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Development of Foam-Free Biosurfactant Production Processes Using *Bacillus licheniformis*

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Abstract: Microbial biosurfactants are considered environmentally friendly alternatives to synthetic surfactants in numerous applications. One of the main bottlenecks to their widespread use is the lack of effective processes for their production on an industrial scale. Biosurfactant production using conventional aerated bioreactors results in excessive foaming due to the combination of air injection and their tensioactive properties. A not widely explored approach to overcome this problem is the development of foam-free production processes, which require the identification and characterization of appropriate microorganisms. *Bacillus licheniformis* EL3 was evaluated for biosurfactant production under oxygen-limiting conditions in a bioreactor, using a mineral medium containing glucose as a carbon source and $NaNO_3$ and NH_4Cl as nitrogen sources. After optimizing the operational conditions, glucose concentration, and inoculum strategy, *B. licheniformis* EL3 produced 75 ± 3 mg biosurfactant/L in 43 h. The purified biosurfactant exhibited exceptional surface active properties, with minimum surface tension values (29 mN/m) and a critical micelle concentration (27 mg/L) similar to those achieved with commercial surfactin. Furthermore, biosurfactant yield per substrate ($Y_{P/S}$ = 0.007 g biosurfactant/g glucose) was similar to the figures reported for *Bacillus subtilis* strains grown in similar conditions, whereas biosurfactant yield per biomass ($Y_{P/X}$ = 0.755 g biosurfactant/g biomass) and specific biosurfactant productivity (q_{BS} = 0.018 g biosurfactant/(g biomass × h)) were almost three times higher when compared to previous reports. The results obtained indicate that *B. licheniformis* EL3 is a promising candidate for the development of foam-free biosurfactant production processes at an industrial scale.

Keywords: foaming; lichenysin; lipopeptide; surfactant; surfactin

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1. Introduction

Reducing our dependence on crude oil is one of the main objectives of the United Nations Sustainable Development Goals. In order to achieve that, it is necessary to replace chemicals commonly used not only by the industry but also in our daily lives with more environmentally friendly compounds. Among those chemicals, surfactants represent an important class. Surfactants are widely used in different industries (textile, food, pharmaceutical, or agriculture, among others), but also in our daily lives, where they are key ingredients of household detergents and cleaners, cosmetics, and personal care products [1,2]. Surfactant properties arise from their amphiphilic structure (they contain a hydrophilic and a hydrophobic domain in the same molecule), which promotes their accumulation at interfaces with different polarities, changing their properties [3,4]. Accordingly, surfactants show the ability to stabilize emulsions and solubilize hydrophobic compounds in aqueous phases (and vice versa) [1]. The world market value of surfactants, whose production reached 14 million tons in 2019, is around 40,000 million USD and is estimated to grow up to 52,000 million USD by 2025 [5].

According to their origin, surfactants can be classified as synthetic, semi-synthetic, and biological. Synthetic and semi-synthetic surfactants are obtained from fossil or natural (e.g.,

animal or vegetal fat) resources, respectively, through chemical synthesis. Synthetic and semi-synthetic surfactants have been widely used as they are cheaper than biological surfactants; however, they are usually associated with detrimental effects on the environment and human health due to their toxicity and low biodegradability. Furthermore, as they are widely used in household and personal care products, they are generally discarded without control, which increases their detrimental effects [1,5].

Biological surfactants are surface-active compounds produced by microorganisms, plants, and animals; those of microbial origin (biosurfactants) are the most widely studied. Biosurfactants are classified according to their chemical structure, being the main classes of glycolipids and lipopeptides [1–3,6,7]. Nowadays, a great variety of biosurfactant-producing microorganisms have been identified. Biosurfactants are potential candidates to replace chemical surfactants in numerous applications, as some of them exhibit remarkable surface-active properties as well as stability at high temperatures and ionic strength. Furthermore, they exhibit higher biodegradability and environmental compatibility than synthetics [2,3]. Despite all these advantages, the main limitation of biosurfactants when compared to their chemical counterparts is their high production costs, mainly associated with low productivities, and the cost of the substrates used for their biosynthesis, which is between 3 and 11 times higher than that of chemical surfactants [1,2]. However, this can be partially circumvented by using agro-industrial wastes and by-products for their biosynthesis, which also contributes to the development of the circular bio-economy [2,8].

Among lipopeptide biosurfactants, surfactin, produced mainly by *Bacillus subtilis* strains, stands out due to its remarkable surface activity, being considered the most powerful biosurfactant known so far. The surfactin structure comprises a seven-amino acid cyclic peptide chain linked to a β-hydroxy fatty acid chain of variable length (12–16 carbon atoms) [8,9]. Besides the limitations described above, another issue associated with surfactin production in bioreactors is the generation of excessive foam due to its surface activity and the usually high agitation and aeration rates required for its appropriate production by *B. subtilis* [5,10]. Excessive foaming is challenging for surfactin production in bioreactors on an industrial scale. For that reason, different strategies have been evaluated in order to manage it, as recently reviewed by Gudiña and Teixeira [5], although most of them exhibit limited success. The use of mechanical foam disruptors, besides being unfavorable due to energy consumption, can be detrimental to the cells, causing mechanical damage and stress [11]. The use of chemical antifoaming agents, which are usually effective in avoiding foam production, can interfere with biosurfactant purification and be detrimental for bacterial growth and surfactin production [12]. As an alternative, different bioreactor designs have been explored to avoid foaming during surfactin production, where unconventional oxygen transfer models are used. These include rotating disc bioreactors, membrane-aerated bioreactors, or biofilm bioreactors, among others, which allow foam-free surfactin production, although surfactin titers are usually low [5,13–15]. Another approach to avoid foaming is solid-state fermentation; although in this case high surfactin titers have been reported, the main limitation is its extraction when applied at an industrial scale [16]. As a completely different strategy, in situ foam fractionation takes advantage of foaming to recover and purify surfactin produced, as it is concentrated in the foam; however, it is difficult to scale up for industrial applications [10,17]. A not widely explored alternative is surfactin production under anaerobic or oxygen-limited conditions, which has been reported in a few works [11,18,19].

Lichenysin is a lipopeptide biosurfactant produced by *Bacillus licheniformis*, whose structure is almost identical to that of surfactin, with the only difference being the first amino acid in the peptide ring (glutamic acid in the case of surfactin and glutamine in the case of lichenysin) [20–22]. Due to their almost identical structures, surfactin and lichenysin share almost the same properties. Despite this, lichenysin has been scarcely studied when compared to surfactin [5,21,22]. However, *B. licheniformis* seems to be better adapted to anaerobic growth than *B. subtilis*, making it a better candidate for the development of anaerobic biosurfactant production processes [5].

The objective of this study was to study biosurfactant production by *B. licheniformis* in bioreactors under oxygen-limited conditions for the development of foam-free biosurfactant production processes.

2. Materials and Methods

2.1. Microbial Strain

Bacillus licheniformis EL3 was isolated from a Brazilian oil reservoir and previously evaluated for biosurfactant production under oxygen-limited conditions by our research group. The strain was stored at −80 °C in LB medium supplemented with glycerol (20%, *v*/*v*).

