistical values were considered significant at p < 0.05.

3. Results

3.1. Petroleum Hydrocarbons Utilization

The ability of the tested strains to grow using kerosene or gasoline as a unique carbon source is reported in Figure 1.

In the presence of kerosene, 15 strains (18.75%) grew well, and 47 strains (58.75%) showed low growth. Only 10 strains (12.50%) showed high growth with gasoline, and 35 (43.75%) exhibited low growth (Figure 1). Strains showing high growth levels in the presence of kerosene (15) and gasoline (10) were selected for further investigation. The selected thermophilic bacilli, their growth characteristics, utilization of kerosene or gasoline (2% v/v) as only carbon sources, and lipase production are reported in Table 2. The full list of 80 tested strains is reported in Table S1.
Table 2. Thermophilic bacilli isolated from hydrothermal vents of Eolian Islands, optimal characteristics for growth, utilization of kerosene (Ker) or gasoline (Gasol) (2% v/v) as only carbon sources, and enzymatic production on selected substrates.

| Strain              | Opt T (°C) | Opt pH | Opt NaCl (%) | Ker (2%) | Gasol (2%) | Tween 20 | Tween 80 | Esterase C4 | Esterase/Lipase C8 | Lipase C14 |
|---------------------|------------|--------|--------------|----------|------------|----------|----------|-------------|--------------------|------------|
| Bacillus sp. A1-3   | 55         | 6      | 2            | +        | +          | +        | +        | +           | +                  | −          |
| B. licheniformis B3-15 a | 45         | 7      | 2            | +        | −          | +        | +        | +           | +                  | +          |
| Bacillus sp. B3-18  | 65         | 7      | 2            | +        | −          | +        | +        | +           | +                  | −          |
| Bacillus sp. B3-24  | 65         | 7      | 2            | +        | −          | +        | −        | +           | −                  | −          |
| Bacillus sp. B3-28  | 50         | 6      | 2            | +        | −          | −        | −        | +           | +                  | −          |
| G. thermodenitrificans B3-72 b | 65         | 7      | 0            | +        | +          | −        | −        | +           | +                  | +          |
| Bacillus sp. B3-75  | 55         | 8      | 2            | +        | +          | +        | +        | +           | +                  | −          |
| Bacillus sp. B3-76  | 50         | 5.5    | 1            | +        | +          | −        | +        | +           | +                  | −          |
| B. licheniformis md4-1-1 | 50        | 7.5    | 5            | +        | +          | −        | −        | +           | +                  | +          |
| Bacillus sp. md4-1-2 | 70         | 5.5    | 1            | +        | +          | −        | −        | +           | +                  | −          |
| Bacillus sp. T1-3   | 60         | 8      | 1            | +        | +          | +        | −        | +           | +                  | −          |
| G. thermodenitrificans S1 | 65         | 8      | 2            | +        | −          | +        | +        | +           | +                  | +          |
| Bacillus sp. S1-1   | 50         | 5.5    | 2            | +        | +          | +        | +        | +           | +                  | +          |
| B. licheniformis s7s-1 | 50        | 7      | 2            | +        | +          | +        | +        | +           | +                  | +          |
| B. horneckiae SBP3 DSM 103063 c | 45     | 8      | 2            | +        | +          | +        | +        | +           | +                  | +          |

a [28]; b [24]; c [17,36].

Five strains were moderately acidophilic (optimal pH values ≤ 6) and four isolates were alkalophilic (optimal pH 8). At their optimal growth conditions, 12 strains were lipolytic on Tween 20 and 9 on Tween 80. All strains possessed esterase C4 and esterase/lipase C8, and eight strains were positive for lipase C14. Six strains (B3-15, T1-3, S1, S1-1, s7s-1, and SBP3) possessed all the tested esterases and lipases and were lipolytic on Tween 20 and Tween 80.

3.2. Screening for Biosurfactants/Bioemulsifiers Production

The presence of biosurfactants/bioemulsifiers, as detected by the BM assay of the 15 selected hydrocarbon-utilizing strains grown in NSW + Ker (2% v/v) after 48 h of incubation and the emulsifying activity (E24) of CFS thereof are reported in Table 3.
Table 3. Screening of biosurfactants/bioemulsifiers production of 15 selected hydrocarbon-utilizing bacilli on methylene blue agar plates (BM) and emulsifying index ($E_{24}$) of their cell-free supernatants in NSW + KER (1:1 v/v), in comparison with the industrially synthesized surfactant Triton X-100. Sterile NSW + KER was used as negative control. (+) presence of halo in BM. Data are expressed as averages and standard deviations (n = 3).

| Strain    | BM | $E_{24}$ (%) |
|-----------|----|--------------|
| A1-3      | +  | 42.1 ± 2.3   |
| B3-15 $^a$| +  | 51.4 ± 1.4   |
| B3-18     | +  | 42.2 ± 2.7   |
| B3-24     | +  | 40.1 ± 1.9   |
| B3-28     | +  | 41.4 ± 1.8   |
| B3-72 $^b$| +  | 41.3 ± 1.8   |
| B3-75     | +  | 32.3 ± 1.5   |
| B3-76     | +  | 43.5 ± 1.8   |
| md4-1-1   | +  | 42.3 ± 1.9   |
| md4-1-2   | −  | 18.2 ± 1.5   |
| T1-3      | −  | 22.3 ± 1.8   |
| S1        | +  | 35.6 ± 1.4   |
| S1-1      | +  | 44.3 ± 1.8   |
| s7s-1 $^c$| +  | 32.2 ± 1.6   |
| SBP3      | +  | 55.2 ± 1.6   |
| Triton X-100 | nd | 74 ± 1.8 |
| Sterile NSW + KER | nd | 0 |

$^a$ [28]; $^b$ [24]; $^c$ [17,36].

Most of the hydrocarbon-utilizing bacilli (13/15) were positive for biosurfactants/bioemulsifiers production according to the BM test. CFSs from the majority of them (10/15) showed an $E_{24} \geq 40\%$, with the highest values observed for strains B3-15 (51\%) and SBP3 (55\%). Based on the results obtained from the prescreening tests, *Bacillus licheniformis* B3-15 and *B. horneckiae* SBP3 were selected for further optimization of biosurfactant production.

