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***Research paper***

# Comparative study: bench-scale surfactin production from bacillus subtilis using analytical grade and concentrated glycerol from the biodiesel industry

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**Abstract**

The market price of glycerol worldwide tends to decrease, since it is a by-product of biodiesel production. Thus its biotechnological use might lead to significant reduction in the cost of fermentations. The aim of this study was to compare the production of surfactin in peptone culture media supplemented with analytical grade glycerol (AGG) and concentrated glycerol from biodiesel production (CGBP). Differences were observed between the two processes including cell growth and dissolved oxygen consumption. Surfactin yield was 325.19 mg/L with AGG and 71.13 mg/L with CGBP, which proves the impact and importance of the purity of glycerol on the yield of surfactin. In addition, five surfactin homologous were identified by ESI-Q-TOFMS, which were composed by two amino acid sequences ELLMDLD and ELLLDLL. Therefore, as surfactin is a high value-added product, the use of glycerol with high purity is fundamental to achieve higher productivity.

***Keywords***: *Bacillus Subtilis; Biodiesel; Biosurfactant; Glycerol; Surfactin.*

## 1. Introduction

Brazil ranks among the top 5 world's largest producers and consumers of biodiesel, which produced ≈ 2,696.00 $m^3$ and 2,741.115 $m^3$ in 2011 and 2012, respectively [1-2]. Glycerol is the main by-product of biodiesel production. It represents approximately 10% of the volume of a reaction [1], [3]. However, glycerol from the biodiesel industry has a low aggregate value due to the presence of impurities [3-4]. Thus, in years to come, due to increasing biodiesel production the price of glycerol will tend to decrease.

Glycerol is a fermentable polyol (sugar alcohol) nutrient for most bacteria and yeasts [5-6]. In addition, depending on the source of triglycerides used in biodiesel production, raw glycerol can contain nutritional elements such as phosphorous, sulfur, magnesium, calcium, nitrogen and sodium, which can be used by microorganisms in the fermentation process [7]. Thus, the by-product from biodiesel industry can be used as a low-cost substrate for bioproduction of high added value products such as biosurfactants [1], [3], [6-7]. It is known that a wide variety of microorganisms produce biosurfactants, including Bacillus subtilis which synthesize lipopeptides (e.g. surfactin) [3], [8]. Surfactin (98% purity) is available from Sigma Chemical Company at approximately $ 191.5/10 mg or $ 724/50 mg (priced on 13 January, 2016). Makkar et al. [9] suggested that the perfect scenario would be to have biosurfactants priced at ≈ $ 0.011/mg, which would make the biosurfactants economically equivalent to surfactants.

About 21 cyclic lipopeptides families have already been studied and identified [10]. The genus Bacillus traditionally produces three out of those 21 families: surfactin, iturin and fengycin. Each family has a specific number of amino acids, but with different residues at specific positions. It also has different lengths and isomery of β-hydroxyl fatty acids (cis, trans, iso and anteiso, etc), that is, lipopetides have a remarkable heterogeneity of molecular weight and isomery [10-14].

One way of reducing bioproduction cost is by using low cost nutrients as culture medium (fermentation) such as industrial waste or by-product, for instance, glycerol from the biodiesel industry. At the same time the use of glycerol from biodiesel industry could improve the profitability of biodiesel in a broader sense for biorefineries. However, a few papers have detailed surfactin production from Bacillus subtilis using glycerol from biodiesel production as carbon source such as Sousa et al. [5], Sousa et al. [3], Sousa et al. [4], De Faria et al. [13]. In particular, there is a lack of knowledge on the effect of glycerol purity on productivity.

We speculate that the purity of glycerol from industrial biodiesel production has significant effect on the productivity of surfactin. Thus, the aim of this study was to evaluate the surfactin production from Bacillus subtilis LB2b, mainly, on a bench-scale bioreactor using glycerol of two different purities: (1) concentrated glycerol from biodiesel production (by-product of biodiesel industry after removal of methanol) (CGBP), (2) analytical grade glycerol (AGG).

## 2. Materials and methods

### 2.1. Chemicals

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The chemicals used: acetonitrile (Synth ≈ 99.8%), analytical grade glycerol (Sigma-Aldrich ≈ 86-89%), bicinchoninic acid kit (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich ≥ 98%), chloroform (Synth ≈ 99.8%), hydrochloric acid (Lafan ≈ 37%), methanol (Sigma-Aldrich ≥ 99.6%), periodic acid (Vetec ≥ 99%), potassium dichromate (Impex ≥ 99%), phosphoric acid (Sigma-Aldrich ≥ 85%), sodium hydroxide (Sigma-Aldrich ≥ 97%), sodium iodide (Synth – analytical grade), sodium thiosulfate (Synth–0.05 M), sulfuric acid (Merck 98%), surfactin (Lipofabrik ≥ 99%), and trifluoroacetic acid (Sigma-Aldrich ≥ 99%).

## 2.2. Experimental strategy

A preliminary study with three different culture media composed by (1) AGG, (2) CGBP and (3) crude glycerol at flask-scale was conducted and the growth and of B. subtilis LB2b and production of surfactin (by surface tension measurement) were assessed over the fermentation time. Then, based on the results at flask-scale, bench-scale batch fermentations were conducted in a 7.5L bioreactor to investigate in more detail the effect of glycerol purity on surfactin productivity and production of surfactin homologous. For this, two glycerol types were investigated separately: (1) AGG and (2) CGBP.

## 2.3. Microorganisms and inoculum

Bacillus subtillis LB2b pertaining to laboratory collection of Bioflavour/Fea/UNICAMP collection, previously identified as biosurfactants producer was used [15]. The inoculum was standardized according to Andrade et al. [16].

## 2.4. Culture media

The culture media were prepared with the following compositions (g/L in distilled water): bacto-peptone 10.0 and glycerol 10.0. In view of the objectives of this study, glycerol from three different sources was used separately: analytical grade glycerol (AGG), concentrated glycerol from biodiesel production (CGBP) and raw glycerol from biodiesel industry. Raw glycerol was used only in the flask-scale fermentations. Raw glycerol was produced by the base-catalyzed transesterification (NaOH) of soybean oil with methanol, obtained at BrasBio Industry (Rio Claro-SP, Brazil).
Regarding bench-scale batch fermentations, a volume of 3.5 L of both culture media described above were adjusted to pH 7 with NaOH 0.05 M, placed into the bench-scale bioreactor (Bioflo® & Celligen® 310-New Brunswick Scientific-7.5 L) and sterilized (121 °C for 20 minutes).

