

Ultrafiltration based purification strategies for surfactin produced by bacillus subtilis lb5a using cassava wastewater as substrate

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Accepted Version

De Andrade, C. J., Barros, F. F. C., De Andrade, L. M., Roco, S. A., Sforca, M. L., Pastore, G. M. and Jauregi, P. (2016) Ultrafiltration based purification strategies for surfactin produced by bacillus subtilis lb5a using cassava wastewater as substrate. *Journal of Chemical Technology and Biotechnology*, 91 (12). pp. 3018-3027. ISSN 1097-4660 doi: <https://doi.org/10.1002/jctb.4928> Available at <https://centaur.reading.ac.uk/67256/>

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To link to this article DOI: <http://dx.doi.org/10.1002/jctb.4928>

Publisher: Wiley

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1 **ULTRAFILTRATION BASED PURIFICATION STRATEGIES FOR**
2 **SURFACTIN PRODUCED BY *Bacillus subtilis* LB5A USING CASSAVA**
3 **WASTEWATER AS SUBSTRATE**

4
5 **Short title:** Ultrafiltration of surfactin produced using cassava wastewater

6
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15
16 **Abstract**

17 BACKGROUND: *Bacillus subtilis* synthesizes surfactin, a powerful surface-active agent. It
18 has interesting potential applications. However, due to its high cost of production, commercial
19 use is impracticable. The downstream processing represents $\approx 60\%$ of production costs and the
20 culture medium $\approx 30\%$. Many reports focused, separately, on production of surfactin using by-
21 products (reduced cost) or the purification using synthetic medium. Therefore, the aim of this
22 work was to evaluate, for the first time, the impact of using a by-product as fermentation
23 medium on the downstream processing based on membrane filtration.

24 RESULTS: Membranes of PES-100-kDa efficiently retained surfactin micelles - the first step
25 of ultrafiltration, whereas, the second step required membranes of 50-kDa to separate

1 surfactin monomers from proteins. Ultrafiltration of crude biosurfactant was associated with
2 fouling and/or concentration polarization resulting in lower purity than when synthetic
3 medium was used. Further improvement in purity was achieved by partial removal of proteins
4 prior to ultrafiltration by precipitation and extraction. The RMN and MALDI-TOFMS
5 analyses identified 11 potential surfactin homologous composed by two amino acid
6 sequences.

7 **CONCLUSION:** Production of surfactin using cassava wastewater as a low-cost culture
8 medium and its purification by the 2-step ultrafiltration process is feasible, nevertheless, the
9 higher protein content of this medium as compared to the synthetic one leads to a lower purity
10 product; further increase in purity can be achieved by applying additional purification steps
11 prior to ultrafiltration with the subsequent increased in process cost.

12

13 Key terms: fermentation, purification, residues, ultrafiltration, wastewater.

14

15 **INTRODUCTION**

16 A wide variety of microorganisms produce biosurfactants including *B. subtilis*
17 that synthesizes lipopeptides such as surfactin, iturin, fengycin, etc. These compounds have
18 high surface activity and resistance to extreme conditions.¹⁻³ They have raised a lot of interest
19 due to their remarkable properties such as: high emulsification index in a wide range of
20 hydrophobic substrates, and maintenance of surface activity under extreme conditions of
21 temperatures, pH and ionic strength.²⁻³

22 Biosurfactants can be produced using industrial wastes and by-products as culture
23 medium. In the production of surfactin from *B. subtilis*, the use of cassava wastewater is well-
24 known; this waste seems to be an ideal match, since it is available in large amounts
25 throughout the year and in all regions of Brazil and any country producer of cassava.¹

1 However there is a lack of knowledge about technical feasibility of the downstream process,
2 which uses industrial wastes as culture medium, that is, the production of biosurfactant using
3 wastes is widely known, but the purification of the products obtained from those production
4 are rarely reported. Downstream, is also the most important economical factor, since it
5 represents about $\approx 60\%$ of the total production cost. ⁴⁻⁵

6 Conventional methods for purification of surfactin produced by *B. subtilis* include
7 acid precipitation followed by extraction with organic solvents and/or adsorption. ⁴⁻⁵

8 In the past ten years the ultrafiltration (UF) based downstream processing and,
9 specifically, the two-step UF ^{4,6-11} has shown to be the most promising both in terms of the
10 yields and purity and its scalability and it is currently being applied in the manufacturing of
11 lipopeptides. In the first step of UF, surfactin micelles are recovered as retentate. Then,
12 methanol or ethanol is added to the retentate in order to disrupt the micelles, and a second
13 step of UF is carried out, however in this case, the surfactin is in solution as monomers and it
14 is recovered in the permeate, whereas proteins remain in the retentate as they form aggregates
15 (>100 nm) in the presence of ethanol/methanol (refer to Isa et al 2007). Table 1 compiles the
16 parameters and yields of surfactin UF.

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Table 1

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In most cases, the fermentation process is carried out using a synthetic culture medium. However, there have been no reports about the UF of surfactin produced using cassava wastewater as a culture medium. Cassava wastewater is a remarkable culture medium for many biotechnological processes, since cassava wastewater is composed by wide range of both macro and micronutrients (dextrose, fructose, saccharose, magnesium $[Mg^{+2}]$, calcium $[Ca^{+2}]$, manganese $[Mn^{+2}]$, iron $[Fe^{+2}]$, zinc $[Zn^{2+}]$ and nitrogen compounds. ¹

1 13), was collected from a flour industry and transported to the laboratory at room temperature.
2 Next, it was boiled (3 min at 100 °C), centrifuged (10^4 g during 10 min at 5 °C (Beckman
3 Coulter, Allegra X-22r), and the supernatant was stored (-18 °C).