3.3. Biosurfactant Production and Activity

3.3.1. Biosurfactant Production

The growth of B3-15 and SBP3 strains and the emulsifying activity of their CFSs were evaluated in different nutritional conditions (nitrogen sources), and saccharose or glycerol (3\%) as carbon sources, after incubation for 48 h under optimal conditions (temperature 45 ± 1 °C and pH 8) (Figure 2).

B3-15 and SBP3 grew in all the tested culture media (Figure 2a and Table S2), and their growth was the highest in MB + SAC (OD$_{600nm}$ = 1.4 and 2.1, respectively), (groups d and e, respectively), followed by MB or MB + GLY (groups c and d, respectively), and it was double compared to NSW + KER (OD$_{600nm}$ = 0.7 for both strains) (group a). The lowest growth of both tested strains were observed in SWY + GLY and SWY + SAC (OD$_{600nm}$ ≤ 0.4) (groups b) (Figure 2a).

Lower emulsifying activities were observed in the CFSs of B3-15 and SBP3 in the SWY medium (Figure 2b and Table S2). The SWY was the only medium that resulted in significant differences in $E_{24}$ between the two CFSs irrespective of the carbon source used (groups b and c). $E_{24}$ values were higher only for MB + SAC (group d) compared to NSW + KER and MB + GLY or MB, indicating that the emulsifying activity is greatly influenced by the nitrogen form present in the media (yeast extract + peptone in MB or the inorganic N in NSW + KER compared to low $E_{24}$ in SWY medium containing only yeast extract). Furthermore, higher $E_{24}$ values observed in MB + SAC suggest that the increasing production of emulsifiers depends not only on the nitrogen source but rather on its combination with a specific carbon source.
When the emulsifying activity of CFSs was normalized for the growth values of the strains, higher values were observed for B3-15 in SWY + GLY and SBP3 in NSW + KER (Table S2), suggesting that emulsifying activity might not be directly correlated to higher growth. Among all the used culture media, we selected MB + SAC for further optimization experiments, as it was the medium in which both the highest growth (OD_{600\,nm}) and emulsifying activity were observed.

To assess the optimal conditions for recovery of the biosurfactants, the growth curves of B3-15 and SBP3 strains in MB + SAC and the E_{24} with kerosene of their CFSs during selected incubation times were evaluated (Figure 3).

Figure 2. Growth of B3-15 and SBP3 strains (OD_{600\,nm}) (a) and emulsifying index (E_{24}%) of their cell-free supernatants (CFSs) (b) with kerosene (1:1 v/v), obtained in different media after incubation at 45 °C for 48 h. NSW + KER, natural seawater plus 2% kerosene; SWY + GLY, seawater plus yeast extract and glycerol (3%); SWY + SAC, seawater plus yeast extract and saccharose (3%); MB, marine broth; MB + GLY, MB plus glycerol (3%) and MB + SAC, marine broth plus saccharose (3%). Data are expressed as averages and standard deviations (n = 3). The lowercase letters above the bars denote groups significantly different by ANOVA (p < 0.05).

Figure 3. (a) Growth curves of B. licheniformis B3-15 and B. horneckiae SBP3 in MB + SAC incubated at 45 °C for 48 h and (b) trend of stable emulsion of supernatants with kerosene (1:1) during the fermentation. Data are expressed as averages and standard deviations (n = 3). * Significantly different (p < 0.05).
SBP3 strain grew better than B3-15, reaching the maximal growth \( \text{OD}_{600 \text{nm}} = 2.1 \) after 20 h of incubation (Figure 3a). The \( E_{24} \) increased during the selected incubation period and reached the maximum value after 48 h when both CFSs exhibited similar emulsifying activity (Figure 3b). These results indicated that the selected nutritional conditions are responsible for fast cell growth, resulting in the production of a great abundance of cells which, once they entered the stationary phase, produced a lot of BSs.

### 3.3.2. CFSs Biosurfactant Activities

The CFSs (MB + SAC) from SBP3 and B3-15 strains were positive for the oil drop-collapse assay, leading to the expansion of the water droplet, by reducing the interfacial tension (Table 4). The ST reduction values are expressed as the difference between the ST value at the beginning and at the end of the incubation period for all isolates. ST was reduced in the presence of both CFSs from B3-15 and SBP3, with the highest reduction for the SBP3 strain (Table 4).

Table 4. Surface activities of (CFSs (MB + SAC) from B3-15 and SBP3 strains, using the oil drop-collapse and ST assays. Data are expressed as averages and standard deviations \((n = 3)\). * Significantly different \((p < 0.05)\); ** \((p < 0.01)\).

| Time (h) | CFSs (MB + SAC) | ST (mN m\(^{-1}\)) | Sterile MB + SAC |
|---------|-----------------|----------------------|------------------|
|         | B3-15 | SBP3 | B3-15 | SBP3 | B3-15 | SBP3 |
| 24      | nd    | nd   | 57 ± 1.3 | 53 ± 1.1 * | 68.3 ± 1.2 |
| 48      | +     | +    | 52 ± 1.4 | 39 ± 1.1 ** | 68.3 ± 1.6 |

The ability of CFSs of B3-15 and SBP3 strains in MB + SAC to emulsify different hydrocarbons and vegetable oils (chloroform, ethyl acetate, decane, hexadecane, castor oil, olive oil, mineral oil, gasoline, and kerosene) \( (1:1, v/v) \), is reported in Figure 4.

![Figure 4](image_url)

**Figure 4.** Ability of CFSs from B3-15 and SBP3 strains to emulsify \( E_{24} \) different hydrocarbons \( (1:1, v/v) \) and vegetable oils. Data are expressed as averages and standard deviations \((n = 3)\). * Significantly different \((p < 0.05)\).
The CFSs from B3-15 and SBP3 showed a higher E_{24} index for kerosene (62 and 64%, respectively), decane (64 and 58%, respectively), followed by olive oil (53 and 55%), and hexadecane (51 and 56%, respectively).

3.4. BSs Extraction and Characterization by ATR-FTIR

After acid precipitation from CFSs MB + SAC of B3-15 and SBP3 strains, the crude BSs yields were 910 ± 15.23 and 950 ± 16.17 mg L^{-1}, respectively. ATR-FTIR analysis was used to determine the structural features of these crude biosurfactants. The peak wavenumbers were assigned as reported in Table 5.