### 2.4.1. Concentration of raw glycerol

The raw glycerol was adjusted to pH 3 by phosphoric acid (0.66 M) and then it was left to rest for 24 h. Subsequently the solution was separated into three phases. According to Rivaldi et al. [7] the intermediate part has the highest concentration of glycerol; thereby, it was isolated using a separating funnel. Then, methanol was removed from the intermediate part by a rotary evaporator at 50 ºC for 4 h. The material (glycerol) was collected from rotary evaporator and used as the culture medium in the bench-scale bioreactor experiments [3-4], [7].

## 2.5. Fermentation procedures and sampling

### 2.5.1. Flask fermentation

The flask-scale fermentations were carried out 3 times as a preliminary screening to evaluate the fermentation process using three culture media, (1) peptone plus raw glycerol from biodiesel industry, (2) peptone plus AGG; (3) peptone plus CGBP. All culture media were adjusted to pH 7 with NaOH 0.05 M. The erlenmeyer flasks, containing each culture medium (100 mL), were inoculated and then incubated at 150 rpm and 30 ºC. Samples (≈ 12 mL) of the culture medium were collected on a 12-hour basis and centrifuged at $10^4$ g for 10 minutes at 5 ºC. Finally, the viable cell count, surface tension (ST) of the samples and their dilutions were analyzed [15-17].

### 2.5.2. Bench-scale fermentation

All experiments were carried out at least 3 times. The process conditions were: 150 rpm, 30 ºC and an aeration rate (air) of 0.266 vvm (maximum aeration pump capacity available) [16-17]. The dissolved oxygen (DO) sensor (Mettler Toledo - INPRO 6830/12/320) was set to measure every thirty seconds during the entire fermentation process. Samples (≈ 30 mL) of the culture medium were collected on a 24-hour basis, and subsequently the viable cell count, ST dilutions and consumption of glycerol were used as process parameters [2], [15-17]. Foam was collected during production from the top of the bench-scale bioreactor (foam overflow) into a Büchner flask through a hose [15-16]. The foam volume was measured on a 24-hour basis, centrifuged ($10^4$ g for 10 minutes at 5 ºC) and had its surface activity (ST and its dilution) measured.

## 2.6. Purification of surfactin

Two purification methods were applied: (1) acid precipitation method and (2) acid precipitation followed by solvent extraction (chloroform: methanol-81:19) and solvent evaporation [15-17]. The resulting product (in powder form) from (1) was named crude biosurfactant and the product from (2) semi-purified biosurfactant. The yield in both methods was calculated as mass of surfactin (g) over total volume of culture medium (3.5 L).

## 2.7. Analytical methods

### 2.7.1. Determination of methanol in raw glycerol and CGBP

The free methanol content of CGBP and raw glycerol were determined by HPLC-Shimadzu Prominence (Kyoto, Japan), using a LC-20AD HPLC system (Shimadzu, Columbia, USA) equipped with a RID-20A refractive index detector and HPX-87H column of dimensions 300 mm × 7.8 mm, and a particle size of 9 µm (Aminex, London, England). The analyses were performed using 5 mM $H_2SO_4$ as mobile phase and the flow rate was 0.6 mL/min. The total run time was 25 min. All the samples were previously filtered through a 0.45 µm teflon membrane (Millipore). The samples were injected (10 µL) at the temperature 4 °C. The column and RID temperatures were maintained at 60 and 50 °C, respectively.
The chromatograms were analyzed and integrated by the LCSolutions data acquisition software, version 5.73 (Shimadzu, Columbia, USA). An external calibration curve was constructed by analyzing standard methanol solutions at different concentration levels and the methanol contents of samples were determined.

### 2.7.2. Fermentation process

Viable cellular growth curves were plotted using CFU/mL data [15-17]. The data of DO were obtained from a probe submerged in the culture medium. Additionally, the glycerol concentrations were measured by titration of a centrifuged culture medium [2]. The concentration of micronutrients in the culture medium comprised of peptone and CGBP was analyzed by ICP-OES, the Kjeldahl' method (N), distillation (ammonia and nitrate) and the Walkley-Black' method (organic carbon).

### 2.7.3. Measurement of surface activity and critical micelle concentration

The ST measurements were carried out by using the plate method at room temperature in a Krüss GmbH K-12 tensiometer (K-12 model, Krüss GmbH) [15-17].

The surface activity was measured in culture media, collapsed foam and solutions (1 mg/mL) of crude and semi-purified biosurfactants. The ST, critical micelle dilution (CMD), and critical micelle concentration (CMC) were determined. The CMD corresponds to the surface tension value of a sample diluted 10 times (CMD-1) and 100 times (CMD-2). The CMC was determined by a serial dilution from 0.006 to 0.3 mg/L, where the objective was to identify the curve inflection point, that is, the CMC [18]. The CMC determination was carried-out using semi-purified biosurfactants from all experiments with the same medium.

#### 2.7.4. Determination of surfactin concentration

Semi-purified biosurfactants (AGG-23.42 mg/50 mL and CGBP-7.95 mg/50 mL) were analyzed by reverse phase-HPLC using a Gilson 306 (Rockford, IL, USA), with a C18 column of dimensions 250 mm × 4.6 mm, and a particle size of 5 µm; and PDA100 diode array detector. The sample was eluted with a binary gradient at a flow rate of 1.1 mL/min. The following gradient solvent system was used: Solvent A: Acetonitrile (0.1% trifluoroacetic acid) and solvent B: Deionized water (0.1% trifluoroacetic acid). First step was carried out by using a linear gradient from 50 to 80% acetonitrile (0.1% trifluoroacetic acid) during 15 min, remaining at 80% for additional 20 min. The column was then re-equilibrated to the initial conditions by increasing solvent A concentration (4%/min) from 80% to 100% for 5 min as a washing step before returning (6%/min) back to 50% and remained for 10 min. A 50 µL of sample was injected in each run, which lasted for 60 min, and eluent absorbance was monitored at 214 nm. The system was calibrated using standard surfactin (>99.8%) [19-20]. the surfactin concentration was determined by HPLC and the purity was determined in terms of mass of surfactin over the total dry weight mass.