4

5 Microorganism and *inoculum*

6 *B. subtilis* LB5a was used as a surfactin producer. The *inoculum* was standardized
7 according to Barros et al. (2008).¹

8

9 Fermentation parameters and sampling

10 Cassava wastewater (3.0 liters working volume) was placed in a bioreactor
11 (Bioflo® & Celligen® 310 - New Brunswick Scientific). The culture medium was sterilized
12 at 121 °C for 20 min. Fermentation parameters used included: 100 rpm and aeration rate of
13 0.4 vvm (1 vessel volume per minute) maintained in the first 24 h, and then 150 rpm and 0.8
14 vvm from 24 to 72 h. The sensor (Mettler Toledo - INPRO 6830/12/320) of DO was
15 programmed to measure every 30 seconds during the entire fermentation processes; the DO
16 probe was calibrated according to the manual of BioFlo 310. Samples of the culture medium
17 and foam were collected on a 12 h basis to analyze viable cell count, content of glucose,
18 volume of foam and surface tension (ST). In order to obtain enough surfactin for the
19 purification experiments, seven fermentations were carried out.

20

21 Volumetric oxygen transfer coefficient

22 Volumetric oxygen transfer coefficient (*K_{la}*) was measured by dynamic methods.
23 Measurements of DO were carried out using a probe (INPRO 6830/12/320). The medium (3
24 liters of cassava wastewater) was bubbled with nitrogen to remove oxygen. Then, aeration
25 was started ($2 \text{ L}\cdot\text{min}^{-1}$) and DO values were used to calculate the *K_{la}*.¹²

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Biosurfactant recovery

The foam was collected from the top of the bioreactor during its production, as described by Barros et al. (2008).¹ The foam was collapsed and its volume was measured, and then centrifuged at 10^4 g for 20 min. Afterwards, the ST was measured in the supernatant phase using a tensiometer (Krüss GmbH K-12) by plate method.¹

Pre-purification (ultrafiltration) – crude and semi-purified biosurfactant

The collapsed foam was acidified with HCl solution (2 and 0.1 N) to pH = 2, and solution stand for 24 h at room temperature; then it was centrifuged at 10^4 g for 20 min. The precipitate was collected, neutralized with NaOH solution (2 and 0.1 N) and dried at 50 °C; the powder was named crude biosurfactant.

The crude biosurfactant (obtained from the seventh fermentation) was dissolved in chloroform: methanol 65:15 (v.v⁻¹) and filtered 0.22 µm. The filtrate was recovered and dried at room temperature.¹ The resulting powder was classified as semi-purified biosurfactant. Yields were calculated by dividing total mass obtained of crude or semi-purified biosurfactant by the volume of culture medium (3 L). Yields were also calculated dividing total mass obtained of crude or semi-purified biosurfactant by the volume of collapsed foam (foam overflow).

Analytical procedures – Production stage

Measurement of surface activity

Critical micelle dilutions (CMDs) are the ST values of the sample diluted at 10-times (CMD-1) and 100-times (CMD-2). The ST measurements (CMDs) were carried out on

1 the centrifuged culture medium and foam samples (12 h basis) by using the plate method at
2 room temperature in a Krüss GmbH K-12 tensiometer (K-12 model, Krüss GmbH).¹

3 4 **Purification of surfactin by two-step ultrafiltration process**

5 Process overview

6 First, the purity of surfactin in crude and semi-purified biosurfactant (see surfactin
7 concentration analysis) was measured. Then, an aqueous solution (Tris-buffer pH 8.5 -
8 optimum solubilization of surfactin)^{4,5,9} was made at 100 mg.L⁻¹ of pure surfactin, filtered
9 (0.45 µm) and used as a feed in the first UF step (UF-1).⁶ UF-1 retained the surfactin micelles
10 and proteins (retentate), while salt and small molecules were recovered as permeate. From the
11 retentate of UF-1, a solvent solution was prepared (ethanol 75%), followed by the second UF
12 step (UF-2). Since ethanol solution disrupts surfactin micelles to monomers, this process
13 aimed to retain proteins, so the surfactin can be recovered as permeate (Figure 1). After these
14 two UF steps, high recovery and purity are expected as shows the Table 1. Basically, three
15 analyses were carried out in all samples feed, permeate and retentate UF-1, and permeate and
16 retentate UF-2 to evaluate the UF processes including: nanoparticle size (Dynamic Light
17 Scattering - DLS), concentration of surfactin (High Performance Liquid Chromatography -
18 HPLC) and protein (Bicinchininic Acid Method - BCA).

19 The two-step ultrafiltration process was applied following three different
20 strategies i, ii and iii (Figure 1). In all strategies polyethersulfone (PES) membranes were
21 used. The first strategy (i) used the crude biosurfactant solution (feed) and membranes of 100
22 kDa in both UF-1 and UF-2, whereas the second strategy (ii) used a crude biosurfactant
23 solution (feed), 100 kDa in the UF-1 and 50 kDa in the UF-2. The third strategy (iii) used the
24 semi-purified biosurfactant solution (feed), 100 kDa in the UF-1 and 50 kDa in the UF-2.
25 Therefore, the comparasion between strategies i and ii allowed to evaluate the effect of

1 membrane size in the UF-2, whereas the comparasion between the strategies ii and iii the effet
2 of initial solution of biosurfactant, crude or semi-purified.

3
4 Centrifugal device of ultrafiltration in two steps

5 The procedures were completed using Vivaspin 20 with PES – 50 and 100 kDa,
6 containing membrane of 6 cm² of active area. For UF-1, biosurfactant solution (crude or semi-
7 purified) at 100 mg.L⁻¹ of pure surfactin (see surfactin concentration analysis) was used as
8 feed, in which 20-15 mL was added to the filter unit (100 kDa), centrifuged at 2205 g (10 or
9 20 min) and 20 °C. Next, the retentate (from UF-1 ≈0.7 mL) was dissolved in 20-15 mL of
10 ethanol (75%) and centrifuged once again (10 or 20 min). The retentate (UF-2) was dissolved
11 in 15-20 mL of tris-buffer (8.5). Finally, all solutions (retentate and permeate of UF-1, -2)
12 were analyzed for surfactin concentration-HPLC, DLS and BCA.

13 The rejection of surfactin or protein by a membrane was defined as the rejection
14 coefficient (R) shown below: ⁶

$$15 \quad \text{Equation(1)} R = (C_f - C_p / C_f)$$

16
17
18 Where C_F and C_P are the concentration of surfactin (C_s) or protein (C_p) in the feed
19 and permeate, respectively.