2.2. Culture Media Composition

LB medium was used to prepare pre-cultures of *B. licheniformis* EL3. Its composition was NaCl 10 g/L, tryptone 10 g/L, and yeast extract 5 g/L at pH 7.0. The culture medium was sterilized at 121 °C for 15 min. For biosurfactant production, the mineral salt medium (MSM) reported by Willenbacher and co-workers [19] for anaerobic surfactin production by *B. subtilis* DSM10 was used. For the preparation of MSM, four different solutions were prepared separately. The first solution contained the nitrogen sources (NH_4Cl 0.1317 M; $NaNO_3$ 0.155 M) and buffer components (KH_2PO_4 0.0395 M; Na_2HPO_4 0.0527 M). The second one consisted of the carbon source (glucose, 100 g/L). The third solution consisted of $MgSO_4$ (4×10^{-2} M). Finally, a trace element solution composed of $CaCl_2$ 7×10^{-3} M, $FeSO_4$ 4×10^{-3} M, Na_2EDTA 4×10^{-3} M, and $MnSO_4$ 1×10^{-3} M. The pH of the nitrogen buffer solution was adjusted to 7.0, whereas the pH of the other solutions was not adjusted. The nitrogen buffer solution, the carbon source, and $MgSO_4$ solutions were sterilized at 121 °C for 15 min, while the trace element solution was filter-sterilized using a 0.22 µm PES filter. Once sterilized, appropriate volumes of the different solutions were mixed to form the MSM medium, with the following composition: NH_4Cl 0.1 M, $NaNO_3$ 0.1177 M, KH_2PO_4 0.03 M, Na_2HPO_4 0.04 M, $MgSO_4$ 8×10^{-4} M, $CaCl_2$ 7×10^{-6} M, $FeSO_4$ 4×10^{-6} M, Na_2EDTA 4×10^{-6} M, and $MnSO_4$ 1×10^{-6} M. The glucose concentration ranged between 20 and 40 g/L, depending on the experiments.

2.3. Pre-Cultures Preparation

Pre-cultures of *B. licheniformis* EL3 were prepared by inoculating a 50 mL flask containing 20 mL of LB medium with 100 µL from a frozen glycerol stock (first pre-culture). Pre-cultures were incubated for 24 h at 37 °C and 200 rpm under aerobic conditions. Subsequently, 1 mL from this pre-culture was transferred to a 250 mL flask containing 49 mL of MSM, which was incubated for 24 h at the same conditions (second pre-culture). The second pre-culture was used to inoculate a third pre-culture, which was performed under oxygen-limiting conditions in 120 mL serum flasks containing 49 mL of MSM. For that purpose, the second pre-culture was centrifuged (2700× *g*, 15 min), the cell-free supernatant was discarded, and the cells were washed with 50 mL of PBS (K_2HPO_4 0.05 M; KH_2PO_4 0.05 M; NaCl 0.15 M; pH 7.0) and centrifuged again at the same conditions. This procedure was repeated twice in order to remove biosurfactants present in the culture medium that could interfere with the subsequent assessment of biosurfactant production. Subsequently, the washed cells were resuspended in 5 mL of PBS, and the optical density at 600 nm ($OD_{600\,nm}$) of the cell suspension was measured. Serum flasks were inoculated with the required volume of cell suspension according to the initial $OD_{600\,nm}$ desired, and the remaining volume up to 1 mL was completed with sterile demineralized water. After inoculation, the serum flasks were closed with a rubber lid and an aluminum cap, and N_2 was injected using a needle coupled to a 0.22 µm PES syringe filter (in order to guarantee sterile conditions) at 0.5 bar for 5 min, whereas another needle was used to allow the gas to outflow through the rubber lid. Inoculated serum flasks were immediately incubated at 37 °C and 200 rpm in a vertical position for 24 h (third pre-culture).

2.4. Bioreactor Assays

Biosurfactant production under oxygen-limited conditions by *B. licheniformis* EL3 was studied in a 3.7 L bioreactor (RALF Advanced, Bioengineering AG, Zürich, Switzerland) containing 1.5 L of MSM and provided with pH, pO_2, and temperature probes. The bioreactor was autoclaved at 121 °C for 60 min, and the culture medium MSM was prepared following the same steps described in Section 2.3. Depending on the experiment, the culture medium was saturated with N_2 or O_2 at the beginning of the assay. Assays were performed at 37 °C, and the stirring speed was 500 rpm.

Three different approaches were used to inoculate the bioreactor, using pre-cultures grown under aerobic or oxygen-limited conditions. In the first case, the bioreactor was inoculated using the cells from a second pre-culture grown under aerobic conditions, taking into account the initial $OD_{600\,nm}$ desired, and the culture medium inside the bioreactor was saturated with N_2 immediately after the inoculation (approach used by Hoffmann and co-workers [11]). In the second approach, the bioreactor was inoculated as in the first case, but the culture medium inside the bioreactor was saturated with O_2 before the inoculation (approach used by Hoffmann and co-workers [18]). In the third case, a third pre-culture (grown under oxygen-limited conditions, as described in Section 2.3) was used directly to inoculate the bioreactor, whose culture medium was previously saturated with N_2 (approach used by Willenbacher and co-workers [19]). In this case, a N_2 stream was used to push the cells inside the bioreactor in order to avoid the incorporation of O_2 in the culture medium during the inoculation.

In all the bioreactor assays, the initial $OD_{600\,nm}$ was adjusted to around 0.15 (corresponding to a biomass concentration of around 0.008 g/L). The pH was monitored over time but was not controlled. No gas injection was performed during the cultivation process. Samples were taken over time in order to assess bacterial growth, biosurfactant production, glucose consumption, and metabolite production, as described in the following sections. Each assay was performed in duplicate.

2.5. Analytical Techniques

Bacterial growth was determined by measuring the $OD_{600\,nm}$ of the samples and performing the appropriate dilutions (using PBS) whenever required. Subsequently, the samples were centrifuged (2700× *g*, 15 min), and the cell-free supernatants were stored at 4 °C or −20 °C for further analysis. The $OD_{600\,nm}$ values obtained were transformed into biomass concentration (g/L) through a calibration curve constructed for *B. licheniformis* EL3:

$$Biomass\ concentration\ (g/L) = \frac{OD_{600\,nm} - 0.023}{14.186} \quad (1)$$

In order to assess biosurfactant production, the surface tension (ST) of the cell-free supernatants was measured using a KRÜSS K6 tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a du Noüy platinum ring [23]. In some cases, the cell-free supernatants were diluted 10 times with demineralized water, and the ST (ST^{-1}) was also measured. Each sample was measured at least twice. All measurements were performed at room temperature, and demineralized water (ST ≈ 70–72 mN/m) was used as a control.