#### 2.7.5. Protein concentration

The concentration of protein in the solutions of semi-purified biosurfactant was determined by the bicinchoninic acid method [19-20].

#### 2.7.6. Identification of surfactin homologous by electrospray ionization mass spectrometry (ESI-Q-TOFMS)

Approximately 10 mg of each semi-purified bisurfactant (AGG and CGBP) were solubilized in 1.5 mL (acetonitrile:deionized water:methanol; 1:1:1) The samples were injected into electrospray transport solvent by using a micro syringe (500 □L) coupled to a micro infusion pump (KD Scientific) at a flow rate of 180 µL/h. The identification of surfactin homologous was performed by ESI-Q-TOFMS Impact II Bruker Daltonics). The mass spectrometer was calibrated with low concentration tuning mix (Agilent Technology) and its typical cone-voltage induced fragments to operate at ultra-high resolution (50,000 Full-Sensitivity Resolution).

A mass spectrum was acquired over the range of 50–1150 m/z in the positive mode, a potential of 7eV at the sample cone and the capillary voltage was 4000 V, desolvation gas temperature at 180 °C, nebulizer pressure 0.4 Bar and drying gas (nitrogen) 4 L/min.

The precursor ion mass was set to 1043.55 m/z and the isolation window width was 10 Da. The collision gas was nitrogen and the fragment energy was 55 eV, and a ramping factor ranging from 100% to 150% (i.e., the real fragment energy ranged from 55 eV to 82.5 eV) was used to achieve improved fragmentation. The MS data were processed with DataAnalysis 4.1 SP3, and the de novo sequencing analysis was performed using BioTools 3.2 (both from Bruker Daltonics) [21].

## 3. Results and discussion

### 3.1. Flask fermentation

Surface tension measurements can be used to monitor production of biosurfactants during the fermentation. The surface tension value and its dilution are inversely proportional to the biosurfactants concentration [3], [15-16].

The total cell count in Fig. 1 showed a relative good microbial growth between 0-9 h, followed by a growth phase (the highest microbial growth rate) up to 36 h and a stationary phase up to 72 h. It is worth noting that the lag phase took place within the interval of 0-9 h, probably during the 1$^{st}$ or 2$^{nd}$ hour of fermentation. A strong reduction in the ST occurred in the first hours of fermentation, where the value dropped from ≈ 40 mN/m to ≈ 27 mN/m. The same behavior was observed in CMD-1 and CMD-2, the first of which showed significant reduction from ≈ 59 mN/m to ≈ 50 mN/m. On the other hand, most cell growth was observed between 9 and 23 h, where cells count increased from $1.71 \times 10^7$ CFU/mL to $1.12 \times 10^8$ CFU/mL. A subtle increase in all parameters (ST and CMDs) was observed after 9 h.

The CMD-2 data were similar to the surface tension of distilled water (72 mN/m). In other words, due to the high dilution (100 times), no significant content of surfactin was observed. On the other hand, CMD-1 showed values around 55 mN/m, which is lower than that of distilled water, indicating a relevant content of surfactin even when it was diluted 10 times. It is worth noting that the highest difference of CMD-1 values took place between 0 and 9 h (Δ ≈ 10 mN/m), which is aligned with the ST data. Thereby, when comparing samples collected subsequently, that period had the highest production of biosurfactants. After that, subtle changes occurred until the 70 hours, which indicates the maintenance of surfactin concentration. Therefore, the culture medium composed only by two compounds, AGG and peptone, was relative suitable to B. subtilis LB2b growth and biosurfactants production.

Then, experiments evaluated the microbial growth and biosurfactants using a culture medium composed by raw glycerol and peptone (Fig. 2).

The fermentation using a culture medium composed by peptone and raw glycerol from biodiesel industry showed significant lower microbial growth rate and biosurfactant production (Fig. 2) than with AGG. Contrary to what was observed in the fermentation with AGG, the characteristic ST value of surfactin at concentrations equal or higher than CMC (≈ 27 mN/m) were not obtained. The CMD-1 was also higher (≈ 65 mN/m), that is, a lower biosurfactants production was achieved using raw glycerol. Salakkam and Webb [22] studied the effect of methanol on Cupriavidus necator DSM4058. It was found that methanol at any concentration (up to 125 g/L) had a negative influence on microbial growth. Thus, we speculate that the difference in biosurfactant production was mainly due to the high concentration of methanol in the raw glycerol.

Thus, based on the experimental data obtained with AGG and raw glycerol from biodiesel industry, further experiments were carried out using treated raw glycerol, CGBP - (Fig. 3).

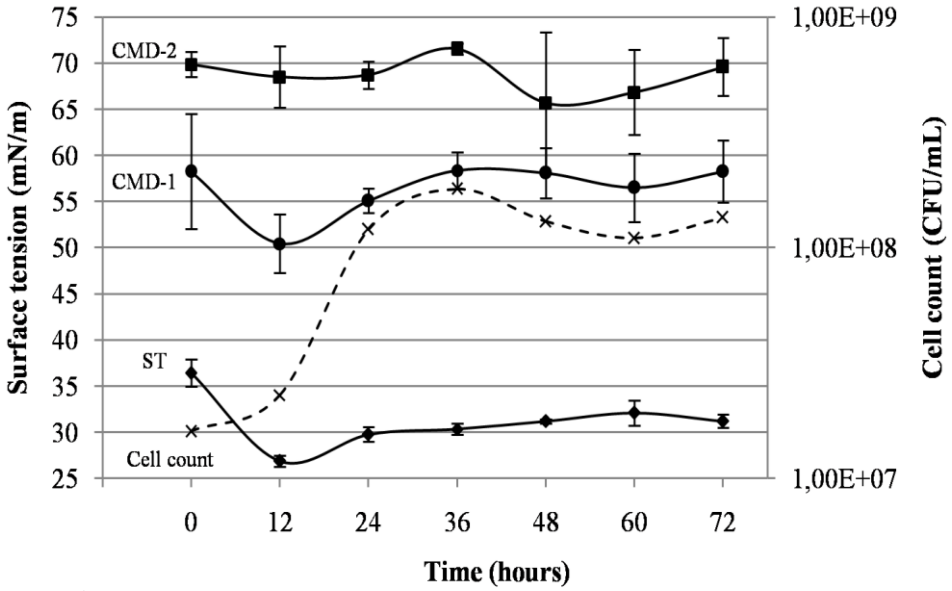


**Fig. 1:** Growth Curve and Surface Activity in the Culture Medium Composed by AGG Plus Peptone at Flask Fermentation Scale; (× - Cell Count); (■ – ST); (● – CMD-1); (♦ - CMD-2).