20 It was also calculated the purity in terms of protein as mass fraction of surfactin in
21 relation to sum of mass of surfactin and protein (P_p) in the UF-1 and UF-2 as shown below: ⁶

$$22 \quad \text{Equation(2)} Pp = ((C_s / C_s + C_p)) * 100$$

23
24 The equation 2 was applied to calculate the purity in the feed, retentate and
25 permeate.

1 Finally, it was calculated the total recovery of surfactin (TRS) by the equation 3,
2 in which M_s is the mass of surfactin.

$$3 \text{ Equation(3) } TRS = (M_{si} / M_{sii}) * 100$$

4
5
6 For the UF-1 (TRS_i), M_{si} is the mass of surfactin in the retentate whereas M_{sii} is
7 the mass of surfactin in the feed. For the UF-2 (TRS_{ii}), M_{si} is the mass of surfactin in the
8 permeate whereas M_{sii} is the mass of surfactin in the feed. It was also calculated the TRS_t in
9 the UF-1 and UF-2, which M_{si} is the mass of surfactin in the initial feed (UF-1) and M_{sii} is the
10 mass of surfactin in the permeate (UF-2). The M_s was obtained multiplying C_s by the volume of
11 solution.

12
13 Analytical procedures - purification

14 Protein concentration

15 The total amount of protein present at each stage of the purification procedure was
16 determined by the BCA. A calibration curve was produced, using bovine serum albumin as
17 the protein standard solution. ⁹

18
19 Surfactin concentration analysis

20 Surfactin concentration was determined by reverse phase HPLC from a filtered
21 (0.45 μm) solution (tris buffer pH 8.5 – 10 mM) of crude biosurfactant ($\approx 1200 \text{ mg.L}^{-1}$). The
22 system used was a Gilson 306 (Rockford, IL, USA) with a C-18 column of dimensions 250
23 mm \times 4.6 mm, and a particle size of 5 μm . The flow rate of the mobile phase was 1.1 mL.min⁻¹
24 with the initial gradient starting from 50 to 80% acetonitrile in 0.1% trifluoroacetic acid in
25 the first 15 min. The gradient remained at 80% for 20 min before increasing to 100% for 5

1 min as a washing step, returning to 50% once again. A 50 μ L sample was injected into each
2 run, which lasted 60 min, and eluent absorbance monitored at 214 nm. The system was
3 calibrated using pure surfactin ($\geq 98\%$) obtained from Sigma-Aldrich. The area of the peaks
4 (samples) eluting at 23.18 and 27 min were identified as having the same retention times as
5 those peaks eluting from the standard, which were added to give the total surfactin peak area.⁹

6

7 Particle size measurements - micelles

8 The nanoparticle sizes were evaluated by DLS, using a Zetasizer Nano ZS system
9 (Malvern, UK).⁹ All samples (feed; permeate UF-1; UF-2 and retentate UF-1; UF-2) were
10 analyzed at least two times, and information about the size distribution by volume was used as
11 a parameter.

12

13 **Chemical structure identification of produced surfactin (strategy iii)**

14 Three different approaches, Infrared Spectroscopy (IR), Matrix Assisted Laser
15 Ionization Time-of-flight (MALDI-TOFMS) and Nuclear Magnetic Resonance (NMR), were
16 used in order to investigate the chemical structure identification of produced surfactin
17 (strategy iii). The sample was prepared for infrared analysis (FTLA2000) by mixing
18 approximately 1 mg of produced surfactin (strategy iii) with 100 mg of KBr and pressing the
19 mixture into the form of a pellet at 134 MPa for 2–3 min to obtain transparent pellets. The IR
20 spectrum of the pellet was collected from 400 to 4000 wavelengh (cm^{-1}).¹³ MALDI-TOFMS
21 spectra were performed using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker)
22 operating in the reflection mode at an accelerating voltage of 22.5 kV. Mass spectra were
23 acquired in m/z range of 700-3500 with ions generated from SmartbeamTM laser irradiation
24 using a frequency of 2000 Hz, a lens 7 kV and the delay time was 110ns. Matrix-suppression
25 was set to 500 Da. External calibration was performed by using the peptide calibration

1 standard (Bruker Daltonics). ¹⁴NMR experiments were performed at 298 K using an Agilent
2 DD2 500 MHz spectrometer equipped with a 5 mm triple resonance probe. After
3 lyophilization, 8 mg of the produced surfactin (strategy iii) was dissolved in 600 μL of
4 deuterated dimethyl sulfoxide (²H₆-DMSO CIL-Cambridge Isotope Laboratories Inc.).
5 Resonance peaks were assigned using standard methods including correlation spectroscopy
6 (COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement
7 spectroscopy (NOESY). The TOCSY spectra were acquired using a mixing time of 100 ms.
8 NOESY spectra were recorded with a mixing time of 250 and 350 ms. All two-dimensional
9 experiments were acquired using a spectral width of 6983 Hz, a matrix size of 4096 X 512
10 points and relaxation delay of 1.5 s.

11 Data were processed using the NMRPipe/NMRVIEW software.¹⁵⁻¹⁶ Prior to
12 Fourier transform, the time domain data were zero-filled in both dimensions to yield a 4K X
13 2K data matrix. When necessary, a fifth-order polynomial baseline correction was applied
14 after transformation and phasing.

15

16 **RESULTS AND DISCUSSION**

17 **Surfactin production - fermentation**

18 Fermentation process and recovery of surfactin

19 The production of surfactin from *B. subtilis* LB5a using cassava wastewater as
20 culture medium was already reported at the following scales, Erlenmeyerflask (250 mL) and
21 pilot bioreactor (80 L) ¹. Even with the subtle changes that were implemented, such as the
22 increase of aeration after 24 h rather than 12 h, working volume, bioreactor, etc., similar
23 process parameters were observed with those previously reported by Barros et al. (2008). ¹ In
24 addition, the DO (culture medium) and viable cells in the foam were evaluated for the first
25 time, which enable a more accurate description of the fermentation process.