Glucose consumption and metabolite production were assessed through high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Des Plaines, IL, USA). The system was equipped with a refractory index (RI-2031 Plus, JASCO, Oklahoma City, OK, USA) and a UV detector (K-2501, Knauer, Highwood, IL, USA). Cell-free supernatants were filtered (0.22 µm) before injection. As a mobile phase, 0.005 M H_2SO_4 was used at a flow rate of 0.6 mL/min, and the column was maintained at 60 °C during the analyses. The obtained chromatograms were analyzed using Star Chromatography Workstation software, version 6.3 (Varian, Palo Alto, CA, USA). The concentration of the different compounds present in the samples was calculated from the area of the peaks identified in the respective chromatograms. For this purpose, calibration

curves (area (mV × s) versus concentration (g/L)) were calculated for pure solutions of glucose, lactic acid, and acetic acid.

2.6. Biosurfactant Purification

The biosurfactant produced by *B. licheniformis* EL3 was purified through acid precipitation followed by chloroform:methanol extraction. First, the cultures were centrifuged (9500× *g*, 15 min). Subsequently, the cell-free supernatants were subjected to acid precipitation. For that purpose, HCl (18%, *v*/*v*) was added to the samples until reaching a pH of 2.0; the precipitation was conducted by incubating the samples for 24 h at 4 °C. After that, the samples were centrifuged (9500× *g*, 30 min), the supernatants were discarded, and the precipitate (crude biosurfactant) was resuspended in 20 mL of demineralized water, by adjusting the pH to 7.0 with the addition of 1 M NaOH. The aqueous crude biosurfactant solution was gently mixed for 1 h with a chloroform:methanol mixture (2:1, *v*/*v*) to achieve a final chloroform:methanol:biosurfactant solution ratio of 8:4:3. Subsequently, the mixture was allowed to separate in a separation funnel for 24 h. After that, the organic (inferior) phase, containing the biosurfactant, was recovered and allowed to evaporate in a fume hood. Subsequently, the purified biosurfactant was resuspended in 5 mL of ultrapure water and freeze-dried. At the end of the freeze-drying process, the purified biosurfactant was weighed and stored at −20 °C for further studies.

2.7. Biosurfactant Characterization

The critical micelle concentration (CMC) of the purified freeze-dried biosurfactant produced by *B. licheniformis* EL3 was determined by preparing solutions in PBS at concentrations between 0.039 and 1 g/L. The ST of the different biosurfactant solutions was measured as described in Section 2.5. By plotting the ST as a function of the logarithm of biosurfactant concentration and finding the intersection of the two lines that best fit the data (one of them pre-CMC data and another post-CMC), the CMC was determined. Fourier transform infrared spectroscopy (FTIR) was used to determine the chemical nature of the biosurfactant produced by *B. licheniformis* EL3 through the identification of the functional groups present in the molecule. Purified biosurfactant FTIR spectra were recorded and compared to standard surfactin (99% purity (SIGMA-Aldrich, Saint Louis, MO, USA)). FTIR spectra were recorded in the range of 400–4000 wavenumbers (cm^{-1}) at a resolution of 2 cm^{-1} using an Alpha II FT-IR Spectrometer (Bruker, Bremen, Germany) at room temperature (25 °C).

2.8. Data Analysis

To evaluate the fermentation processes, different parameters were calculated. With the data of biomass, glucose, metabolites, and biosurfactant concentration, it was possible to calculate the parameters biomass yield per substrate ($Y_{X/S}$ (g/g)), product yield per biomass ($Y_{P/X}$ (g/g)), product yield per substrate ($Y_{P/S}$ (g/g)), and specific biosurfactant productivity (q_{BS} [g/(g × h)]).

The biomass yield per substrate ($Y_{X/S}$) was calculated using the maximum biomass produced (Δm_{Xmax}) and the corresponding mass of consumed glucose ($\Delta m_{substrate}$) as follows:

$$Y_{X/S} = \frac{\Delta m_{Xmax}}{\Delta m_{substrate}} \quad (2)$$

The product yield per biomass ($Y_{P/X}$) was calculated using the maximum mass of the selected product ($\Delta m_{ProductMax}$) and the corresponding cell dry weight (Δm_{Xmax}) (Equation (3)) as follows:

$$Y_{P/X} = \frac{\Delta m_{ProductMax}}{\Delta m_{Xmax}} \quad (3)$$

The product yield per substrate ($Y_{P/S}$) was calculated by dividing the mass of the selected product ($\Delta m_{ProductMax}$) by the mass of glucose consumed ($\Delta m_{substrate}$) as follows:

$$Y_{P/S} = \frac{\Delta m_{ProductMax}}{\Delta m_{substrate}} \quad (4)$$

The specific biosurfactant productivity (q_{BS}) was calculated using the maximum mass of biosurfactant produced (Δm_{BSMax}), the corresponding cell dry-weigh mass (Δm_{Xmax}), and cultivation time (Δt) as follows:

$$q_{BS} = \frac{\Delta m_{BSMax}}{\Delta m_{Xmax} \times \Delta t} \quad (5)$$

3. Results and Discussion

3.1. Biosurfactant Production by Bacillus licheniformis EL3 in a Bioreactor under Oxygen-Limiting Conditions

In order to study biosurfactant production by *B. licheniformis* EL3 in a bioreactor under oxygen-limiting conditions, different strategies were assessed. In all the cases, the cultures were performed using 1.5 L of MSM supplemented with 20 g glucose/L (unless indicated otherwise), and the bioreactor was inoculated with an initial $OD_{600\,nm}$ around 0.15 (corresponding to approximately 0.008 g biomass/L).

In the first approach, the cells used to inoculate the bioreactor were obtained from aerobic pre-cultures performed in MSM (second pre-culture), and after inoculation, the bioreactor was saturated with N_2 in order to remove the oxygen present in the culture medium (approach used by Hoffmann and co-workers [11]). The results obtained are summarized in Figure 1.

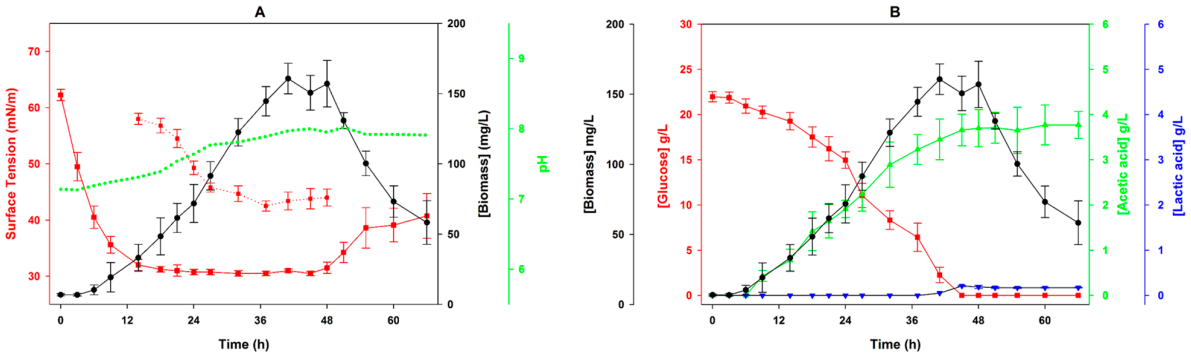

Figure 1. Results obtained in cultures performed with *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in a bioreactor under oxygen-limited conditions at 37 °C and 500 rpm using an aerobic pre-culture grown in MSM. Immediately after inoculation, the culture medium in the bioreactor was saturated with N_2. (**A**) Surface tension values (mN/m) of cell-free supernatants without dilution (ST, continuous line) and 10 times diluted (ST^{-1}, dotted line), pH, and biomass concentration (mg/L). (**B**) Glucose concentration (g/L), metabolite concentration (g/L), and biomass concentration (mg/L). Results correspond to the average of two assays, and error bars represent the standard deviation.