Table 5. FTIR band assignments for the functional groups.

| Wavenumber Values (cm\(^{-1}\)) | Assignment | References |
|----------------------------------|------------|------------|
| 3300–3200                        | Amide A    | [37,38]    |
| 3000–2800                        | CH\(_2\) and CH\(_3\) of lipids | [39] |
| 1690–1618                        | Amide I peptidic conformation | [37] |
| ~1548                            | Amide II peptidic conformation | [38] |
| 1456–1453                        | -CH\(_2\) of lipids | [38] |
| 1400–1380                        | CH\(_2\) and CH\(_3\) of lipids, dipicolinic acid, amide III | [38] |
| ~1066                            | (R-O-p-O-R) from ring vibrations of carbohydrates | [38] |
| ~1250                            | CH-NH stretching | [40] |
| 1055–1050                        | Phosphate groups | [41] |
| 1035–1030                        | Stretching vibrations of the C-O group in esters | [42] |

The FTIR characterization of crude biosurfactants from *B. licheniformis* B3-15 and *B. horneckiae* SBP3 is reported in Figure 5.

3.5. Oil Removal Test

The oil removal ability of BSs from B3-15 and SBP3 strains is shown in Table 6.

Table 6. The removal rate (%) of mineral, crude, and castor oil from cotton sections by BS solution (1 g L\(^{-1}\)) obtained from B3-15 and SBP3 strains. Data are expressed as averages and standard deviations (n = 3).

| Washing Solution | Mineral Oil | Crude Oil | Castor Oil |
|------------------|-------------|-----------|------------|
| Tap water        | 0.52 ± 0.04 | 0.42 ± 0.01 | 0.87 ± 0.02 |
| BS B3-15         | 53.0 ± 0.04 | 45.0 ± 0.04 | 70.0 ± 2.20 |
| BS SBP3          | 48.0 ± 0.02 | 80.0 ± 2.50 | 32.0 ± 0.07 |

The removal was negligible with tap water in control tests, conversely positive effects were observed in the cotton section in the presence of both BSs, although with different levels (from 32 to 80%). When BS B3-15 (1 g L\(^{-1}\)) was dispersed in tap water, the castor oil removal rate was the highest (70%) and about two-fold higher than BS SBP3, followed by mineral oil (53%) and crude oil (45%). Differently, the aqueous mixture with BS SBP3 showed the highest removal rate of crude oil (80%), about two-fold higher than BS B3-15, followed by mineral oil (48%) and castor oil (32%).

Figure 5. ATR-FTIR spectra of crude BSs from B3-15 (blue) and SBP3 (black) strains.

In the FTIR spectra of BSs from the two strains, the peaks observed at 3301 cm\(^{-1}\) were assigned to CH and OH groups of Amide A. The peaks observed at 2944 and 2927 cm\(^{-1}\)
were attributed to the aliphatic (-CH3 and -CH2) stretching vibrations of lipids, indicating the presence of alkyl chains (Figure 5). The peaks at 1641 and 1636 cm\(^{-1}\) were attributed to Amide I, indicating a peptide structure of BSs. According to other studies [43–45], the characteristic stretching frequency of amides in the region 3300–3250 cm\(^{-1}\) (Amide A) and 1650–1500 cm\(^{-1}\) (Amide I) is specific for surfactin-like lipopeptides of biosurfactant. Moreover, the shifts on the peaks attributed to lipids and peptides suggested that the two lipopeptides possessed different structures.

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### 4. Discussion

Biosurfactants and bio-emulsifiers are gaining increasing attention in several fields of applications, such as bioremediation, pharmaceutics, and cosmeceuticals because they are eco-friendly alternatives to their industrially made counterparts.

In this study, 80 thermophilic bacilli isolated from shallow hydrothermal vents of the Eolian Islands were screened for their ability to utilize kerosene or gasoline as the only carbon source. Among them, 15 bacilli showed high growth in a mineral medium supplemented with kerosene or gasoline (2%, v/v) at 45 °C for 48 h. This prompted us to verify the surfactant production capability of these bacteria. Biosurfactants, by reducing the ST of oil droplets, make them easier for bacterial uptake [44]. Although the relationship between the production of lipases and biosurfactants has not been established yet [45], the synthesis of lipases and biosurfactants by microorganisms may occur in the metabolization of oil substrates [46].

The thermophilic bacilli utilizing hydrocarbons were able to hydrolyze Tween 20 and Tween 80, produced esterase (C4) and/or esterase lipase (C8), and interestingly, only eight strains also produced lipase (C14). Many studies reported that the lipases are produced by hydrocarbon-utilizing Bacillus, Geobacillus, and Pseudomonas species [47–51]. Such hydrolytic enzymes play a role in the utilization and/or degradation of hydrocarbons, since they are induced when the intracellular content of alkane catabolism intermediates, such as alkanols, long-chain, and short-chain fatty acids, increases [52]. Furthermore, thermostable lipases are among the most interesting enzymes in the food and pharmaceutical field and are promising tools as potential biocatalysts in biodiesel production and other biotechnology applications [53]. Since most of the thermophilic hydrocarbon-utilizing strains displayed both lipolytic and good emulsifying activity (E\(_{24}\) ≥ 40%), their CFSs could be used, without any further extraction procedure, as detergent agents, such as surface cleaning agents for oil pipelines, bilge tanker or industrial silos.
BSs production from B3-15 and SBP3 (DSM 103062) strains, displaying the highest $E_{24}$ values in the presence of kerosene, was then investigated under different nutritional conditions. It has been reported that the choice of nitrogen sources, as an inorganic or organic nutrient, and carbon sources could have a major influence on the yield of BSs [54,55]. CFs of B3-15 and SBP3, from the medium containing organic nitrogen (peptone in MB) and saccharose, showed the highest $E_{24}$ values, then it can be concluded that the selected nutritional conditions allowed the production of a lot of BSs, as proven by the oil drop-collapse assay and the reduction in ST, with SBP3 being the most efficient in reducing ST.