1 Figure 2 represents the viable cell counts in the culture medium and foam during
2 the fermentation process.

3
4 As expected a similar profile between viable cell count in culture medium and
5 foam was found since higher cell concentration in the medium favors carrying cells in foam.
6 The analysis of cells in the foam enabled to establish that a significant number of cells were
7 removed during the process, for instance, at 36 h $\approx 4 \times 10^4$ viable cells per mL of foam; thus,
8 from 330 mL (volume of foam produced at 36 h) $\approx 10^6$ cells were removed from the
9 bioreactor. This data supports a more accurate understanding of microbial growth.
10 Nevertheless, it only relates to viable cells, and the high surfactin and low nutrient
11 concentration (foam) will most likely lyse some cells; therefore, we speculate that the results
12 were underestimated. Finally, high viable cell count reached $\approx 10^8$ CFU when the stationary
13 phase was between 24 and 48 h.

14 The ST of culture medium showed a decrease in the first 24 h, in other words, the
15 biosurfactant content increased. As already expected, the recovery of surfactin increased due
16 to the change in the aeration rate from 0.4 to 0.8 vvm (at 24 h). As a result, the ST values in
17 the beginning and at the end of fermentation were similar, which indicated a high recovery of
18 surfactin.

19 The surface activity of the foam was remarkable, from basically 12 h until the end
20 of fermentation, the ST and its CMD-1 remained around $27 \text{ mN}\cdot\text{m}^{-1}$. Taking into account that
21 the critical micellar concentration (CMC) of surfactin is $\approx 10 \text{ mg}\cdot\text{L}^{-1}$ and CMD-1 values
22 remained at $27 \text{ mN}\cdot\text{m}^{-1}$, it is easy to conclude that the surfactin concentration was at least 100
23 $\text{mg}\cdot\text{L}^{-1}$. In addition, CMD-2 data showed ST around the CMC $\approx 30 \text{ mN}\cdot\text{m}^{-1}$. A more accurate
24 determination of the concentration was obtained by HPLC analysis which enabled the

determination of the exact purity of the biosurfactant in the foam (crude biosurfactant) (see purification of surfactin by two-step UF).

We believe that the recovery by foam overflow is a good strategy, when it is used in a particularly complex culture medium such as cassava wastewater. This technique is advantageous since it primarily separates surfactin and proteins from the culture medium into the foam. In addition, the high concentration of surfactin in the culture medium may act as an inhibitor on the microorganism itself leading to reduced growth and yields.

However the recovery of surfactin by foam overflow requires relatively high aeration rate in order to produce the foam. On the other hand, depleted oxygen conditions⁹ and micro-aeration conditions, $\approx 30\%$ of DO lead to better yields of surfactin production.¹⁷ During the fermentation using cassava wastewater, the DO remained at 0% (Figure 3) and it was found the Kla 102.02 h^{-1} . In this context, Fahim et al. (2012)¹² described that the optimum Kla for the production of surfactin was 216 h^{-1} ($0.04\text{-}0.08 \text{ s}^{-1}$). Hence, the fermentations were operated at optimum aeration conditions, as demonstrated also by the high surfactin production (according to ST measurements and high volume of foam collected $\approx 1000 \text{ mL} \pm 84$). However, productivity could potentially be optimized further by increasing DO and Kla to the optimum values reported by Fahim et al. (2012).¹²

Figure 3 shows the profile of DO and dextrose content during the fermentation.

Figure 3

The DO profile indicates that microorganisms hardly sense the change of culture medium (due to inoculation) from nutrient broth to cassava wastewater, and based on DO, the lag phase took place within the first two hours. Then, it abruptly decreased to 0% and remained so for most of the time (from ≈ 3 to 68 h). This behavior is extremely good because

1 the microorganism growth happen at aerobic and anaerobic conditions (0%) and the
2 fermentation happened mainly at oxygen depleted conditions which favors the production of
3 surfactin. ^{9,12,18,19} Also, as mentioned above, the aeration rate was enough to generate foam
4 and in this way facilitate the recovery of surfactin in the foam. Finally, at 68 h, the DO
5 increased, indicating the death phase.

6 It was found that the highest volume of foam was reached at 36 h, which is
7 aligned with the viable cell profile. It shows that surfactin production was growth-associated.
8 On the other hand the pH increased from ≈ 5.5 to 7.5, this sort of fermentation (alkaline) is
9 characteristic of *B. subtilis*. ¹

10 All seven bioprocesses showed a low relative standard deviation and very
11 similarity with previously studies of *Bacillus subtilis* LB5a using cassava wastewater as
12 substrate. ¹ In each fermentation and its collected foam, 2.80 (+/- 0.6 g) of crude biosurfactant
13 was obtained, in other words, 0.93 g per liter of culture medium.

14

15 Purification of surfactin by a two-step ultrafiltration process

16 The HPLC analysis showed that crude biosurfactant had 36.14 (+/- 9.05% w.w⁻¹)
17 pure surfactin; thereby, ≈ 1010 mg.L⁻¹ of surfactin was in the foam, and a total of 1.01 g of
18 pure surfactin was produced from each batch (3 liters of culture medium) or 336.66 mg.L⁻¹.
19 This yield was lower than that reported by Isa et al. (2007) ⁹, which achieved 583 mg of
20 surfactin per liter of culture medium and recovered surfactin directed from the culture
21 medium. It is worth noting that the optimization of the production of surfactin was not the
22 focus of this study; also it was considered that 100% of surfactin was recovered in the foam
23 however a small fraction of surfactin possibly remained in the culture medium and bioreactor
24 walls.

25

1 Strategy i

2 A feed solution (312 mg.L^{-1}) was elaborated based on the results of purity of
3 surfactin (36.14%). This solution was analyzed by HPLC, and surfactin concentration was
4 determined as 105.85 mg.L^{-1} (Table 2). The DLS analysis indicated that surfactin micelles
5 had a diameter (d) of 71.4 nm (Table 3). As in the feed, the permeate of UF-1 showed
6 micelles of $d=129 \text{ nm}$. This together with the determination of surfactin concentration (Table
7 2, 19.21 mg.L^{-1}) shows that part of the surfactin was not retained by the membrane but
8 majority of the surfactin was rejected ($R_s=0.82$) whereas for proteins $R_p=0.68$. Thus, the UF-
9 1 separated most of the surfactin in micellar form from small molecules (e.g., peptides, acids,
10 etc.) together with a large proportion of proteins.