As it can be seen, the ST values decreased from 62.3 ± 1.0 mN/m to 31.2 ± 0.5 mN/m in 18 h and then remained almost constant until 45 h of culture, indicating biosurfactant production. According to the ST^{-1} values, the highest biosurfactant production was achieved at 37 h of culture (Figure 1), although similar ST^{-1} values were observed up to 45 h. A subsequent increase in the ST values was observed after 45 h, when glucose was exhausted (Figure 1). Regarding bacterial growth, a lag phase was observed during the first 3 h of culture, probably due to the adaptation of the cells from aerobic to oxygen-limiting conditions; the highest biomass concentration (around 0.16 g/L) was achieved between 41 and 48 h of culture, matching the highest biosurfactant production (Figure 1).

Bacterial growth was associated with glucose consumption, and biomass concentration decreased after glucose was exhausted (45–48 h). A considerable production of acetic acid was observed, which was almost parallel to bacterial growth and glucose consumption, achieving 3.8 g/L after 66 h, whereas lactic acid production was scarce (0.2 g/L), and started only after 41 h of culture, corresponding to glucose depletion (Figure 1). However, despite the considerable acid production, pH values increased during bacterial growth until 45 h of culture, from values around 7.2 to almost 8.0.

Subsequently, a set of assays similar to the previous ones was performed. As in the previous case, the cells used to inoculate the bioreactor were obtained from an aerobic pre-culture performed in MSM (second pre-culture), but the bioreactor was saturated with O_2 before inoculation (approach used by Hoffmann and co-workers [18]). The results obtained are shown in Figure 2.

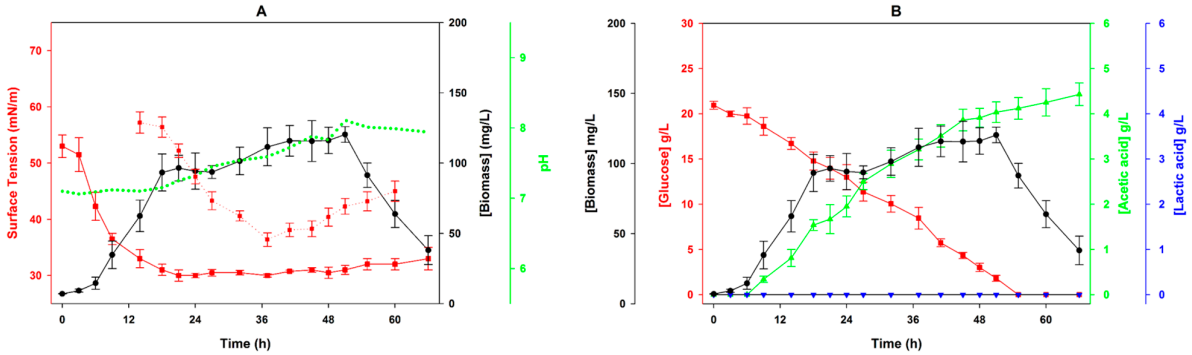

Figure 2. Results obtained in cultures performed with *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in a bioreactor under oxygen-limited conditions at 37 °C and 500 rpm, using an aerobic pre-culture grown in MSM. Before inoculation, the culture medium in the bioreactor was saturated with O_2. (**A**) Surface tension values (mN/m) of cell-free supernatants without dilution (ST, continuous line) and 10 times diluted (ST^{-1}, dotted line), pH, and biomass concentration (mg/L). (**B**) Glucose concentration (g/L), metabolite concentration (g/L), and biomass concentration (mg/L). Results correspond to the average of two assays, and error bars correspond to the standard deviation.

As it can be seen in Figure 2, using this approach, the ST decreased from 53.0 ± 2.0 mN/m to values around 30–31 mN/m in 18 h and after that remained almost constant until 51 h of culture, when glucose was almost exhausted. According to the ST^{-1} values, the highest biosurfactant production was achieved at 37 h of culture (Figure 2). As in the previous assays, the decrease in ST to values around 30 mN/m highlights the biosurfactant-producing ability of this strain under oxygen-limiting conditions. A further increase in ST and ST^{-1} values was observed between 50 and 66 h, although lower than in the previous strategy. An increase in biomass concentration up to 0.096 g/L was observed in the first 21 h of culture and then remained at values around 0.1 g/L up to 51 h, corresponding to glucose exhaustion, followed by an abrupt decrease (Figure 2). A lag phase was not observed in this case, as the cells were transferred from an aerobic pre-culture to a culture medium saturated with oxygen, although oxygen was completely exhausted in the first 3 h of culture. Acetic acid was continuously produced until 66 h, achieving 4.4 g/L, whereas lactic acid was not detected (Figure 2). Despite acetic acid production, once again, the pH increased from an initial value of around 7 to 8.1 after 51 h of culture (Figure 2).

Although the bioreactor assays were performed under oxygen-limiting conditions, the incorporation of an initial aerobic phase in this case can be beneficial in order to allow a faster development of the culture when pre-cultures grown under aerobic conditions are used, allowing a faster adaptation to the oxygen-limiting conditions, and as it can be concluded from the results obtained (Figure 2), it was not detrimental for biosurfactant production. Using this approach, the oxygen available to the cells progressively decreases, allowing a better adaptation to oxygen-limited conditions, as previously demonstrated by

Hoffmann and co-workers [18], who observed an abrupt biomass concentration decrease as the *B. subtilis* JABs24 cells were transferred from aerobic to oxygen-limited conditions too fast [18].

In the last approach assayed, the cells used to inoculate the bioreactor were obtained from pre-cultures grown under oxygen-limiting conditions in MSM (third pre-culture), and the bioreactor was saturated with N_2 in order to remove the oxygen present in the culture medium before inoculation (approach used by Willenbacher and co-workers [19]). The results obtained are shown in Figure 3.