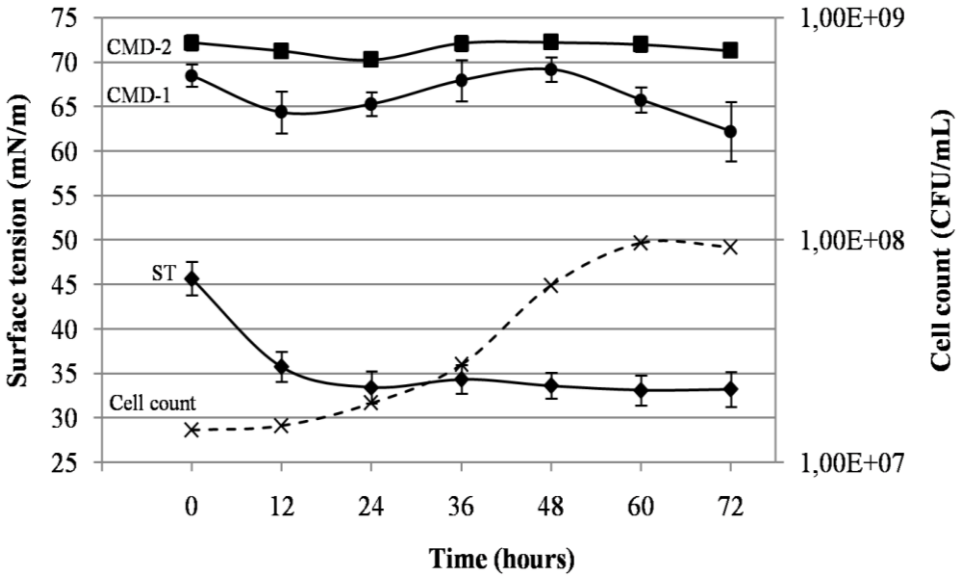


**Fig. 2:** Growth Curve and Surface Activity in the Culture Medium Composed by Raw Glycerol from Biodiesel Industry Plus Peptone at Flask Fermentation; (× - Cell Count); (■ – ST); (● – CMD-1); (♦ - CMD-2).

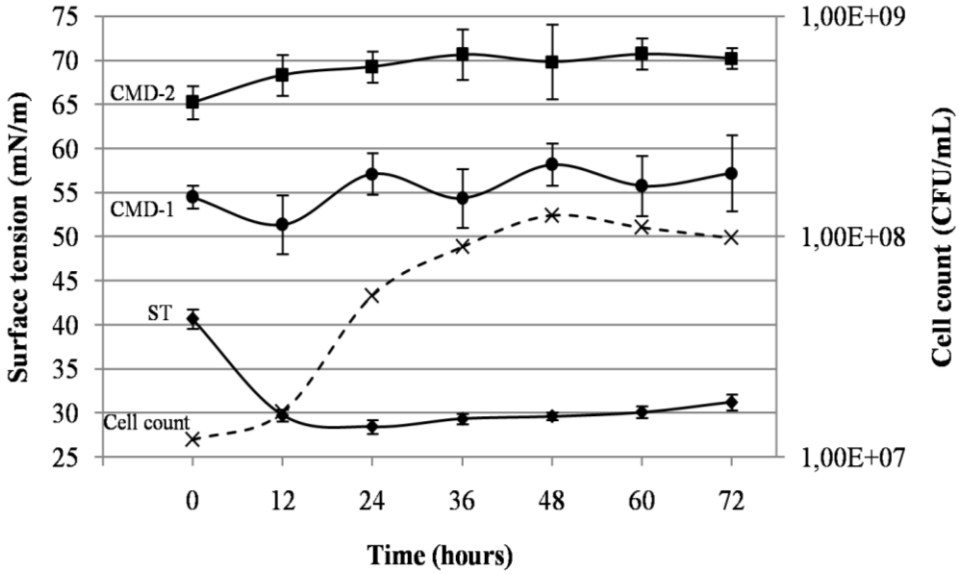


**Fig. 3:** Growth Curve and Surface Activity in the Culture Medium Composed by CGBP Plus Peptone at Flask Fermentation Scale; (× - Cell Count); (■ – ST); (● – CMD-1); (♦ - CMD-2).

Fig. 3 shows that the B. subtilis Lb2b growth in the medium composed by CGBP and peptone showed similar microbial growth and biosurfactants production compared to AGG plus peptone, that is, ST ≈ 27 mN/m, CMD-1 ≈ 55 mN/m and microbial growth curve.

## 3.2. Composition of culture medium (concentrated glycerol from biodiesel industry)

Fermentation with AGG and CGBP led to good and similar production of biosurfactants. The main difference between raw and CGBP glycerol is the removal of salts, soap, but mainly methanol (32.41% in raw and 4.41% in CGBP, Table 1). On the other hand, the raw glycerol from biodiesel industry experiments showed lower production. Thus, there is strong evidence that B. subtilis Lb2b is very sensitive to the presence of methanol.
Most of the metal present in the culture medium with CGBP was higher than 0.01 ppm (Table 1), However Fe, Mn, Cu and Ca were below the detectable limits of the test. Also, the composition, compared with Cooper's medium, most of the minerals were at a higher concentration [23].