11 Regarding the UF-2, the retentate from UF-1 (solubilized in ethanol 75%) was
12 utilized as a feed solution; a solution mainly comprised by surfactin (monomers) and proteins.

13 Nanoparticles with $d=466 \text{ nm}$ were observed in the retentate of UF-1. Since
14 ethanol 75% efficiently disrupted surfactin micelles ⁶ the presence of these nanoparticles is
15 explained as the aggregation of protein molecules in agreement with previous observations. ⁶

16 The permeate of the UF-2 had nanoparticles of $d=0.739 \text{ nm}$ which demonstrates
17 the disruption of the micelles which led to the recovery of the surfactin in the permeate at a
18 concentration of 65.66 mg.L^{-1} . However, the protein followed the same trend ($R_p=5\%$) and
19 was also recovered in the permeate which resulted in low purity $\approx 44 \text{ g}$ of surfactin/ 100 g
20 surfactin and proteins. The total recovery of surfactin was 62% .

21 Isa et al. (2007) ⁹ demonstrated that surfactin micelles can be effectively
22 recovered using ultrafiltration centrifugal devices with RC or PES membranes of MWCO 10
23 kDa, TRS_i (90%) with a regenerated cellulose membrane of 30 kDa and TRS_{ii} (91%) using a
24 regenerated cellulose membrane of 10 kDa. These values are in agreement with those
25 obtained here (Table 3). and those reported by Jauregi et al. (2013) ⁶ using PES 100 kDa.

1 The size of micelles, relatively, followed the same trend as reported by Jauregi et
2 al. (2013) ⁶ in which concentrations between 50-100 mg.L⁻¹ of surfactin resulted in the largest
3 micelles with d between 100-200 nm. Also, according to Knoblich et al. (1995) ²⁰ surfactin
4 micelles adopt cylindrical form due to parameters such as pH and presence of salts (CaCl₂ and
5 NaCl). As a result, proteins, salts, etc., from the cassava wastewater and/or synthesized from
6 *B. subtilis* may have some influence on the surfactin micelles shape.

7 In conclusion, the size and the rejection of surfactin by the membrane of 100 KDa
8 produced using cassava wastewater as culture medium in the UF-1 were in agreement with
9 previous findings that were described in the literature. ^{10,6}.

10 Consequently, UF-1 was an adequate process. However in UF-2, due to high
11 MWCO (PES-100 kDa) proteins were also permeated which led to no purification. Therefore,
12 strategy (ii) was applied where all parameters of UF-1 were maintained but the MWCO of
13 membrane in the UF-2 was reduced to 50 kDa.

14 15 Strategy ii

16 As shown in Table 2, the feed solution for strategy (ii) (180.17 mg.L⁻¹ of crude
17 biosurfactant) had nanoparticles (micelles) of similar size to those in the feed solution of
18 strategy i (d=72.3 nm and 81.13 mg.L⁻¹ of surfactin). Samples of permeate and retentate (UF-
19 1) and permeate (UF-2) showed similar sizes R_s and R_p to those described in strategy (i)
20 (Table 3). This data indicated good reproducibility of the UF-1 process. However, in the
21 retentate of UF-2, contrary to what was obtained in strategy (i), a high R_p of 49% and a low R_s
22 of 1% was observed which proved to be a better strategy as it led to a better separation of
23 surfactin (in the permeate) from the protein (in the retentate). Thus a higher purity (\approx 59 g of
24 surfactin/ 100 g surfactin and proteins) and higher TRS_t 86.23% were obtained in strategy (ii)

1 However in the UF-2, the flow rate with this membrane was lower than with the
2 higher MWCO membrane as expected and the ultrafiltration took 20 min (instead of 10 min).

3 In an attempt to improve the separation of proteins from surfactin further a third
4 strategy (strategy iii) was developed where prior to the UF process the crude biosurfactant
5 was pre-purified by acid precipitation and extraction (see 'pre-purification' in methods). It
6 was speculated that the reduction of proteins in the feed would facilitate the separation of
7 proteins from the surfactin.

8 9 Strategy iii

10 The feed solution (188.17 mg.L⁻¹ of semi-purified biosurfactant) had 94.24 mg.L⁻¹
11 of surfactin at 50.08% purity (mass of surfactin in semi-purified biosurfactant/mass of semi-
12 purified biosurfactant). Thus, the extraction step increased the purity of surfactin from 36.14%
13 (crude biosurfactant) to 50.08% (semi-purified biosurfactant).

14 Concerning the UF-1, a similar surfactin rejection, $R_s = 0.87$, as strategies (i) and
15 (ii) (0.82 and 0.91 respectively) however the rejection of proteins was lower, $R_p = 0.39$.

16 In UF-2 low surfactin rejection was obtained, $R_s = 0.02$, as in strategies (i) and
17 (ii), indicating that ethanol 75% efficiently disrupted surfactin micelles (crude and semi-
18 purified biosurfactant) consequently surfactin was successfully recovered in the permeate.
19 Similar rejection of protein was obtained here, $R_p = 0.5$ as in strategy (ii), $R_p = 0.49$ (Table
20 3). However, in strategy (iii) this process took only 10 min, indicating that fouling and/or
21 concentration polarization was reduced due to a reduction in protein content; the feed in UF-2
22 (which was the retentate of UF1) for strategy (ii) contained much higher concentration of
23 protein (93.65 mg/L) than (iii) (36,31 mg/L); the first step of ultrafiltration determined the
24 effectiveness of the protein separation in the final second step as it is at this stage where
25 protein deposition (fouling) hinders the permeation of protein which results in more protein

1 being retained in strategy (ii) than in (iii) ($R_p = 0.75$ vs $R_p = 0.39$). This effect by proteins is
 2 well documented and it has been also reported for surfactin by Chen et al. (2008) ⁸ who
 3 described that the flux declined during cross-flow UF with PES 100 membranes and it was
 4 predominantly caused by the concentration polarization, as well as weak adsorption of small
 5 amino acids and the formation of a gel layer on the membrane surface. In summary the
 6 reduction of the protein in the feed led to an improved separation of the protein from surfactin
 7 leading to a final surfactin product with increased purity (≈ 80 g of surfactin/ 100 g surfactin
 8 and proteins).