Moreover, CFs from B3-15 and SBP3 strains in these nutritional conditions exhibited significant potential in emulsifying hydrocarbons and vegetable oils, with the highest values obtained with kerosene ($E_{24} = 62$ and $64\%$, respectively), (Table 3 and Figure 4), and the emulsion layers were stable for over thirty days. CFs from several Bacillus strains capable of forming emulsions with kerosene ($E_{24}$) have been reported previously, such as B. subtilis ATCC 6633 (57.9%) [56], B. subtilis DSVP23 (86.5%) [57], B. subtilis LB5a (70.4%) [58], and B. licheniformis NIOT-AMKV06 (64.3%) [59] under different nutritional conditions. To the best of our knowledge, this is the first report of surfactant production by a strain belonging to B. horneckiae. In comparison with the emulsification indices ($E_{24}$ with kerosene) of undiluted Triton X-100 (74%), sodium dodecyl sulfate (74.4%), and Tween 80 (73.2%) [59], our CFs possessed emulsifying activity that could compete with these industrially manufactured surfactants. Moreover, the ability to emulsify undiluted hexadecane of CFs from B3-15 ($51.2 \pm 1.4\%$) and SBP3 ($55.5 \pm 3.5\%$) appeared similar to that of commercial surfactants, such as linear alkylbenzene sulfonates ($51.7 \pm 2.4\%$), Findet®1214N/23 (61.8 ± 0.1%) and Glucopone®650 (55.8 ± 1.3%) [60]. According to several studies, the capacity of CFs to emulsify hydrocarbons suggests a potential use in bioremediation and as industrial detergents for cleaning petrol-contaminated surfaces, such as oil pipes, bilge tankers, or industrial silos [61,62]. Furthermore, CFs from both strains were able to emulsify vegetable oils, such as castor and olive oils, then they may be used as cleaning solutions in the food industry, as green alternatives to synthetic surfactants [63].

After optimization of BSs production and acid extraction, the highest yield of crude BSs was 910 mg L$^{-1}$ from B3-15 and 950 mg L$^{-1}$ from SBP3 strains, after incubation at 45 °C for 48 h. It is well known that bacilli are able to produce a variety of exoproducts depending on the culture conditions and the extraction procedures. Using an equal volume of cold ethanol, EPSs were previously extracted from CFs of several Eolian bacilli, including B. licheniformis B3-15 [13], B. licheniformis T14 [25], G. thermodenitrificans B3-72 [24] and SBP3 strain [17,36], with different yields (165, 70, 70 and 70 mg L$^{-1}$, respectively). As also reported for several bacterial EPSs [64,65], the EPS-T14 was able to emulsify two immiscible liquids, but possessed low surface activity, characteristics that were related to its antiadhesive properties against the bacterial adhesion and biofilm formation [25].

The ATR-FTIR spectra of BSs showed signals attributed to Amide A, Amide I, and aliphatic stretching vibrations of lipids, specific for surfactin-like lipopeptides [43–45,66–68]. Although similar, the spectra of BSs slightly differed from each other in the peaks attributed to Amide A and lipids.

A wide range of lipopeptides with surfactant activity have been described from marine bacilli, such as pumilacidin from B. pumilus and B. stratosphericus [69,70], surfactin and lichenysin from B. licheniformis NIOT-06 [71], fengycins from B. circulans [72], iturins from B. megaterium and Bacillus sp. KCB145006 [73,74], and surfactin and bacillomycin F from B. siamensis [75]. When compared with other marine thermophilic and halophilic bacilli the yield of crude BSs from B3-15 and SBP3 was higher than that produced by both B. licheniformis BAS50 (160 mg L$^{-1}$) and B. licheniformis BNP29 (90 mg L$^{-1}$), obtained in the aerobic, carbohydrate-based medium [76]. However, the yields of these surfactants greatly depended on the cultivation conditions, since the anaerobic surfactant production was three–five times lower than that of aerobic cultures [76].

The oil-removal test indicated that BS B3-15 (1 g L$^{-1}$) efficiently removed castor oil (70.0%), whereas the BS SBP3 was more effective in removing crude oil (80.0%) from a
cotton matrix, and the different activity may be ascribable to the observed differences in the chemical structure of the two lipopeptides. As previously reported, the difference in the efficacy of BSs from several bacterial strains to remove oil from the cotton matrix can be explained on the basis of their chemical properties, specifically in the distribution of polar head groups, leading to their classification as anionic, cationic and non-ionic surfactants [77]. Compared to physical methods and/or chemical products, BSs from B3-15 and SBP3 could be used for the removal of hydrocarbons from contaminated soil and cotton cloth, properties that have not been adequately explored until now [78,79].

Actually, BSs receive a great interest for oil recovery and remediation purposes, detergent products such as household, personal care products, and industrial detergents, and other possible medical applications, such as antimicrobial and antiadhesive agents. Further studies will be performed on our BSs to evaluate agro-industrial waste and by-products, such as crude glycerol from biodiesel production, and exhausted vegetable oils such as feedstocks for large-scale BSs production.

5. Conclusions

Thermophilic hydrocarbon utilizing bacilli from shallow hydrothermal vents of the Eolian Islands represents a valuable source of biomolecules that could be suitable for several industrial applications. CFSs of the 15 strains, able to grow with kerosene or gasoline, which harbor both lipolytic and emulsifying activity, could be used as green detergents, as well as in bioremediation. Two selected strains, *B. licheniformis* B3-15 and *B. horneckiae* SBP3 (DSM 103062), have been investigated for their ability to produce BSs. Data indicated that BSs production is dependent on nutritional factors and that the copresence of peptone and saccharose gave the best results in terms of $E_{24}$ values. Their CFSs exhibited significant potential in emulsifying both hydrocarbons and vegetable oils, suggesting a potential use in bioremediation and for cleaning petrol contaminated surfaces, such as oil pipelines, bilge tankers, or industrial silos, or as cleaning solutions in the food industry. As determined by ATR-FTIR, extracted BSs were two chemically distinct lipopeptides that can be considered for high-value biotechnological applications, such as the cosmeceutical and pharmaceutical industries.

In conclusion, CFSs from *B. licheniformis* B3-15 and *B. horneckiae* SBP3 (DSM 103062) can be employed without further chemical treatments, and BSs could be considered green detergents, as their production does not release any toxic byproducts and, differently from industrially made surfactants, does not require the use of petrochemicals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10081077/s1, Table S1: Enzymatic profile of the 80 thermophilic strains isolated from the hydrothermal vents of Eolian Islands (Italy); Table S2: Growth of B3-15 and SBP3 strains (OD$_{600nm}$) in different culture media and emulsifying index ($E_{24}\%$).