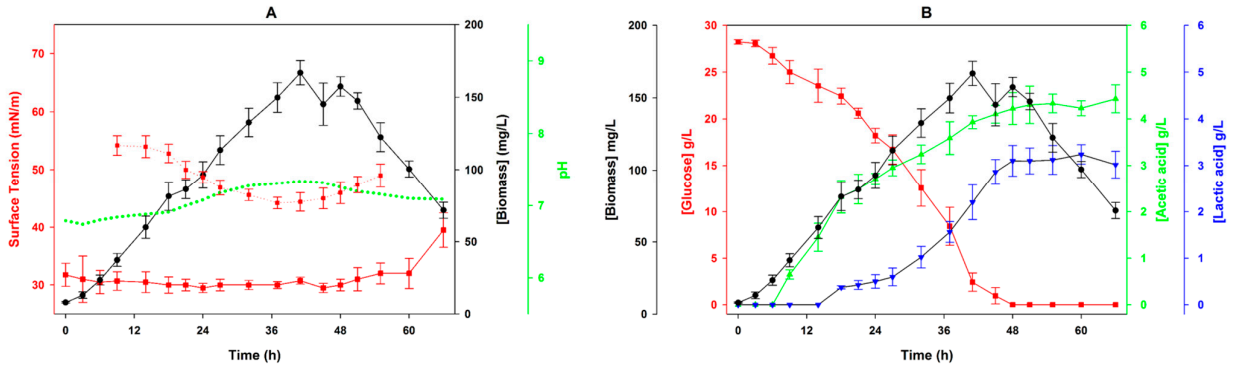

Figure 3. Results obtained in cultures performed with *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in a bioreactor, under oxygen-limited conditions at 37 °C and 500 rpm using a pre-culture grown in MSM under oxygen-limited conditions. Before inoculation, the culture medium in the bioreactor was saturated with N_2. (**A**) Surface tension values (mN/m) of cell-free supernatants without dilution (ST, continuous line) and 10 times diluted (ST^{-1}, dotted line). pH, and biomass concentration (mg/L). (**B**) Glucose concentration (g/L), metabolite concentration (g/L), and biomass concentration (mg/L). Results correspond to the average of duplicate assays, and error bars represent the standard deviation.

Although a similar bacterial growth profile was observed when compared to the first set of experiments (Figure 1), in this case, a lag phase was not observed, as bacterial growth was observed just 3 h after inoculation (Figure 3), similar to the second approach (Figure 2). The highest biomass concentration (around 0.16 g/L) was achieved between 41 and 48 h of culture. In this case, the ST values were low (between 30 and 32 mN/m) from the beginning of the assay due to the biosurfactant produced by *B. licheniformis* EL3 during the pre-culture, as in this case, pre-cultures (300 mL) were used directly to inoculate the bioreactor in order to keep the cells under oxygen-limiting conditions. However, a progressive decrease in the ST^{-1} values was observed, indicating biosurfactant production, which achieved its maximum between 37 and 41 h of culture. ST and ST^{-1} values started to increase after 48–51 h of culture, when glucose was exhausted (Figure 3). In this case, the initial glucose concentration was higher than 20 g/L due to the incorporation of glucose present in the pre-culture in the bioreactor. As in the first assay (Figure 1), glucose consumption and biomass concentration displayed a parallel profile, and biomass concentration started to decrease as glucose was exhausted (48 h) (Figure 3). Also, acetic acid production exhibited a similar profile to bacterial growth achieving 4.3 g/L after 51 h (Figure 3). The main difference when compared to the previous approaches is the considerable lactic acid production herein observed, also showing a similar profile to bacterial growth and achieving 3.1 g/L at 48 h. Interestingly, substantial lactic acid production occurred only in this approach when the pre-culture was grown under oxygen-limiting conditions. However, as in the previous assays, the pH increased from an initial value of 6.7 to 7.3 in 45 h, and as glucose was depleted and bacterial growth stopped (48 h), a slight pH decrease occurred (Figure 3).

In the absence of oxygen, *B. subtilis* and *B. licheniformis* can grow through nitrate respiration or fermentative metabolism. In the absence of terminal electron acceptors (nitrate and nitrite), both species can grow under anaerobic conditions through glucose

fermentative metabolism. In the presence of nitrate, nitrate respiration is preferred for anaerobic growth as it is more energetically favorable. The main metabolites identified during anaerobic growth in *B. subtilis* and *B. licheniformis* cultures are lactate, acetate, acetoin, and 2,3-butanediol. Acetate and acetoin are mainly produced during nitrate respiration, whereas lactate and 2,3-butanediol are the most abundant end products of fermentative metabolism [11,24–27]. In the first and second approaches herein studied, when *B. licheniformis* EL3 was grown in a bioreactor under oxygen-limiting conditions, acetic acid was the predominant metabolite identified, with almost no production of lactic acid (Figures 1 and 2), which is characteristic of growth through nitrate respiration [11,24,26,27]. However, although nitrate respiration suppresses the fermentative growth, low concentrations of lactate can be observed even during nitrate respiration, which indicates the existence of various processes during the anaerobic growth, as reported by Hoffmann and co-workers [11] for anaerobic cultures of *B. subtilis*. Ramos and co-workers [26] demonstrated that the expression of the gene encoding the enzyme lactate dehydrogenase (which catalyzes the reduction of pyruvate to lactate) is rapidly induced in *B. subtilis* after a shift from aerobic to anaerobic conditions, both in the presence or absence of nitrate. However, in the presence of nitrate, the expression of this gene is lower than in its absence. Accordingly, even in the presence of nitrate under anaerobic conditions, lactate production occurs in *B. subtilis* [26]. In contrast, in the third approach assayed, when the bioreactor was inoculated with a pre-culture grown under oxygen-limiting conditions, a considerable production of lactic acid was observed together with acetic acid (Figure 3), which may indicate a more active fermentative metabolism [11,24,26].

Previous studies performed using *B. subtilis* strains reported acetate production (2.3–2.5 g/L) simultaneous to nitrate respiration in bioreactor assays performed under strict anaerobic conditions, both using batch and fed-batch approaches; furthermore, even reducing the initial glucose concentration (from 10 to 2.5 g/L), acetate production was not reduced [11]. Other studies have demonstrated that when the anaerobic synthesis of acetate is reduced, the anaerobic growth of *B. subtilis* through nitrate respiration is significantly reduced [26]. On the other hand, Hoffmann and co-workers [11] demonstrated that acetate concentrations higher than 5 g/L reduced the overall growth rate in *B. subtilis* JABs24 grown in mineral medium under anaerobic conditions, which could be explained by the negative effect of acetate on the expression of nitrate and nitrite reductases [11]. Accordingly, the role of acetate during the anaerobic growth of *B. subtilis* is not completely understood.

According to the ST and ST^{-1} values obtained (Figures 1–3 and Tables S1–S3), it can be concluded that the bioreactor assay that used a pre-culture grown under aerobic conditions and in which the culture medium was saturated with O_2 before inoculation (Figure 2 and Table S2) was more favorable for biosurfactant production. Previous assays performed during the study of biosurfactant production by *B. licheniformis* EL3 under oxygen-limiting conditions in serum flasks using the culture medium MSM showed that, if the serum flasks were inoculated with cells obtained from pre-cultures performed in LB medium (first pre-culture), neither growth nor biosurfactant production occurred. However, when a second pre-culture prepared in MSM under aerobic conditions was performed, biosurfactant production occurred in the serum flasks under oxygen-limiting conditions. For that reason, that inoculum strategy was also used in the bioreactor assays. However, in an attempt to simplify the inoculation process, it was studied if *B. licheniformis* EL3 was capable of producing biosurfactant in a bioreactor under oxygen-limiting conditions when the bioreactor was inoculated with cells obtained from a pre-culture grown in LB medium under aerobic conditions. As in the previous assays, the culture medium used in the bioreactor was MSM supplemented with 20 g glucose/L, and it was saturated with oxygen before inoculation. The results obtained are shown in Figure 4.