**Table 1:** Nutritional Composition of the Culture Medium Comprised of Bacto-Peptone and Concentrated Glycerol from Biodiesel Production.

| Nutrient | [mg/L] | Nutrient | [mg/L] |
|---|---|---|---|
| P | 0.3 | Zn | 0.8 |
| K | 0.1 | $NH_3$ | 43.1 |
| Ca | <0.01 | Mg | 0.02 |
| C* | 9.1 | S | 0.1 |
| $NO_3^-$ | 4.3 | B | 8.0 |
| N* | 1.2 | Mn | <0.01 |
| Cu | <0.01 | MeOH† | 4.41 |
| Fe | <0.01 | | |

* - g/Kg
† - %

The C/N ratio ≈ 7.52 was very similar to Cooper's medium, which was one of the first papers on the content of minerals and production of surfactin by Bacillus subtilis [23]. Obviously, this result is due to the positive combination of glycerol and peptone, since both are carbon sources. In addition, the peptone could also be a nitrogen source.
Peptides can be absorbed into the cell and metabolized into amino acids. Then, by deamination or oxidative deamination, these amino acids are converted into intermediates of tricarboxylic acid cycle such as serine → pyruvate, aspartate → oxaloacetate, glutamate → 2-oxoglutarate [24-25]. However, the catabolic pathways of many amino acids remain unknown or only partially characterized. In this context, arginine and histidine are known to provide energy [24-25]. Yan et al. [25] evaluated the aflatoxin production from Aspergillus flavus using a culture medium comprised by salts and peptone as sole carbon source. They indicated that Aspergillus flavus preferred peptone as a sole carbon source for growth rather than traditional fermentable sugars. Thus, peptone can be used by microorganisms as carbon source.

The first reports on biosurfactant production using glycerol from biodiesel production were carried out with Pseudomonas sp., which synthesizes rhamnolipids. To the best of our knowledge, De Faria et al. [13] published the first relevant report on the production of lipopeptides: surfactin ($C_{14}$/$Leu_7$) from B. subtilis using raw glycerol (5% v/v) from biodiesel production as the sole carbon source.
Sousa et al. [3] neutralized the raw glycerol and then removed the methanol by evaporation. Finally, the remaining product was added to the culture medium. As a result, 4 out of 7 strains of B. subtilis reached ST values around 27 mN/m. Thus, there are differences in glycerol metabolism, even among the same species of a microorganism [3].
In summary, results above confirmed that both culture media peptone plus AGG and peptone plus CGBP are better suited for B. subtilis LB2b growth and biosurfactant production than raw glycerol plus peptone. Further experiments were carried out with culture media containing either AGG or CGBP at bench scale and a comparative study was carried out in terms of biosurfactant production.

## 3.3. Bench-scale fermentation

### 3.3.1. Fermentation parameters

In the experiments with AGG, DO dropped to 0% at ≈ 4.5 h of fermentation and started to increase at ≈ 30 h (Fig. 4). On the other hand, tests with CGBP, DO decreased to 0% at ≈ 9 h and maintained this level until 72 h (Fig. 4). In both cases, the experiments remained stable at 0% DO for the majority of the time, 25.5 and 63 h, respectively. It is worth noting that, after 54 h of fermentation, there was a great difference in DO between both fermentations, AGG and CGBP.
In the experiments with AGG, the number of viable cells increased from $1.6x10^8$ (+/- $2*10^8$) at 0 h to $1.3x10^{11}$ CFU/mL (+/- $4*10^{11}$) after 48 h; then, at 72 h, this value was ≈ $7x10^{10}$ (+/- $5*10^8$). On the other hand, when CGBP was used, the count reached only $3.5x10^{10}$ (+/- $7*10^{10}$) CFU/mL after 48 h, and at 72 h, it was ≈ $8.3x10^9$ (+/- $6*10^9$) In experiments with CGBP, there was a delay in overall cellular development, in a similar way to that reported by Salakkam and Webb [22]. This difference is consistent with the curves of the DO (Fig. 4), which has a direct relationship with cellular growth. Low DO values during cellular growth indicated high oxygen consumption per cell and high cell content. It is worth mentioning that after 48 hrs the number of cells decreased in both cases. The bench-scale bioreactor is a semi-closed system in which the foam was collected during its production. Thus, many cells were removed from the bioreactor by foam overflow. This hypothesis was supported by the fact that the DO levels rose strongly after this time in the fermentation in which AGG was used.

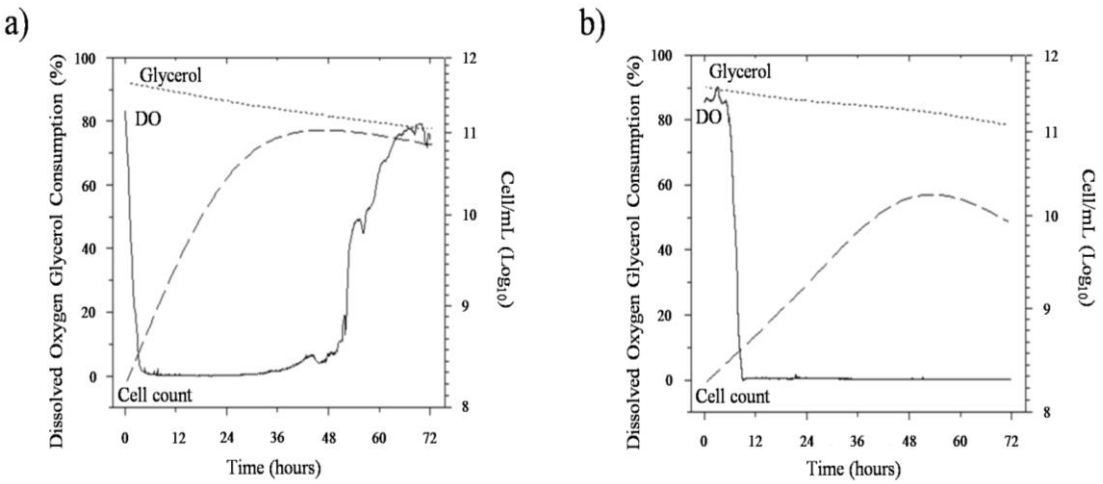


**Fig. 4:** Comparative Fermentation Parameters between Two Culture Media: (A) AGG and (B) CGBP at Bioreactor Scale; (Solid Line - DO); (Dashed Line - Cell Count); (Dotted Line - Glycerol).

Raw glycerol contains typically ≈ 5% NaCl and up to ≈ 30% methanol and even though most of salt and methanol were removed from the by-product from biodiesel industry (raw glycerol → CGBP), their presence, even at low concentrations, may have had a significant effect upon the metabolism of B. subtilis [22].