10 **Table 2**

11 Comparison and evaluation of strategies i, ii and iii

12 Similar rejection (separation) of surfactin was obtained following the three strategies
 13 however the main difference was in the separation of protein in the second step, ie: in the
 14 purity. The worst separation of proteins from surfactin was obtained by strategy (i) as both
 15 surfactin and protein were rejected similarly in the UF-2 leading to low purity (44% purity
 16 and little improvement from that in the feed, 35%). The best results of purification were
 17 obtained with the strategy (iii) P_{pii} 80% (from 53% in the feed). The strategy (ii) showed also
 18 relative good results P_{pi} 59% (from 41% in the feed). Jauregi et al. (2013) ⁶ described the
 19 ultrafiltration of surfactin using a PES 100 kDa in both steps after the production using
 20 synthetic culture medium, in which the P_{pi} was higher than 92% and P_{pii} 94% and Isa et al.
 21 (2008) ¹⁰ obtained P_{pi} 88% and P_{pii} 96% using a PES 10 kDa. Better results were obtained
 22 with the synthetic culture medium than with the cassava water which may be due to lower
 23 protein content in the culture (feed) of the former (75 mg/L) which facilitates the purification.
 24 Regarding the yield of the entire process (UF-1 and UF-2), high TRS_t was observed for the
 25 three strategies, i (62%), ii (86%) and iii (78).

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Table 3

The proteins from cassava wastewater and *B. subtilis* LB5a are capable to form foam or be incorporated into the biosurfactant foam, and consequently will be recovered in the foam overflow (see item 2.2.5. - biosurfactant recovery). The production of surfactin using cassava wastewater (or any other waste) followed by the UF, perhaps is a feasible process only when associated with recovery of surfactin by the foam overflow (as a pre-purification process, previous to UF), that is, in general waste waters will have so many impurities that will make it very hard to apply UF directly, membrane fouling being one of the main problems. However foam overflow will facilitate the UF by separating first in the foam overflow the foam-forming compounds, such as surfactin and some proteins.

It is worth noting that only with the strategy (iii), where acid precipitation followed by solvent extraction (semi-purified biosurfactant) were applied prior to UF, both, high surfactin recovery (TRS_t 78.25%), and effective separation from proteins were achieved and at high flux (Table 2 and Table 3). The strategy (iii) is a remarkable process since it removed majority of the proteins (concentration of proteins in the feed at 83 mg/L reduced to 18 mg/L in the permeate) and 78% of surfactin was recovered. However, the strategy (iii) added an extra purification step (solvent extraction), which would increase the cost of production.

Thus, cassava wastewater is a low-cost culture medium comprised of carbohydrates, minerals and proteins. On the other hand, considering the two-step UF of surfactin, the proteins from cassava wastewater make the purification harder, requiring solvent extraction (crude biosurfactant → semi-purified biosurfactant). The removal of proteins (e.g. precipitation) in the cassava wastewater - as previous treatment (before

1 fermentation) – may be considered a feasible option to improve the process without having to
2 add so many pre-purification steps. However, the protein is a valuable nitrogen source which
3 has a significant effect on the production of surfactin from *B. subtilis* (preferably organic
4 nitrogen); the lower the nitrogen source - the lower the surfactin production. ²¹

5 Results above bring about some interesting issues concerning production of
6 surfactin using cassava wastewater and other biotechnological processes, which use industrial
7 waste as culture medium. Since, on one hand the use of industrial waste as culture medium
8 could reduce the cost of production, but on the other hand this makes the separation and
9 purification of the products more complicated as a larger number of steps will need to be
10 applied. Thus, this will need to be taken into consideration in the costing of the process.

11

12 **Chemical structure identification of produced surfactin (strategy iii)**

13 *Bacillus* produces lipopeptides, which are classified in three families: surfactin,
14 iturin and fengycin. Each family has a specific number of aminoacids, but with different
15 residues at specific position. It also has different length and isomery of β -hydroxyl fatty acid,
16 that is, lipopeptides have a remarkable heterogeneity of molecular weight. The analysis of
17 MALDI-TOFMS data showed the presence of compounds within/near the range of surfactin
18 homologous (1045-1080 m/z): (i) 1043.53; (ii) 1049.57; (iv) 1065.57; (v) 1066.58; (vi)
19 1068.58; (vii) 1079.60; (viii) 1082.57; (ix) 1093.55; (x) 1096.62 and (xi) 1109.60 (m/z).
20 These molecules were clearly separated in three groups (\approx 1066, 1079 and 1093 m/z). These
21 groups probably are related to length of β -fatty acids. ¹⁴ Thus, potentially, at least 11 surfactin
22 homologous were produced by *B.subtilis* LB5a using cassava wastewater as culture medium.

23 The IR analysis of produced surfactin (strategy iii) was similar to reported by
24 Faria et al. (2011) ¹³, that is, strongly absorbing band at 1639 cm^{-1} , which correspond to
25 peptide.