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Abbreviations

ATR-FTIR: attenuated total reflectance Fourier transform infrared; BM: methylene blue agar assay; BSs: biosurfactants; CFS: cell-free supernatant; E24: emulsifying index after 24 h; EPS: exopolysaccharide; MB: Marine Broth; MB + GLY: Marine Broth plus glycerol (3%); MB + SAC: Marine Broth plus saccharose (3%); NSW: natural seawater, NSW + KER: natural seawater supplemented with kerosene (2% v/v); SAMs: surface-active molecules; ST: surface tension; SWY + GLY: seawater plus yeast extract (0.1%) and glycerol (3%); SWY + SAC: seawater plus yeast extract (0.1%) and saccharose (3%).

References

1. Kaczorek, E.; Pachholak, A.; Zdarta, A.; Smulek, W. The impact of biosurfactant on microbial properties leading to hydrocarbon biodegradability. Colloids Interfaces 2018, 2, 35. [CrossRef]
2. Rosenberg, E. Surface active and drag-reducing bacterial polymers. In Biotechnology: Bridging Research and Applications; Kamely, D., Chakrabarty, A.M., Kornguth, S.E., Eds.; Kluwer Academic Publishers: Boston, MA, USA, 1991; pp. 231–248.
3. Banat, I.M.; Franceschi, A.; Gandolfi, I.; Bestetti, G.; Martinotti, M.G.; Fracchia, L.; Smyth, T.J.; Marchant, R. Microbial biosurfactants production, applications and future potential. Appl. Microbiol. Biotechnol. 2010, 87, 427–444. [CrossRef]
4. Gudiña, E.J.; Rodríguez, A.I.; Alves, E.; Domingues, M.R.; Teixeira, J.A.; Rodrigues, L.R. Bioconversion of agro-industrial by-products in rhamnolipids toward applications in enhanced oil recovery and bioremediation. Bioresour. Technol. 2015, 177, 87–93. [CrossRef]
5. Franceschi, A.; Gandolfi, I.; Bestetti, G.; Smyth, T.J.; Banat, I.M. Production and applications of trehalose lipid biosurfactants. Eur. J. Lipid. Sci. Technol. 2010, 112, 617–627. [CrossRef]
6. De Almeida, D.G.; Soares da Silva, R.D.; Luna, J.M.; Rufino, R.D.; Santos, V.A.; Banat, I.M.; Sarubbo, L.A. Biosurfactants: Promising molecules for petroleum biotechnology advances. Front. Microbiol. 2016, 7, 1718. [CrossRef]
7. Janek, T.; Łukaszewicz, M.; Krasowska, A. Identification and characterization of biosurfactants produced by the Arctic bacterium Pseudomonas putida BD2. Colloids Surf. B Biointerfaces 2013, 110, 379–386. [CrossRef]
8. De França, I.W.L.; Lima, A.P.; Lemos, J.A.M.; Lemos, C.G.F.; Melo, V.M.M.; de Sant’ana, H.B.; Gonçalves, L.R.B. Production of a biosurfactant by Bacillus subtilis ICA56 aiming bioremediation of impacted soils. Cad. Today 2015, 255, 10–15. [CrossRef]
9. Perfumo, A.; Rancich, I.; Banat, I.M. Possibilities and challenges for biosurfactants use in petroleum industry. In Biosurfactants. Advances in Experimental Medicine and Biology; Sen, R., Ed.; Springer: New York, NY, USA, 2010; pp. 135–145.
10. Smyth, T.J.P.; Perfumo, A.; Marchant, R.; Banat, I.M. Isolation and analysis of low molecular weight microbial glycolipids. In Handbook of Hydrocarbon and Lipid Microbiology; Timmis, K.N., Ed.; Springer: Berlin, Germany, 2010; pp. 3705–3723.
11. Sekhon-Randhawa, K.K.; Rahman, P.K. Rhamnolipid biosurfactants—past, present, and future scenario of global market. Front. Microbiol. 2014, 5, 1–8. [CrossRef]
12. Venkateswaran, K.; Iwabuchi, T.; Matsui, Y.; Toki, H.; Hamada, E.; Tanaka, H. Distribution and biodegradation potential of oil-degrading bacteria in North Pacific coastal waters. FEMS Microbiol. Ecol. 1991, 86, 113–122. [CrossRef]
13. Maugeri, T.L.; Gugliandolo, C.; Caccamo, D.; Panico, A.; Lama, L.; Gambacorta, A.; Maugeri, T.L. New bacilli from shallow hydrothermal vents of Panarea Island (Italy) and their biotechnological potential. J. Appl. Microbiol. 1993, 39, 123–126. [CrossRef]
14. Nazina, T.N.; Tourova, T.P.; Poltaraus, A.B.; Novikova, E.V.; Grigoryan, A.A.; Ivanova, A.M.; Lysenko, A.M.; Lysenko, A.M.; Osipov, G.A.; Belyaev, S.S.; et al. Taxonomic study of aerobic thermophilic bacilli: Descriptions of Geobacillus subterraneus gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus thermodenitrificans, Bacillus thermodenitrificans, Bacillus thermodenitrificans, Bacillus kaustophilus, Bacillus thermoelukasialisus and Bacillus thermocatenulatus to Geobacillus as the new combinations Geosthermobacillus, Geothermocatenulatus, Geothermoleukasialisus, Geokauthophilus, Geothermocatenulatus and Geothermoleukasialisus. Int. J. Syst. Evol. Microbiol. 2001, 51, 433–446. [PubMed]
15. Sorkhoh, N.A.; Ibrahim, A.S.; Ghannoum, M.A.; Radwan, S.S. High-temperature hydrocarbon degradation by Bacillus steathomophilus from oil-polluted Kuwaiti desert. Appl. Microbiol. Biotechnol. 1993, 39, 123–126. [CrossRef]
16. Nazina, T.N.; Tourova, T.P.; Poltaraus, A.B.; Novikova, E.V.; Grigoryan, A.A.; Ivanova, A.M.; Lysenko, A.M.; Osipov, G.A.; Belyaev, S.S.; et al. Taxonomic study of aerobic thermophilic bacilli: Descriptions of Geobacillus Subterraneus gen. nov., sp. nov. and Geobacillus Uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus Thermodenitrificans, Bacillus Thermodenitrificans, Bacillus Thermodenitrificans, Bacillus Kaustophilus, Bacillus Thermoelukasialisus and Bacillus Thermocatenulatus to Geobacillus as the new combinations Geosthermobacillus, Geothermocatenulatus, Geothermoleukasialisus, Geokauthophilus, Geothermocatenulatus and Geothermoleukasialisus. Int. J. Syst. Evol. Microbiol. 2001, 51, 433–446. [PubMed]
17. Gugliandolo, C.; Lentini, V.; Spanò, A.; Maugeri, T.L. New bacilli from shallow hydrothermal vents of Panarea Island (Italy) and their biotechnological potential. J. Appl. Microbiol. 2012, 112, 1102–1112. [CrossRef] [PubMed]
18. Calanchi, N.; Capaccioni, B.; Martini, M.; Tassi, F.; Valentini, L. Submarine gas-emission from Panarea Island Aeolian Archipelago: Distribution of inorganic and organic compounds and inferences about source conditions. Acta Vulcanol. 1995, 7, 43–48.
19. Wang, W.; Li, Z.; Zeng, L.; Dong, C.; Shao, Z. The oxidation of hydrocarbons by diverse heterotrophic and mixotrophic bacteria that inhabit deep-sea hydrothermal ecosystems. ISME J. 2020, 14, 1994–2006. [CrossRef]
20. Maugeri, T.L.; Gugliandolo, C.; Caccamo, D.; Stackebrandt, E. A polyphasic taxonomic study of thermophilic bacilli from shallow, marine vents. Syst. Appl. Microbiol. 2001, 24, 451–468. [CrossRef]
21. Lentini, V.; Gugliandolo, C.; Maugeri, T.L. Identification of enzyme-producing thermophilic bacilli isolated from marine vents of Aeolian Islands (Italy). Ann. Microbiol. 2007, 57, 355–361. [CrossRef]
22. Spanó, A.; Gugliandolo, C.; Lentini, V.; Maugeri, T.L.; Anzelmo, G.; Poli, A.; Nicolaus, B. A novel EPS-producing strain of Bacillus licheniformis isolated from a shallow vent of Panarea Island (Italy). *Curr. Microbiol.* 2013, 67, 21–29. [CrossRef]