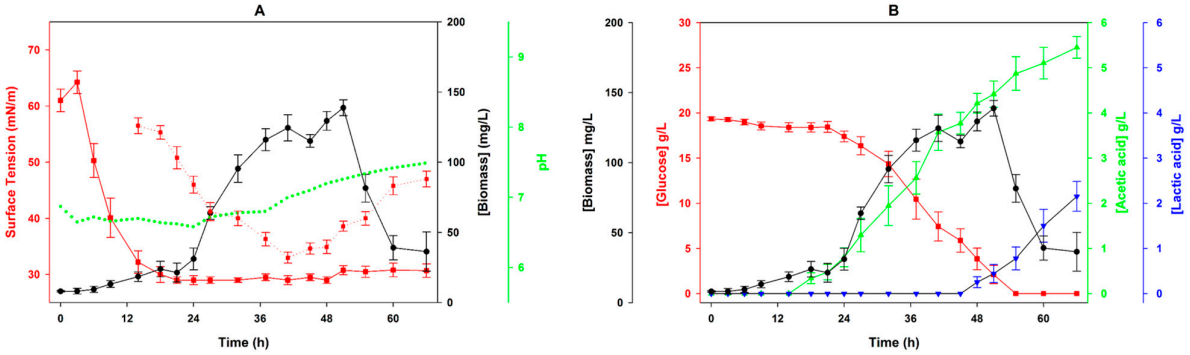

Figure 4. Results obtained in cultures performed with *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in a bioreactor under oxygen-limited conditions at 37 °C and 500 rpm using a pre-culture grown in LB medium. Before inoculation, the culture medium in the bioreactor was saturated with O_2. (**A**) surface tension values (mN/m) of cell-free supernatants without dilution (ST, continuous line) and 10 times diluted (ST^{-1}, dotted line), pH, and biomass concentration (mg/L). (**B**) Glucose concentration (g/L), metabolite concentration (g/L), and biomass concentration (mg/L). Results correspond to the average of three assays, and error bars correspond to the standard deviation.

As it can be seen from the results obtained, in this case, bacterial growth and biosurfactant production were observed (Figure 4), in contrast to the results obtained in serum flasks using the same strategy. After an extended lag phase, probably due to the adaptation of the cells to the mineral medium, biomass concentration increased, mainly from 20–24 h to 48–50 h of culture, achieving values around 0.13 g biomass/L, and after that an abrupt decrease was observed, corresponding to the consumption of most of the glucose present in the culture medium, which was exhausted after 55 h (Figure 4). In this case, oxygen depletion was achieved after 6 h of culture. The ST decreased from 61.0 mN/m to 30.0 mN/m after 18 h, achieving a minimum value of 29.0 mN/m at 21 h and then remained almost constant until 48 h of culture (corresponding to the consumption of most of the glucose present in the culture medium), followed by a slight increase. According to the ST^{-1} values, the highest biosurfactant production was achieved between 41 and 48 h of culture (Figure 4). Acetic acid production exhibited the same profile as bacterial growth until 48 h of culture, achieving 5.4 g/L at 66 h, whereas lactic acid production started at 48 h and achieved 2.1 g/L at the end of the assay (66 h). The pH remained almost constant until 41 h of culture, and after that, it started to increase up to 7.5 at 66 h of culture (Figure 4).

When compared to the previous bioreactor assays performed at the same conditions but using a second pre-culture grown in MSM (Figure 2), it can be concluded that slightly higher bacterial growth and ST reduction were achieved using the pre-culture prepared in LB (Tables S2 and S4), although in this case, an extended lag phase was observed, probably due to the adaptation of the cells to the new culture medium. The main difference was the production of lactic acid when the bioreactor was inoculated with cells obtained from the pre-culture performed in LB medium, which was not observed when the second pre-culture grown in MSM was used. That could be explained by a transition from nitrate respiration to fermentative metabolism with the subsequent production of lactic acid, as previously reported by other authors for *B. subtilis* [11,24].

3.2. Effects of Glucose Concentration on Bacterial Growth and Biosurfactant Production

Considering the positive results obtained in the last assays and the fact that glucose was completely consumed, a further experiment was performed at the same conditions, but increasing the initial glucose concentration in MSM from 20 to 30 g/L in order to study if increasing the amount of glucose available in the culture medium was favorable for biosurfactant production. The results obtained are presented in Figure 5.

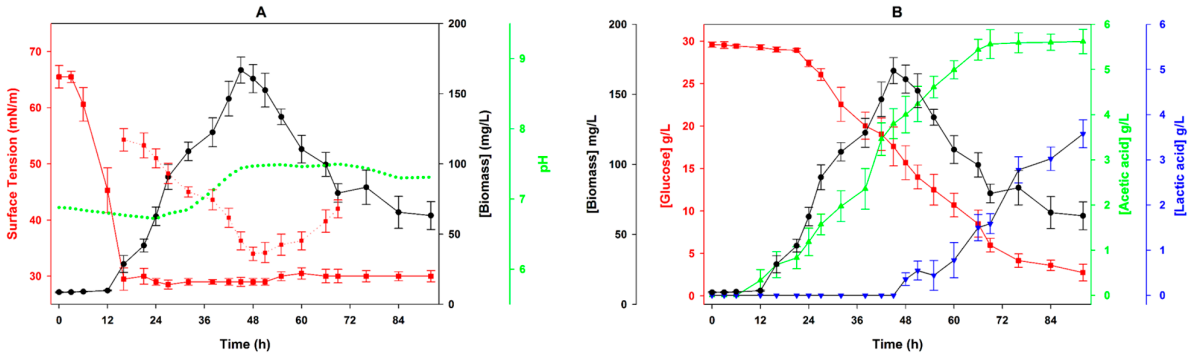

Figure 5. Results obtained in cultures performed with *Bacillus licheniformis* EL3 grown in MSM supplemented with 30 g glucose/L in a bioreactor under oxygen-limited conditions at 37 °C and 500 rpm using a pre-culture grown in LB medium. Before inoculation, the culture medium in the bioreactor was saturated with O_2. (**A**) Surface tension values (mN/m) of cell-free supernatants without dilution (ST, continuous line) and 10 times diluted (ST^{-1}, dotted line), pH, and biomass concentration (mg/L). (**B**) Glucose concentration (g/L), metabolite concentration (g/L), and biomass concentration (mg/L). Results correspond to the average of two assays, and error bars correspond to the standard deviation.

MSM supplemented with 30 g glucose/L was favorable for bacterial growth and biosurfactant production. Regarding bacterial growth, the highest biomass concentration achieved with 30 g glucose/L (0.166 ± 0.009 g/L) was slightly higher than using 20 g glucose/L (0.138 g biomass/L) (Figure 5). ST values decreased from 65.5 ± 2.0 mN/m to 29.5 ± 2.0 mN/m after 16 h and then remained almost constant until the end of the assay (92 h). According to the ST^{-1} values, the highest biosurfactant production was achieved between 48 and 51 h of culture (Figure 5). Glucose was not completely consumed (2.8 g/L remained in the culture medium even after 92 h of culture). Acetic and lactic acid production displayed the same time profile observed in the previous assay. Comparing both bioreactor assays regarding acetic and lactic acid production, it can be concluded that increasing the initial glucose concentration from 20 to 30 g/L did not result in an increase in acetic acid production (5.4–5.6 g/L); however, lactic acid production increased, from 2.2 to 3.6 g/L (Figures 4 and 5), associated with higher glucose consumption, and the same transition from nitrate respiration to fermentative metabolism could be speculated here. Accordingly, acetic acid yield per substrate decreased as the initial glucose concentration increased from 20 to 30 g/L, whereas for lactic acid, a slight increase was observed. As in the previous assay, pH values remained almost constant until 27 h and then increased from 6.7 to 7.5 at 69 h (Figure 5).