1 The NMR analysis identified three sequences of amino acids. One of them was
2 not considered due to the very low signal intensity. Thus, 14 strong NH-signals correlations
3 were detected between 7.207 and 9.681 ppm, in which they correspond to the two sequences
4 of amino acids, defined in this study as S and S' - Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7
5 and Glu1'-Leu2'-Leu3'-Val4'-Asp5'-Leu6'-Val7' (Figure 4, Figure 5 and Table 4). All
6 protons from leucine residues (4 in S and 3 in S') were identified by βCH_2 ($\omega I \approx 1.66$ to 1.33
7 ppm), γCH ($\omega I \approx 1.47$ to 1.33 ppm) and δCH_3 ($\omega I \approx 0.8$ ppm). Aspartic acids (S and S') were
8 identified by two βCH_2 crosspeaks (S - 2.62 and 2.17 ppm; S' - 2.66 and 2.11). Glutamic acid
9 (S and S') was identified by a single pattern with two βCH_2 signals ($\omega I \approx 1.95$ to 1.75 ppm)
10 and two for γCH_2 ($\omega I \approx 2.04$ to 1.98 ppm). All valines residues showed common pattern with
11 a single βCH ($\omega I \approx 2.0$ ppm) and γCH_3 ($\omega I \approx 0.8$ ppm) which sometimes were superposed to
12 the δCH_3 of the leucines. The identification of proton resonances of C3H C2H C2H' C4H
13 $(\text{CH}_2)_n \text{CH}_3$, were found to be similar in S and S'; and indicated (overlapping signals) that
14 length of β -fatty acid (from 13 to 15 – expected), which is bonded to the amino acids. It also
15 confirmed the presence of glutamic acid.

16
17 **Table 4**

18
19 **Figure 4**

20
21 **Figure 5**

22
23 It was already reported that the 3rd and 6th amino acids show D stereo
24 configuration. ²²⁻²³ On natural abundance basis, L stereo configuration is significantly higher

1 than D stereo one. The D stereo configuration of surfactin is one of key surfactin properties
2 such as antimicrobial.

3 Comparing the sequences of amino acids, previously reported, there is a trend that
4 only the 2nd, 4th and 7th amino acids are changeable, while the 1st (Glu), 3rd (Leu), 5th (Asp)
5 and 6th (Leu) are unchangeable. For instance, Grangemard et al. (1997) ²² Ile2, Ile4, Ile7 and
6 Leu2, Val4, Leu7, Korenblum et al (2012) ²⁴ Leu2, Val4, Leu7 and also the two obtained
7 sequences S - Leu2-Val4-Leu7 and S' - Leu2'-Val4'-Val7'.

8 Cassava wastewater was already explored in many biotechnological processes, for
9 instance biotransformation. ²⁵ In this study we evaluated the biosurfactant production, which
10 based on MALDI-TOFMS and NMR analysis indicated that there are at least 11 surfactin
11 homologous, with two main amino acid sequences, resulting in a remarkable heterogeneity of
12 molecular structure, which will potentially have different properties (surface activity,
13 antimicrobial, etc.).

14

15 CONCLUSION

16 For the first time, the UF process was applied to the production of surfactin using
17 cassava wastewater. Solutions of crude and semi-purified biosurfactant at 100 mg L⁻¹ of
18 surfactin result in larger surfactin micelles, which can be retained in UF-1. In UF-2, the 100
19 kDa membrane led to poor purification whereas high purity was achieved with the 50 kDa
20 membrane. Therefore the best results were obtained with strategies (ii) and (iii) however the
21 highest purity in terms of protein was obtained with strategy (iii). These results and also the
22 comparison with our previous results obtained in the production of surfactin in synthetic
23 medium show that the higher the protein content in the culture (feed) the more complicated
24 the purification and therefore a larger number of steps will need to be added if a high purity
25 product is required. Furthermore the RMN and MALDI-TOFMS analyses identified 11

1 potential surfactin homologous, which are composed by different β -fatty acids and two amino
2 acid sequences – S and S’.

3

4 **Acknowledgments**

5 The authors are grateful to the São Paulo State Research Foundation (Fapesp) for
6 their financial support, Brazilian Bioscience National Laboratory (institution of CNPEM) for
7 RMN analysis and Polytechnic School of University of São Paulo for MALDI-TOFMS
8 analysis.

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Table 1. Parameters and yields of ultrafiltration – surfactin – in two steps

| First step of ultrafiltration – retentate | | | | | | |
|--|--|-----------------|------------------|-----------------------------------|-----------|-------------------|
| Flux (L(h.m²)⁻¹) | [†]C₀ (mg.L⁻¹) | Membrane | TMP (psi) | Rejection of surfactin (%) | pH | References |
| 25 | 1530 | PES (100) | 12.5 | 87 | 11 | 4 |
| 175 | 400 | PES (100) | 12.5 | 93 | 7 | 7 |
| 120 | 4020 | CE (100) | 8.7 | 97 | 7 | 8 |
| *NM | 583 | RC (10) | 29 | 98 | 7 | 9 |
| 83 | 596 | PES (10) | 29 | 83 | *NM | 10 |
| *NM | 250 | RC (30) | 30.45 | 97.9 | *NM | 11 |
| Second step of ultrafiltration – permeate | | | | | | |

| Micelle-destabilizing conditions | $\dagger C_0$ (mg.L ⁻¹) | Membrane | TMP (psi) | Recovery (%) | Purity (%) | Flux (L(h.m ²) ⁻¹) | References |
|------------------------------------|-------------------------------------|-----------|-----------|--------------|------------|--|------------|
| ethanol (33%) and ammonium sulfate | 2,054 | PES (100) | 12.5 | 81 | 78 | 5 | 4 |
| methanol (33%) | 2,054 | PES (100) | 12.5 | 87 | 85 | *NM | 7 |
| ethanol (50%) | 2,550 | CE (100) | 8.7 | 80 | 74 | 220 | 8 |
| methanol (50%) | 571 | RC (10) | 29 | 96 | 93 | 30 | 9 |
| methanol (50%) | 560 | PES (10) | 36.5 | 94 | 96 | 118 | 10 |
| methanol (50%) | 5,000 | RC (30) | 30.45 | 95 | 98 | *NM | 11 |

* Not mentioned

$\dagger C_0$ = initial concentration

Table 2. Concentration of protein (PC) and surfactin (SC) in the feed, retantate (R) and permeate (P) of the first and second ultrafiltration steps (UF1 and UF2) for strategies i, ii and iii.