There are very few works where the relation between microbial kinetics and toxicity of methanol is evaluated, in particular for bacteria. One of these was developed by Salakkam and Webb [22], who studied the effect of methanol on microbial growth rate of bacteria using glycerol as carbon source. They reported that the effect of methanol on microbial growth rate (inversely proportional) and lag phase (proportional) were due to the following changes in the cell: (i) reduction of membrane stability, (ii) denaturation of protein, including enzymes and (iii) changes in fatty acid and acid nucleic composition. Thus, Sallakam and Webb [22], strongly recommended to eliminate methanol from any culture medium.

Some species of Bacillus are classified as methylotrophic microorganisms and may use methanol as a carbon source via the ribulose monophosphate (RuMP) [26]. The experiments with CGBP contained 4.41% of methanol and probably, due to the absence of the RuMP in this strain, the methanol might have been oxidized to formaldehyde, which could have started alkylation reactions within the cytoplasm. As a result of this, cell metabolism was reduced, and consequently, substrates were consumed at a lower rate, which allowed consumption of oxygen (0% of DO) until 72 h. alternatively, experiments with AGG did not have methanol or other impurities in the medium. Thereby, high oxygen intake (0% of DO) was readily reached after 9 h, hence, a lack of nutrients or excess of secondary metabolites may have occurred after 48 h, which is aligned with increase of DO after that time. Therefore, there is evidence that CGBP, even after the purification steps described above, contained other molecule(s) with significant deleterious effect on growth. In other words, the medium with AGG was the best for microbial growth.

Taking this into account, research on more efficient processes and techniques for glycerol purification can increase cell viability, and, therefore, biosurfactant production.

Glycerol consumption showed similar results in both experiments. Glycerol, when used as a carbon source, is mainly degraded by glycerol kinase pathways, which is better expressed under aerobic conditions [27]. Surfactin produced from B. subtilis is synthesized in the exponential phase. Thereby, considering the process as non-segregated and structured, the highest metabolic rate took place at exponential phase [27]. Therefore, the intake of glycerol should be similar to the oxygen consumption curve (or the opposite of DO), Fig. 4. However, glycerol consumption curves showed linearity (gradually absorbed during the fermentation). Thus, it could mean that glycerol was not used as carbon source, but the presence of glycerol improved the fermentation, for instance by increasing the cellular membrane permeability.

### 3.3.2. Measurement of surface activity – collapsed foam and culture media

Biosurfactant concentration is inversely proportional to the ST; the lower the CMD values are, the higher the biosurfactant concentration. Also, an increase in foam production is expected with higher biosurfactant concentrations (Table 2).

The ST values for the clarified foams from experiments with AGG and CGBP did not show statistical differences at a significance level of 0.05 and were 29.42 mN/m (+/- 3.02) and 29.97 mN/m (+/- 4.27), respectively (Fig. 5). This is most likely due to the fact that in both samples the biosurfactant concentration was higher than its CMC and this resulted in a constant value for ST. This obviously indicates that in both cases good biosurfactant production and recovery was obtained.

For both culture media (AGG and CGBP), the ST remained constant ≈ 34 mN/m, after 24 h, which is similar to reported by Sousa et al. [5] 31.5 mN/m after 72 h of fermentation. In this sense, CMD-2 remained also constant ≈ 72 mN/m data. However, CMD-1 data for the experiments with AGG were lower, indicating a higher biosurfactant production (Fig. 5). These results converged with the results of purity and yields of surfactin (Table 2), viable cells and DO, that is, comparing with CGBP medium, AGG showed higher purity and yield of surfactin, viable cell count and absorption of oxygen.

Finally, the ST data for culture media – higher than surface tension at CMC (27 mN/m) - indicates that the recovery of surfactin by foam is a good strategy, since less than 10 mg of surfactin per liter of culture medium remained in the system during the fermentation. Henceforth, surfactin production was calculated based only in the collapsed foam, that is, it was assumed that 100% (theoretically) of surfactin produced was recovery by foam overflow.

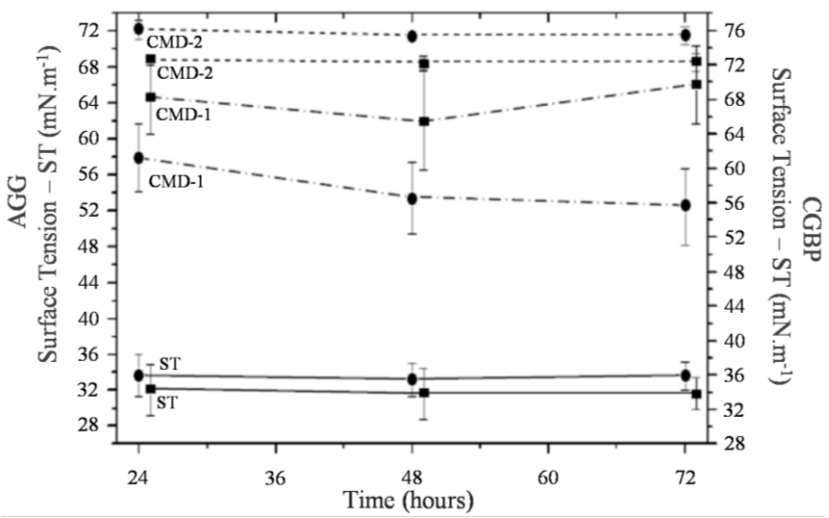


**Fig. 5:** Surface Activity Measurements of Two Culture Media AGG (Left) and CGBP (Right) at Bioreactor Scale. (Solid Line - ST); (Dashed Line - CMD-1); (Dotted Line - CMD-2); (AGG – ●); (CGBP – ■).

### 3.3.3. Volume of collapsed foam, crude and semi-purified biosurfactant yields, protein concentration in semi-purified and purity of surfactin

Table 2 illustrates all yields of collapsed foam produced, crude and semi-purified biosurfactants and the purity of semi-purified biosurfactants. Volumes of foam produced were statistically different (Tukey test 95%) and their yields (foam/culture medium) were 0.18 and 0.10 (v/v) in the experiments with AGG and CGBP, respectively. This difference is clearly related to a higher yield of surfactant.