In a further set of experiments, bioreactor assays were performed using MSM supplemented with 40 g glucose/L. In this case, biomass concentration increased from 0.008 g/L to values around 0.05 g/L in the first 21 h of culture and then started to decrease. In a similar way, the ST of the cell-free supernatants decreased from 56.3 ± 1.3 mN/m to 51.5 ± 0.5 mN/m in the first 24 h and then increased again to values around 60 mN/m. Regarding substrate consumption, less than 10 g glucose/L were consumed in 48 h, indicating an inhibitory effect on growth and biosurfactant production of high glucose concentrations under oxygen-limiting conditions. In contrast, *B. licheniformis* EL3 produced biosurfactants when grown in MSM supplemented with 40 g glucose/L under aerobic conditions, reducing the ST to 29 mN/m after 24 h of culture and maintaining those ST values up to 168 h, whereas glucose was completely consumed in 73 h. As previously discussed, that behavior could be explained by an inhibitory effect of high glucose concentrations in cultures performed under oxygen-limited conditions, as previously reported by other authors. Willenbacher and co-workers [19] reported bacterial growth inhibition in *B. subtilis* DSM10 growing under strict anaerobic conditions in MSM when glucose concentration was higher than 7.5 g/L. In contrast, Hoffmann and co-workers [11] did not observe a negative

effect on biomass concentration when *B. subtilis* JABs24 was grown under strict anaerobic conditions in MSM for glucose concentrations up to 10 g/L, although a decrease in growth rates was observed as glucose concentration increased. Further studies demonstrated that initial glucose concentrations up to 10 g/L did not have a negative effect on nitrate respiration in *B. subtilis* JABs24 [11,28]. The differences observed among the different works can be due to the different *B. subtilis* strains used in them.

According to the lowest ST^{-1} values obtained using MSM supplemented with 20 g glucose/L (33.0 ± 1.0 mN/m (Table S4)) or 30 g glucose/L (34.0 ± 1.2 mN/m (Table S5)), it can be concluded that increasing glucose concentration in MSM from 20 to 30 g/L does not allow the production of a higher amount of biosurfactant. Neither longer culture times allow the production of more biosurfactant, which seems to stabilize after 41 h (MSM 20 g glucose/L) nor 48 h (MSM 30 g glucose/L) (Tables S4 and S5). According to the results obtained, the most favorable condition for biosurfactant production by *B. licheniformis* EL3 in a bioreactor under oxygen-limiting conditions was the use of MSM supplemented with 20 g glucose/L and saturated with oxygen at the beginning of the assay, using a pre-culture prepared in LB medium under aerobic conditions.

3.3. Purification and Characterization of Biosurfactant Produced by Bacillus licheniformis EL3 under Oxygen-Limiting Conditions

In cultures performed under the optimized conditions described above, *B. licheniformis* EL3 produced 75 ± 3 mg of purified biosurfactant/L in 43 h. Hoffmann and co-workers [11] and Willenbacher et al. [19] studied surfactin production by *B. subtilis* JABs24 and *B. subtilis* DSM10, respectively, in a bioreactor under strict anaerobic conditions, using mineral media containing 10 g glucose/L (*B. subtilis* JABs24) and 2.5 g glucose/L (*B. subtilis* DSM10). Surfactin titers achieved were 100 mg/L (*B. subtilis* JABs24, 54 h) and 87 mg/L (*B. subtilis* DSM10, 55 h), slightly higher than those herein obtained for *B. licheniformis* EL3.

Maximum biomass concentrations obtained for *B. subtilis* JABs24 (0.620 g/L) and *B. subtilis* DSM10 (0.320 g/L) in those assays were considerably higher than the value obtained for *B. licheniformis* EL3 (0.108 g/L) (Table 1). Accordingly, $Y_{X/S}$ values reported by those authors (0.051 and 0.120 g biomass/g glucose) were higher than those herein obtained for *B. licheniformis* EL3 (0.009 g/g) (Table 1) [11,19], due to a lower biomass production in the last case, together with a higher initial glucose concentration in the culture medium (20 g/L). However, biosurfactant yields per substrate ($Y_{P/S}$) obtained in those works (0.007 and 0.033 g biosurfactant/g glucose) [11,19] are in the same range as those obtained with *B. licheniformis* EL3 (0.007 g/g) (Table 1).

Table 1. Summary of the results obtained for *Bacillus licheniformis* EL3 grown in a bioreactor under oxygen-limiting conditions in comparison to the results obtained in bioreactor assays performed with *Bacillus subtilis* DSM10 and JABs24 for biosurfactant production under strict anaerobic conditions.

Parameter	*B. licheniformis* EL3	*B. subtilis* DSM10	*B. subtilis* JABs24
$[Biomass]_{max}$ (mg/L)	108	320	620
$[BS]_{max}$ (mg/L)	75 ± 3 (43 h)	87 (55 h)	100 (54 h)
$Y_{X/S}$ (g/g)	0.009	0.120	0.051
$Y_{P/S}$ (g/g)	0.007	0.033	0.007
$Y_{P/X}$ (g/g)	0.755	0.278	0.140
q_{BS} (g/(g×h))	0.018	0.005	0.004
CMC (mg/L)	27–39	-	-
Reference	This study	[19]	[11]

Regarding the amount of biosurfactant produced per gram of biomass ($Y_{P/X}$), the value obtained for *B. licheniformis* EL3 (0.755 g/g) was considerably higher when compared to those reported for *B. subtilis* in similar works (0.140 and 278 g surfactin/g biomass) [11,19] (Table 1). Furthermore, specific biosurfactant productivity ($q_{Biosurfactant}$) values obtained for *B. subtilis* (0.004 and 0.005 g biosurfactant/(g biomass × h)) were lower than those obtained for *B. licheniformis* EL3 (0.018 g/(g × h)) [11,19] (Table 1). In general, these results indicate

that *B. licheniformis* EL3 is more efficient in producing biosurfactant than biomass when grown under oxygen-limited conditions, when compared to previous studies performed with *B. subtilis* under anaerobic conditions [11,19].

The CMC values obtained for the purified biosurfactant produced by *B. licheniformis* EL3 under oxygen-limited conditions were between 27 and 39 mg/L (Table 1), which highlights its purity and efficiency, taking into account that for commercial surfactin (99% purity (SIGMA-Aldrich, Saint Louis, MO, USA)), a CMC value of 14 mg/L has been reported [8].