23. Arena, A.; Gugliandolo, C.; Stassi, G.; Pavone, B.; Iannello, D.; Bisignano, G.; Maugeri, T.L. An exopolysaccharide produced by Geobacillus thermodenitrificans strain B3-72: Antiviral activity on immunocompetent cells. *Immunol. Lett.* 2009, 123, 132–137. [CrossRef]

24. Nicolaus, B.; Panico, A.; Manca, A.C.; Lama, L.; Gambacorta, A.; Maugeri, T.; Gugliandolo, C.; Caccamo, D. A thermophilic Bacillus isolated from an Eolian shallow hydrothermal vent, able to produce exopolysaccharides. *Syst. Appl. Microbiol.* 2000, 23, 426–432. [CrossRef]

25. Spanó, A.; Laganà, P.; Visalli, G.; Maugeri, T.L.; Gugliandolo, C. In vitro antibiofilm activity of an exopolysaccharide from the marine thermophilic Bacillus licheniformis T14. *Curr. Microbiol.* 2016, 72, 518–528. [CrossRef] [PubMed]

26. Caccamo, M.T.; Zammuto, V.; Gugliandolo, C.; Madeleine-Perdrierat, C.; Spanó, A.; Magazù, S. Thermal restraint of a bacterial exopolysaccharide of shallow vent origin. *Int. J. Biol. Macromol.* 2018, 114, 649–655. [CrossRef] [PubMed]

27. Caccamo, M.T.; Gugliandolo, C.; Zammuto, V.; Magazù, S. Thermal properties of an exopolysaccharide produced by a marine thermotolerant Bacillus licheniformis isolated from a shallow vent. *J. Int. J. Biol. Macromol.* 2020, 145, 77–83. [CrossRef]

28. Ibrahim, M.L.; Ijah, U.J.J.; Manga, S.B.; Bilbis, L.S.; Umar, S. Production and partial characterization of biosurfactant produced by crude oil degrading bacteria. *Int. J. Biol. Macromol.* 2016, 88, 886–892. [CrossRef]

29. Spanó, A.; Laganà, P.; Visalli, G.; Maugeri, T.L.; Gugliandolo, C. In vitro antibiofilm activity of an exopolysaccharide from the marine thermophilic Bacillus licheniformis T14. *Curr. Microbiol.* 2016, 72, 518–528. [CrossRef] [PubMed]

30. Siegmund, I.; Wagner, F. New method for detecting rhamnolipids excreted by Geobacillus thermodenitrificans by ATTR-FTIR spectroscopy. *Int. J. Biol. Macromol.* 2020, 145, 77–83. [CrossRef]

31. Cooper, D.G.; Goldenberg, B.G. Surface-active agents from two Bacillus species. *Appl. Environ. Microbiol.* 1987, 53, 224–229. [CrossRef]

32. Tuleva, B.; Christova, N.; Jordanov, B.; Nikolova-Damyanova, B.; Petrov, P. Naphthalene degradation and biosurfactant activity by Bacillus cereus 28BN. *Z. Naturforsch. C. J. Biosci.* 2005, 60, 577–582. [CrossRef]

33. Sharma, N.; Lavana, M.; Kukreti, V.; Rana, D.P.; Lal, B. Laboratory investigation of indigenous consortia TERI]-188 for incremental oil recovery. *Front. Microbiol.* 2018, 9, 2357. [CrossRef]

34. Tripathi, V.; Gaur, V.K.; Dhiman, K.; Gautam, K.; Manickam, N. Characterization and properties of the biosurfactant produced by PAH-degrading bacteria isolated from contaminated oily sludge environment. *Environ. Sci. Pollut. Res. Int.* 2020, 27, 27268–27278. [CrossRef] [PubMed]

35. Chen, C.; Sun, N.; Li, D.; Long, S.; Tang, X.; Xiao, G.; Wang, L. Optimization and characterization of biosurfactant production from kitchen waste oil using Pseudomonas aeruginosa. *Environ. Sci. Pollut. Res. Int.* 2018, 25, 14934–14943. [CrossRef]

36. Zammuto, V.; Fuchs, F.M.; Fiebrandt, M.; Stapelmann, K.; Ulrich, N.J.; Maugeri, T.L.; Pukall, R.; Gugliandolo, C.; Moeller, R. Comparing spore resistance of Bacillus strains isolated from hydrothermal vents and spacecraft assembly facilities to environmental stressors and decontamination treatments. *Astrobiology* 2018, 18, 1425–1434. [CrossRef]

37. Arrondo, J.L.; Goñi, F.M. Special Issue: Infrared spectroscopy of membrane lipids. *Chem. Phys. Lipids* 1998, 96, 1–164.