**Table 2:** Yields of Biosurfactant Production

| | AGG medium | CGBP Medium |
|---|---|---|
| Collapsed foam produced - (mL) | 657 | 360 |
| Crude biosurfactant - (1) acid precipitation method - (g/L of foam) | 7.85 | 4.89 |
| Semi-purified biosurfactant - (2) acid precipitation followed by solvent extraction - (g/L of foam) | 1.58 | 1.13 |
| Purity of surfactin in semi-purified biosurfactant-(%w/w)* | 72.02 | 22.03 |
| Concentration of protein in semi-purified biosurfactant–BCA kit-(%w/w) | 26.52 | 48.08 |

* The surfactin concentration was determined by HPLC and the purity in terms of mass of surfactin over the total dry weight mass.

Differences of crude biosurfactant yields were observed between both culture media. This may be due to the decrease in solubility of peptone residue in the medium during the acidification step, or to peptones and/or proteins synthesized by the strain.

The concentration of protein in the solutions of semi-purified biosurfactant was: 124.23 mg/L (AGG) and 76.45 mg/L (CGBP), that is, 26.52 and 48.08%, respectively. These results follow the same trend as crude and semi-purified yields, in which the products (crude and semi-purified biosurfactant) obtained from AGG showed higher surfactin concentration, that is, lower impurities (mainly proteins) concentration (see Table 2).

Thus, probably the impurities of CGBP decrease the surfactin production and also, increased protein production. A plausible explanation for the higher concentration of protein when using CGBP is that the impurities (toxic molecules) suppressed the metabolic pathway of surfactin production and induced the strain to synthesize more enzymes to keep itself alive or the impurities diverted the metabolic pathway of surfactin.

The CMC of semi-purified biosurfactant from experiments with AGG and CGBP were determined as 11 mg/L and 19 mg/L, respectively. These results converge with the definition that a powerful biosurfactant has a CMC value between 1 mg/L and 2000 mg/L [28]. Even using a new culture medium (reported for the first time), the results are similar to those reported by Nitschke et al. [15] ≈ 11 mg/L, and better than the 14 mg/L reported by Sheppard & Mulligan [18], and 25 mg/L reported by Cooper et al. [23], respectively. However, it is possible to notice that a higher value of CMC was identified for the culture grown in CGBP than in the medium with AGG. This difference in CMC values agrees with the trends observed when monitoring ST (Fig. 2 and Fig. 3).

The literature describes the production of surfactin per liter of culture medium (glucose as carbon source and extracted direct from the culture medium) to be at least 500 mg/L, whereas using glycerol as carbon source, lower yields are usually described (158.14 mg/L after 72 h of fermentation) [5], [19-20]. The production with AGG medium was ≈ 325 mg of surfactin/liter of culture medium, whereas with the CGBP medium was 71 mg/L. De Faria et al. [13] used a synthetic culture medium for surfactin production, then recovered it by foam overflow and purified it by absorption column chromatography. They produced 230 mg surfactin/L of foam, or 89.93 mg surfactin/L of medium. The same fermentative process was used to identify the fengycin homologues (decapeptide attached to a β-hydroxy fatty acid) [29]. In this context, it should be noted that the aim of this study was not to optimize the production of surfactin but the effect of the purity of glycerol on productivity. We speculate the reasons for the relative low production as: (i) glucose is more assimilable carbon source than glycerol; (ii) no optimization experiments were performed (agitation, inoculum, temperature, proportion of glycerol and peptone, etc) and (iii) the recovery of surfactin by foam overflow (collapsed foam) did not recover 100% of surfactin (remainders: in the culture medium, foam (bioreactor), hose, etc). Further studies will be carried to optimize the production of surfactin.

### 3.3.4. Identification of surfactin homologous by ESI-Q-TOFMS

Ayed et al. [12] analyzed lipopeptides from Bacillus mojavensis by ESI-Q-TOFMS with m/z range between 1045-1080. They found that these corresponded to three different surfactin homologous which differ in the length of the fatty acid chain. In this work we have identified five surfactin homologous by ESI-Q-TOFMS (Table 3 and Fig. 6).

A further investigation was carried out in order to identify the chemical structure of surfactin homologous (β-hydroxyl fatty acids and amino acid sequences). For that, MSMS spectra were obtained by the identified peaks (5 peaks AGG and 5 peaks CGBP) as showed in Fig. 6.

The chemical structure of surfactin homologous was identical for AGG and CGBP. Two amino acid sequences were identified, in which the sequence (ELLMDLD) was related to shorter β-hydroxyl fatty acid chains (12-14 carbons), whereas (ELLLDLL)

was related to greater β-hydroxyl fatty acid chains (16-17 carbons) (Table 3).

In order to exemplify the obtained data for identification of the two amino acid sequences, 2 out of 10 peaks were chosen, 1044.521 and 1065.409 m/z (CGBP) as illustrated in Fig. 7, in which at the top it is described the amino acid sequences by ions (b, b-18 and y fragments).