FTIR analysis allowed the identification of functional groups characteristic of lipopeptide biosurfactants in the purified biosurfactant produced by *B. licheniformis* EL3, according to previous works [8,20]: a characteristic absorption peak at 3300 cm^{-1}, corresponding to the N–H stretching mode; an absorption peak at 1645 cm^{-1}, corresponding to the stretching mode of CO–N bond; an absorption peak at 1531 cm^{-1}, corresponding to the deformation mode of N–H bond combined with C–N stretching; absorption peaks at 2956, 2854, and 1397 cm^{-1}, corresponding to aliphatic long hydrocarbon chains (CH_3–; –CH_2–); and a peak at 1736 cm^{-1}, corresponding to carbonyl groups (Figure 6A). Similar FTIR spectra profiles were reported for lichenysin produced by *B. licheniformis* Ali5 [20] and surfactin produced by *B. subtilis* [8]. Furthermore, an almost identical FTIR spectrum profile was obtained for commercial surfactin (Figure 6B). The similarity between both FTIR spectra (Figure 6A,B) is expected, as the only difference between surfactin and lichenysin is the first amino acid in the peptide ring (glutamic acid or glutamine, respectively) [5], confirming that the biosurfactant produced by *B. licheniformis* EL3 belongs to the surfactin family of lipopeptide biosurfactants (that includes surfactin, lichenysin, and pumilacidin).

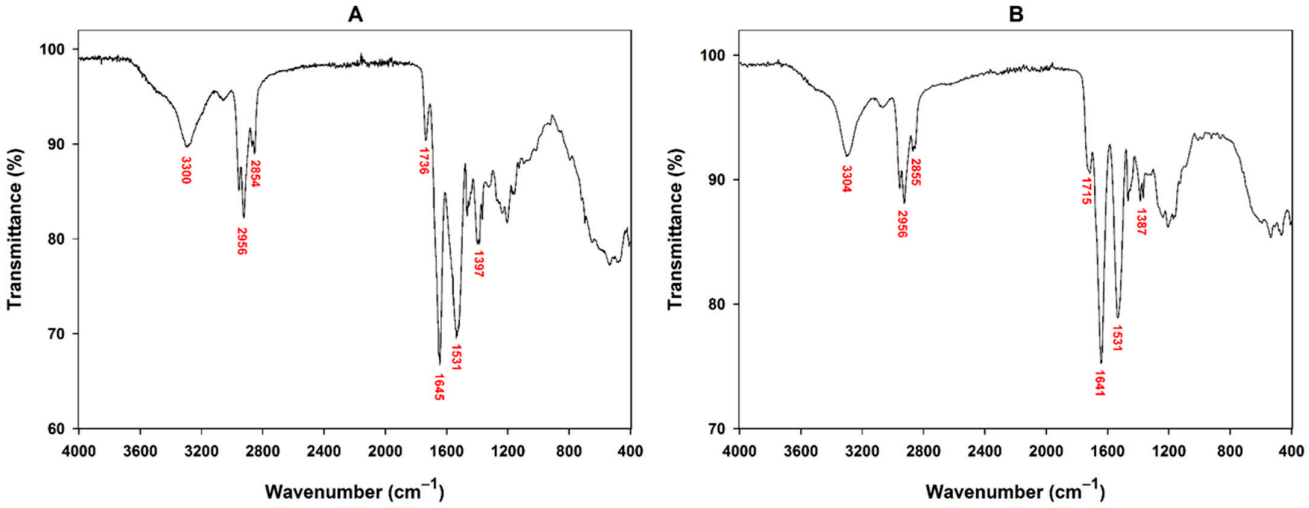

Figure 6. FTIR spectra of the purified biosurfactant produced by *Bacillus licheniformis* EL3 (**A**) and surfactin (99% purity (SIGMA-Aldrich, Saint Louis, MO, USA)) (**B**).

4. Conclusions

Conventional aerobic biosurfactant production processes result in excessive foaming due to the use of high agitation and aeration rates necessary to increase dissolved oxygen concentration to allow microbial growth and biosurfactant production. Foam accumulation in industrial bioreactors causes numerous problems, from the loss of cells and nutrients to contamination. An innovative solution is the development of foam-free processes through the growth of biosurfactant-producing microorganisms under oxygen-limited conditions. In this regard, *B. licheniformis*, which produces the lipopeptide biosurfactant lichenysin, seems to be a good option for this purpose. Biosurfactant titers herein obtained with *B. licheniformis* EL3 were similar to those achieved with *B. subtilis* strains in similar assays, despite its supposed better potential for biosurfactant production under oxygen-limited

conditions, although better figures were obtained regarding biosurfactant yield per biomass ($Y_{P/X}$) and specific biosurfactant productivity (q_{BS}).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10070340/s1. Table S1. Surface tension values of cell-free supernatants without dilution (ST) and 10 times diluted with demineralized water (ST^{-1}) obtained from cultures of *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in bioreactor, under oxygen-limited conditions, at 37 °C and 500 rpm, using an aerobic pre-culture grown in MSM. Immediately after inoculation, the culture medium in the bioreactor was saturated with N_2 (data correspond to results presented in Figure 1 in the manuscript). Results correspond to the average ± standard deviation of two assays. Table S2. Surface tension values of cell-free supernatants without dilution (ST) and 10 times diluted with demineralized water (ST^{-1}) obtained from cultures of *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in bioreactor, under oxygen-limited conditions, at 37 °C and 500 rpm, using an aerobic pre-culture grown in MSM. Before inoculation, the culture medium in the bioreactor was saturated with O_2 (data correspond to results presented in Figure 2 in the manuscript). Results correspond to the average ± standard deviation of two assays. Table S3. Surface tension values of cell-free supernatants without dilution (ST) and 10 times diluted with demineralized water (ST^{-1}) obtained from cultures of *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in bioreactor, under oxygen-limited conditions, at 37 °C and 500 rpm, using a pre-culture grown in MSM under oxygen-limited conditions. Before inoculation, the culture medium in the bioreactor was saturated with N_2 (data correspond to results presented in Figure 3 in the manuscript). Results correspond to the average ± standard deviation of two assays. Table S4. Surface tension values of cell-free supernatants without dilution (ST) and 10 times diluted with demineralized water (ST^{-1}) obtained from cultures of *Bacillus licheniformis* EL3 grown in MSM supplemented with 20 g glucose/L in bioreactor, under oxygen-limited conditions, at 37 °C and 500 rpm, using a pre-culture grown in LB medium. Before inoculation, the culture medium in the bioreactor was saturated with O_2 (data correspond to results presented in Figure 4 in the manuscript). Results correspond to the average ± standard deviation of two assays. Table S5. Surface tension values of cell-free supernatants without dilution (ST) and 10 times diluted with demineralized water (ST^{-1}) obtained from cultures of *Bacillus licheniformis* EL3 grown in MSM supplemented with 30 g glucose/L in bioreactor, under oxygen-limited conditions, at 37 °C and 500 rpm, using a pre-culture grown in LB medium. Before inoculation, the culture medium in the bioreactor was saturated with O_2 (data correspond to results presented in Figure 5 in the manuscript). Results correspond to the average ± standard deviation of two assays.

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