38. Naumann, D.; Fabian, H.; Lasch, P. FTIR spectroscopy of cells, tissues and body fluids. In *Advances in Biomedical Spectroscopy*; IOSPress BV: Amsterdam, The Netherlands; Volume 2, pp. 312–354.

39. Yoshida, S.; Miyazaki, M.; Sakai, K.; Takeshita, M.; Yuasa, S.; Sato, A.; Kobayashi, T.; Watanabe, S.; Okuyama, H. Fourier Transform Infrared spectroscopic analysis of rat brain microsomal membranes modified by dietary fatty acids: Possible correlation with altered learning behavior. *Biospectroscopy* 1997, 3, 281–290. [CrossRef]

40. Ramani, K.; Jain, S.C.; Mandal, A.B.; Sekaran, G. Microbial induced lipoprotein biosurfactant from slaughterhouse lipid waste and its application to the removal of metal ions from aqueous solution. *Colloids Surf. B* 2012, 97, 254–263. [CrossRef]

41. Bezza, F.A.; Chirwa, E.M.N. Production and applications of lipopeptide biosurfactant for bioremediation and oil recovery by Bacillus subtilis CN2. *Biochem. Eng. J.* 2015, 101, 168–178. [CrossRef]

42. Rohman, A.; Man, Y.C. Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. *Food Res. Int.* 2010, 43, 886–892. [CrossRef]

43. Joy, S.; Rahman, K.S.M.P.; Sharma, S. Biosurfactant production and concomitant hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon contaminated environments. *Biochem. Eng. J.* 2017, 317, 232–241. [CrossRef]

44. Lin, S.C.; Minton, M.A.; Sharma, M.M.; Georgiou, G. Structural and immunological characterization of a biosurfactant produced by Bacillus licheniformis JF-2. *Appl. Environ. Microbiol.* 1994, 60, 31–38. [CrossRef]

45. Ibrahim, M.L.; Ijah, U.J.J.; Manga, S.B.; Bilbis, L.S.; Umar, S. Production and partial characterization of biosurfactant produced by crude oil degrading bacteria. *Int. Biodeterior. Biodegrad.* 2013, 81, 28–34. [CrossRef]

46. Paula, A.V.; Barboza, J.C.S.; Castro, H.F. Study of the influence of solvent, carbohydrate and fatty acid in the enzymatic synthesis of sugar esters by lipases. *Quim. Nova* 2005, 28, 792–796. [CrossRef]

47. Desai, J.D.; Banat, I.M. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 1997, 61, 47–64.

48. Yusoff, D.F.; Raja Abd Rahman, R.N.Z.; Masomian, M.; Ali, M.S.M.; Leow, T.C. Newly isolated alkane hydroxylase and lipase producing Geobacillus and Anoxybacillus species involved in crude oil degradation. *Catalysts* 2020, 10, 851. [CrossRef]
49. Mulani, N.; Fulke, A.B.; D’souza, E.; Ram, A.; Maloo, A.; Sayed, F.; Gajbhiye, S.N. Biodegradation of crude oil using marine Bassilus species from Vadinar coast, Gujarat, India. Curr. Sci. 2017, 112, 569–576. [CrossRef]

50. Margesin, R.; Zimmerbauer, A.; Schinner, F. Soil lipase activity—A useful indicator of oil biodegradation. Biotechnol. Technol. 1999, 13, 859–863. [CrossRef]

51. Meng, L.; Li, H.; Bao, M.; Sun, P. Metabolic pathway for a new strain Pseudomonas syxantha LSH-70: From chemotaxis to uptake of n-hexadecane. Sci. Rep. 2017, 7, 39068. [CrossRef] [PubMed]

52. Adlan, N.A.; Sabri, S.; Masomian, M.; Ali, M.S.M.; Rahman, R.N.Z.R.A. Microbial biodegradation of paraffin wax in malaysian crude oil mediated by degradative enzymes. Front. Microbiol. 2020, 11, 565608. [CrossRef]

53. Hamdan, S.H.; Maiangwa, J.; Ali, M.S.M.; Normi, Y.M.; Sabri, S.; Leow, T.C. Thermotable lipases and their dynamics of improved enzymatic properties. Appl. Microbiol. Biotechnol. 2021, 105, 7069–7094. [CrossRef]

54. Abouseouda, M.; Maarchi, R.; Amranec, A.; Bouderguia, S.; Nabi, A. Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. Desalination 2008, 223, 143–151. [CrossRef]

55. Fooladi, T.; Hamid, A.; Yusoff, W.; Moazami, N.; Shafee, Z. Production of biosurfactant by indigenous isolated bacteria in fermentation system. AIP Conf. Proc. 2013, 1571, 197–201.

56. Reis, F.A.; Sárvulo, E.F.; De França, F.P. Lipopeptide surfactant production by Bacillus subtilis grown on low-cost raw materials. Appl. Biochem. Biotechnol. 2004, 115, 899–912. [CrossRef]

57. Pemmaraju, S.C.; Sharma, D.; Singh, N.; Panwar, R.; Cameotra, S.S.; Pruthi, V. Production of microbial surfactants from oily sludge-converted soil by Bacillus subtilis DSVP23. Appl. Biochem. Biotechnol. 2012, 167, 1119–1131. [CrossRef] [PubMed]

58. Nitschke, M.; Pastore, G.M. Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava wastewater. Biorens. Biotechnol. 2006, 97, 336–341. [CrossRef]

59. Anburajan, L.; Meena, B.; Toms Cherith, J.; Dheenan Palaiya, S.; Vinithkumar Nambali, V.; Dharani, G.; Kirubagaran, R. Functional and molecular characterization of a lipopeptide surfactant from the marine sponge-associated eu-bacteria Bacillus licheniformis NIO-AMKV06 of Andaman and Nicobar Islands, India. Mar. Pollut. Bull. 2014, 82, 76–85.