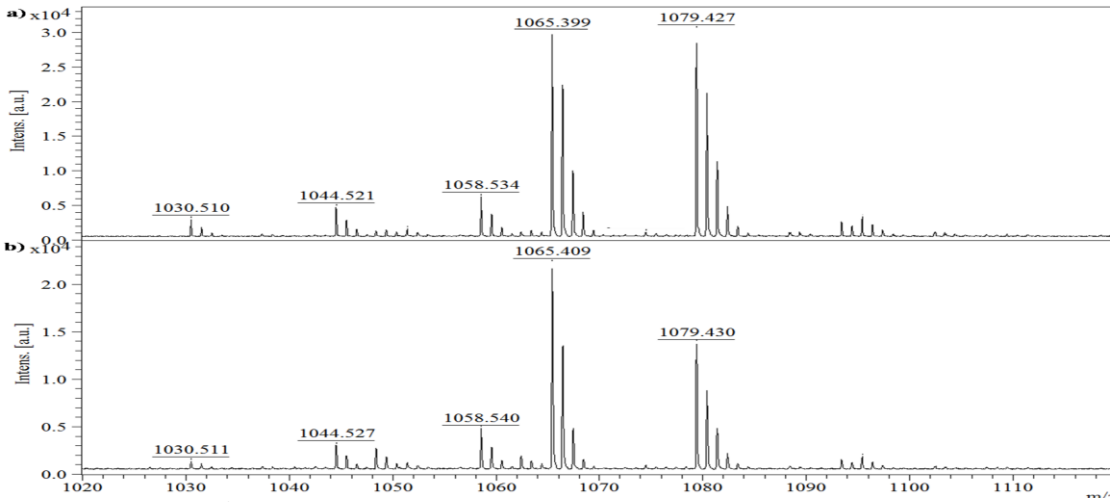


**Fig. 6:** Surfactin Homologous Detected by ESI-Q-TOFMS; Five Main Peaks (A) AGG; (B) CGBP.

**Table 3:** Surfactin Homologous

| | β-HFA* | AGG | CGBP |
|---|---|---|---|
| Homolog I – 1030.510 | C-12 | | |
| Homolog II – 1044.521 | C-13 | ELLMDLD† | ELLMDLD |
| Homolog III – 1058.399 | C-14 | | |
| Homolog IV – 1065.399 | C-16 | ELLLDLL** | ELLLDLL |
| Homolog V – 1079.427 | C-17 | | |

* β-hydroxyl fatty acids

† E - Glutamic acid; L – Leucine; M – Methionine; D – Aspartic acid

** E - Glutamic acid; L – Leucine; M – Methionine

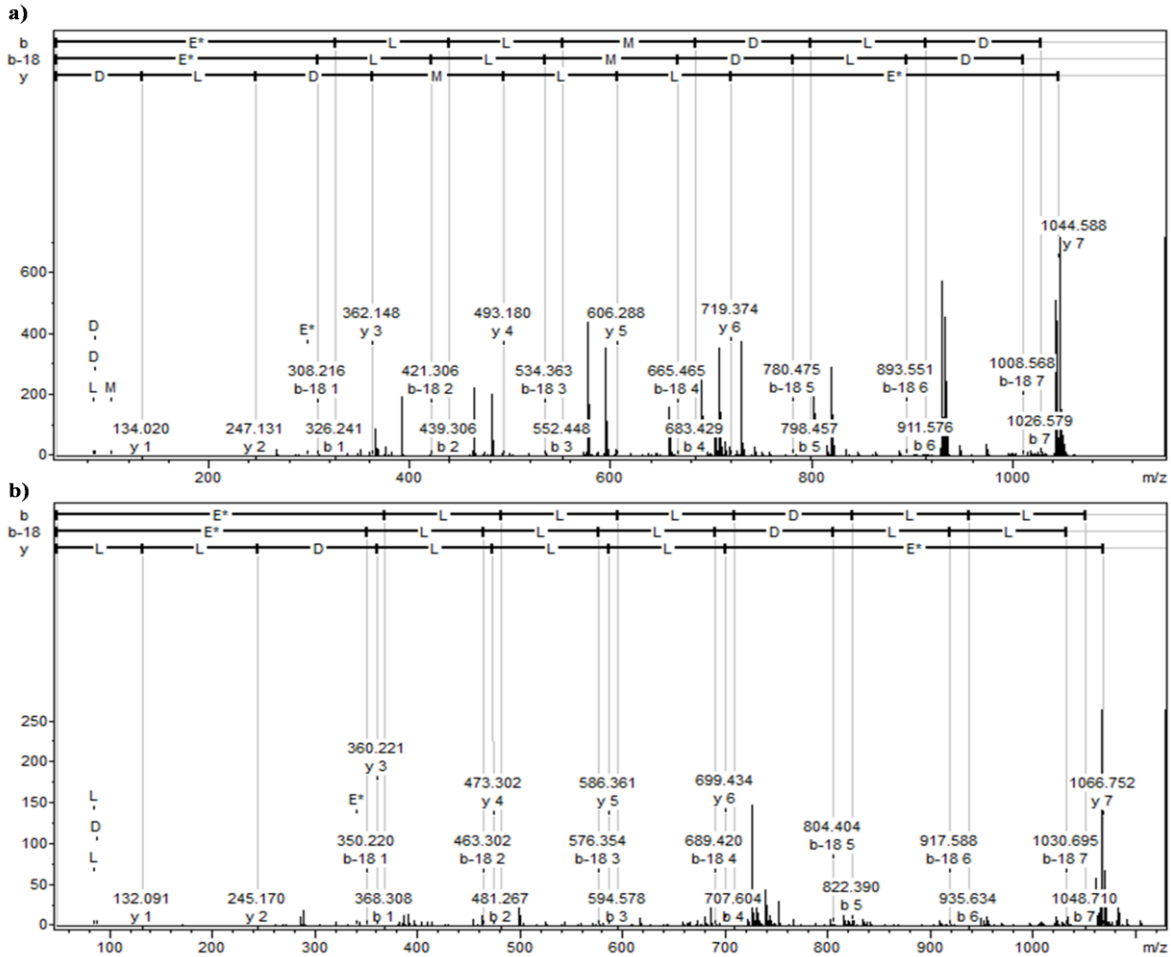


**Fig. 7:** MSMS Spectra By ESI-Q-TOFMS for Determination of Amino Acid Sequences by Using B, B-18 and Y Fragments (A) CGBP 1044.521 (M/Z); (B) CGBP 1065.409.

Our results however suggest that the impurities from biodiesel production (eg: methanol) do affect the productivity of surfactin, however do not affect the production of surfactin homologous.

## 4. Conclusions

Flask experiment data indicated a negative influence of impurities (present on by-product from biodiesel industry) on growth of B. subtilis LB2b. However, good growth and biosurfactant production were obtained using a medium comprised of peptone and AGG, which was scaled up to a bench-scale bioreactor (3.5 L working volume). Higher surfactin production (4.6 times) was obtained with glycerol of highest purity (AGG) than with lower purity glycerol from biodiesel industry (CGBP). However no difference in glycerol consumption was observed. Significant differences were observed on the purity (protein concentration) of the final product, which may be associated to the effect of impurities on metabolic pathways of protein and/or surfactin production. The semi-purified biosurfactant from AGG contained ≈ 4 times more homologous of surfactin than semi-purified biosurfactant from CGBP. Therefore, the downstream processing of biodiesel derived glycerol should provide a product with a purity level equivalent to that of AGG when used as fermentation medium for the production of surfactin to increase productivity.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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