| Ultrafiltration – First Step (UF-1) | | | | | | | | | |
|--------------------------------------|-------------------------------|-------|-------|--------------------------------|-------|-------|---------------------------------|-------|-------|
| | PES - 100 kDa Strategy (i) | | | PES - 100 kDa Strategy (ii) | | | PES - 100 kDa Strategy (iii) | | |
| | Feed | R | P | Feed | R | P | Feed | R | P |
| SC | 105. | 70.12 | 19.21 | 81.13 | 70.73 | 7.02 | 94.24 | 75.54 | 12.35 |
| PC | 194. | 87.41 | 62.85 | 112.7 | 93.65 | 28.66 | 83.14 | 36.31 | 50.64 |
| P _{pi} | 35 | 44 | 23 | 41 | 43 | 19 | 53 | 67 | 19 |
| Ultrafiltration – Second Step (UF-2) | | | | | | | | | |
| | PES - 100 kDa | | | PES - 50 kDa | | | PES - 50 kDa | | |

| | Strategy (i) | | | Strategy (ii) | | | Strategy (iii) | | |
|----------------|--------------|------|-------|---------------|-------|-------|----------------|-------|-------|
| | Feed | R | P | Feed | R | P | Feed | R | P |
| SC | 70.1 | 8.57 | 65.66 | 70.73 | 12.94 | 69.96 | 75.54 | 0.94 | 73.74 |
| PC | 87.4 | 0 | 83.41 | 93.65 | 35.35 | 47.78 | 36.31 | 16.24 | 18.15 |
| P _p | 44 | 100 | 44 | 43 | 26 | 59 | 67 | 5 | 80 |

1 SC – surfactin concentration (mg.L⁻¹); PC – protein concentration (mg.L⁻¹).

2 †P_p – purity of surfactin as mass fraction of surfactin in relation to sum of mass of surfactin
3 and protein (% w.w⁻¹) – P_{pi} (UF-1) and P_p (UF-2).

13 Table 3. UF in two steps; coefficient of rejection and nanoparticle size – strategies i, ii and iii.

| Ultrafiltration – First Step (UF-1) | | | | | | | | | |
|--------------------------------------|---------------|-------|-----|---------------|-------|-----|----------------|-------|------|
| | PES - 100 kDa | | | PES - 100 kDa | | | PES - 100 kDa | | |
| | Strategy (i) | | | Strategy (ii) | | | Strategy (iii) | | |
| | Feed | R | P | Feed | R | P | Feed | R | P |
| d | 71.4 | 466 | 129 | 72.3 | 428 | 123 | 78 | 441 | 60.3 |
| R _s | | 0.82 | | | 0.91 | | | 0.87 | |
| R _p | | 0.68 | | | 0.75 | | | 0.39 | |
| TRS _i | | 66.78 | | | 87.18 | | | 80.16 | |
| Ultrafiltration – Second Step (UF-2) | | | | | | | | | |
| | PES - 100 kDa | | | PES - 50 kDa | | | PES - 50 kDa | | |
| | Strategy (i) | | | Strategy (ii) | | | Strategy (iii) | | |
| | Feed | R | P | Feed | R | P | Feed | R | P |

| | | | | | | | | | |
|-------------------|-----|-------|------|-----|-------|------|-----|-------|------|
| D | 466 | 60.3 | 0.74 | 428 | 20.9 | 20.9 | 441 | 35.8 | 22.5 |
| R _s | | 0.06 | | | 0.01 | | | 0.02 | |
| R _p | | 0.05 | | | 0.49 | | | 0.50 | |
| TRS _{ii} | | 93.64 | | | 98.91 | | | 97.62 | |
| TRS _t | | 62.53 | | | 86.23 | | | 78.25 | |

1 R – retentate; P – permeate

2 *R_s or R_p - Rejection coefficient – equation 1; d – diameter of nanoparticle size (nm)

3 †TRS – Total recovery of surfactin – equation 3. – TRS_i (UF-1), TRS_{ii} (UF-2) and TRS_t (UF-1
4 and UF-2).

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13 Table 4. ¹H chemical shifts of two sequence of produced surfactin (strategy iii) - (²H₆-DMSO
14 at 25°C). For the non-peptide moiety, carbon atoms are numbered

15 as in Figure 5.

| | HN | αH | βH | | γH | γCH ₃ | δCH ₃ | |
|-------------------|-------|-------|-------|-------|-------|------------------|------------------|-------|
| Glu1 | 9.491 | 4.271 | 1.956 | 1.818 | 2.044 | 1.985 | | |
| Leu2 | 9.567 | 4.218 | 1.500 | 1.472 | 1.472 | | 0.828 | 0.787 |
| Leu3 | 7.457 | 4.351 | 1.441 | 1.337 | 1.337 | | 0.865 | 0.798 |
| ^s Val4 | 8.439 | 4.057 | 2.155 | | | 0.891 | 0.829 | |
| Asp5 | 8.305 | 4.287 | 2.622 | 2.172 | | | | |
| Leu6 | 7.291 | 4.177 | 1.556 | 1.474 | 1.474 | | 0.860 | 0.816 |

| | | | | | | | | |
|---|--------------------|-------|------------|------------|-------------|------------|--------------------------|------------|
| | Leu7 | 8.421 | 4.339 | 1.663 | 1.521 | 1.429 | 0.821 | 0.804 |
| | Glu1' | 9.681 | 4.261 | 1.944 | 1.756 | 2.038 | 2.006 | |
| | Leu2' | 9.616 | 4.218 | 1.500 | 1.472 | 1.472 | 0.828 | 0.787 |
| | Leu3' | 7.442 | 4.351 | 1.441 | 1.337 | 1.337 | 0.865 | 0.798 |
| S | Val4' | 8.329 | 4.050 | 2.161 | | | 0.892 | 0.818 |
| | Asp5' | 8.453 | 4.290 | 2.669 | 2.116 | | | |
| | Leu6' | 7.207 | 4.295 | 1.532 | 1.432 | 1.432 | 0.867 | 0.817 |
| | Val7' | 8.275 | 4.039 | 2.021 | | | 0.845 | 0.808 |
| | Lipid chain | | C3H | C2H | C2H' | C4H | (CH2)_n | CH3 |
| S | | | 4.933 | 2.801 | 2.292 | 1.557 | 1.213 | 0.833 |
| S | | | 4.918 | 2.824 | 2.292 | 1.577 | 1.213 | 0.833 |

1 * S (1 - sequence of amino acids)

2 † S' (2 - sequence of amino acids)