60. Vaz, D.A.; Gudiña, E.J.; Alameda, E.J.; Teixeira, J.A.; Rodrigues, L.R. Performance of a biosurfactant produced by a Bacillus subtilis strain isolated from crude oil samples as compared to commercial chemical surfactants. Colloids Surf. B Biointerfaces 2012, 89, 167–174. [CrossRef]

61. Banat, I.M.; Carboué, Q.; Saucedo-Castañeda, G.; de Jesús Cázares-Marinero, J. Biosurfactants: The green generation of speciality chemicals and potential production using Solid-State fermentation (SSF) technology. Bioresour. Technol. 2021, 320, 124222. [CrossRef]

62. Satpute, S.K.; Banat, I.M.; Dhakephalkar, P.K.; Banpurkar, A.G.; Chopade, B.A. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. Biotechnol. Adv. 2010, 28, 436–450. [CrossRef]

63. Campos, J.M.; Stamford, T.L.M.; Rufino, R.D.; Luna, J.M.; Stamford, T.C.M.; Sarubbo, L.A. Formulation of mayonnaise with the enzymatic properties. Biotechnol. Adv. 2006, 28, 1164–1170. [CrossRef]

64. Rendueles, O.; Kaplan, J.B.; Ghigo, J.M. Antibiofilm polysaccharides. Environ. Microbiol. 2012, 15, 334–346. [CrossRef]

65. Uzoigwe, C.; Burgess, J.G.; Ennis, C.J.; Rahman, P.K. Bioemulsifiers are not biosurfactants and require different screening approaches. Front. Microbiol. 2015, 5, 2015. [CrossRef] [PubMed]

66. Yakimov, M.M.; Timmis, K.N.; Wray, V.; Fredrickson, H.L. Characterization of a new lipopeptide surfactant produced by thermostolerant and halotolerant subsurface Bacillus licheniformis BAS50. Appl. Environ. Microbiol. 1995, 61, 1706–1713. [CrossRef] [PubMed]

67. Cho, S.J.; Lee, S.K.; Cha, B.J.; Kim, Y.H.; Shin, K.S. Detection and characterization of the Gloeosporium gloeosporioides growth inhibitory compound iturin A from Bacillus subtilis strain KSO3. FEMS Microbiol. Lett. 2003, 223, 47–51. [CrossRef]

68. Nanjundan, J.; Ramasamy, R.; Uthandi, S.; Ponnusamy, M. Antimicrobial activity and spectroscopic characterization of surfactin class of lipopeptides from Bacillus amyloliquefaciens SR1. Microb. Pathog. 2019, 128, 374–380. [CrossRef] [PubMed]

69. Gandhimathi, R.; Seghal Kiran, G.; Hema, T.A.; Selvin, J.; Rajeetha Raviji, T.; Shanmughapriya, S. Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomyces Nocardiosis alba MSA10. Bioprocess Biosyst. Eng. 2009, 32, 825–835. [CrossRef]

70. Ueno, T.; Chebbi, A.; Hadrich, F.; Frika, I.; Rabanal, F.; Sayadi, S.; Manresa, A.; Chambka, M. Production, characterization and biotechnological potential of lipopeptide biosurfactants from a novel marine Bacillus stratosphericus strain FLU5. Ecotoxicol. Environ. Saf. 2019, 167, 441–449. [CrossRef]

71. Anburajan, L.; Meena, B.; Raghavan, R.V.; Shridhar, D.; Joseph, T.C.; Vinithkumar, N.V.; Dharani, G.; Dheenan, P.S.; Kirubagaran, R. Heterologous expression, purification, and phylogenetic analysis of oil-degrading biosurfactant biosynthesis genes from the marine sponge-associated Bacillus licheniformis NIO-06. Bioproc. Biosyst. Eng. 2015, 38, 1009–1018. [CrossRef]

72. Sikampathasekaran, C.; Mukherjee, S.; Samanta, R.; Sen, R. High-performance liquid chromatography purification of biosurfactant isoforms produced by a marine bacterium. Anal. Bioanal. Chem. 2009, 395, 845–854. [CrossRef]

73. Dey, G.; Bharti, R.; Dhanaranjan, G.; Das, S.; Dey, K.K.; Kumar, B.N.; Sen, R.; Mandal, M. Marine lipopeptide Iturin A inhibits Akt mediated GSK3β and FoxO3a signaling and triggers apoptosis in breast cancer. Sci. Rep. 2015, 5, 10316. [CrossRef]

74. Son, S.; Ko, S.K.; Jang, M.; Kim, J.W.; Kim, G.S.; Lee, J.K.; Jeon, E.S.; Futamura, Y.; Ryu, I.J.; Lee, J.S.; et al. New cyclic lipopeptides of the iturin class produced by salt-derened Bacillus sp. KCB14S006. Mar. Drugs 2016, 14, 72. [CrossRef]
75. Xu, B.H.; Lu, Y.Q.; Ye, Z.W.; Zheng, Q.W.; Wei, T.; Lin, J.F.; Guo, L.Q. Genomics-guided discovery and structure identification of cyclic lipopeptides from the Bacillus siamensis JFL15. *PLoS ONE* 2018, 13, e0202893. [CrossRef] [PubMed]

76. Yakimov, M.; Amro, M.; Bock, M.; Boseker, K.; Fredrickson, H.L.; Kessel, D.G.; Timmis, K.N. The potential of *Bacillus licheniformis* strains for in situ enhanced oil recovery. *J. Petrol. Sci. Eng.* 1997, 18, 147–160. [CrossRef]

77. Bouassida, M.; Fourati, N.; Ghazala, I.; Ellouze-Chaabouni, S.; Ghribi, D. Potential application of *Bacillus subtilis* SPB1 biosurfactants in laundry detergent formulations: Compatibility study with detergent ingredients and washing performance. *Eng. Life Sci.* 2018, 18, 70–77. [CrossRef] [PubMed]

78. Mukherjee, A. Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations. *Lett. Appl. Microbiol.* 2007, 45, 330–335. [CrossRef]

79. Rosenberg, E.; Ron, E.Z. High- and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* 1999, 52, 154–162. [CrossRef]