Bioconversion of low-cost brewery waste to biosurfactant: an improvement of surfactin production by culture medium optimization

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Abstract

Trub is the second-largest residue from brewing industry (250 thousand tons/year) and, due to the presence of high amounts of carbon and nitrogen, it is a potential source of reducing sugars and nitrogen for biological processes. Therefore, this work aimed to enhance surfactin production by *B. subtilis* using Trub as a carbon source. The effect of nitrogen sources on biosurfactant production was investigated by a Full Factorial Design (2^2). Yeast extract and its interaction with peptone presented a statistically significant effect on the response variable of surface tension (ST). ST was described by a linear regression model (R^2 = 0.93). The effect of metals supplementation on the culture medium was also investigated, where the volumetric productivity increased from 2.98 to 7.43 mg L^{-1} h^{-1} for the medium without (M-I) and with metals (M-II), respectively. After 28h, 210.11 mg L^{-1} was obtained in the batch culture with M-II medium, being higher than the maximum surfactin concentration achieved after 52 h (121.80 mg L^{-1}) for M-I medium. The biosurfactant production in bioreactor was successfully performed since a concentrated extract was obtained in the foam collected, reaching the maximum of 1118.20 mg L^{-1} of surfactin.

Keywords: Brewery waste, Trub, surfactin, biosurfactant, bioreactor

1. Introduction

Biotechnology has stood out in the industrial scenario due to the necessity of processes that not only aim for a better production yields with low costs, but which aim at reducing environmental impacts, including the reduction or reuse of waste generated. Hence, the development of new biotechnologies to obtain products that already exist in the market has been intensified since bioprocesses have less environmental impacts compared to classic industrial processes.

Surfactants are a class of chemicals used in various processes in the current industry, involving wetting, dispersing, emulsifying, foaming, and anti-foaming. Most of the commercial surfactants available are derived from petroleum. However, environmental awareness among consumers and stricter legislation have boosted the demand for biological surfactants as potential alternatives to existing products [1].

Biosurfactants have several advantages over synthetics, being environmentally friendly, biodegradable, less toxic, and non-hazardous. In addition, they present better foaming properties and higher selectivity [2]. Due to their properties, biosurfactants present anti-microbial/antifungal activity [3, 4, 5], anti-biofilm activity [6, 7], and applications in bioremediation [8].

The global market of biosurfactants exceeded US$ 1.5 Billion in 2019 and US$ 2.5 Billion is expected for the year 2026 [9]. In addition, it is estimated that Europe becomes a leader in the consumption of microbial biosurfactant products (around 53%) followed by the United States [10].

Although biosurfactants are already inserted on industrial scale, their production is limited due to the high cost of production. Low-cost substrates, high-production strain selecting, culture medium optimization, and fermentation process improvement are alternatives that can be used to reduce production costs [11]. The substrate, which represents 30-50% of the final product value, and purification processes, are the major expense on biosurfactant production [10, 12, 13]. In addition, surfactin, which is a powerful biosurfactant mainly concerning high surface tension reduction capacity, has not been used or produced on a commercial scale yet [12]. Regarding this, low-cost substrates are necessary to enable biosurfactants production, especially surfactin. In

recent years, several studies have focused on the use of agro-industrial waste as an attractive alternative to the traditional substrates [14, 15, 16, 17].

Beer production has great worldwide representation, both in industrial scale and its production in handcrafted form. According to Dias and Falconi [18], Brazil is the third largest manufacturer country in the world, with 14 million kL year^{-1} produced, being only behind China (39.788 kL year^{-1}) and the United States (21.775 kL year^{-1}). Thus, the waste generated during beer production deserves attention, through the reuse in the manufacture of new products and technologies.

Different types of waste are generated during the brewing process, such as brewing spent grain, Trub and residual brewer's yeast. In the fermentation process, Trub is formed during boiling of the wort and, along with the other brewery by-products, it is discarded. This waste is mainly composed of coagulated proteins (between 50 and 70% of the dry mass) and reducing sugars (20%), which are related to the high carbon concentration contained in this residue. In general, between 0.2 to 0.4 kg of wet Trub (80 to 90% humidity) is formed for each hectoliter of beer produced [19]. Since brewery waste has a rich composition in organic compounds with significant nutritional power, its use as a carbon and nitrogen source in the production of biosurfactants becomes a viable proposal to overcome the impasses related to high process costs.

The optimization of the culture medium and the growth conditions is primordial to increase the biosurfactant production yield. Hence, it is relevant to identify the factors that influence production, such as culture medium compounds and operational conditions (temperature, pH, agitation, and aeration), in which the design of experiments (DoE) is a powerful tool to optimize fermentative processes [20, 21], being frequently used in the optimization of biosurfactant production. Mishra et al. [22] evaluated rhamnolipid production using a central composite design (CCD), where carbon source, pH, and incubation time were employed as independent variables. Pathania and Jana [23] investigated rhamnolipid production, where carbon and nitrogen sources were evaluated through a CCD as well.

The presence of metal ions (Mn^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+} and Zn^{2+}) in the culture medium for surfactin production has been investigated in the last years, where Mn^{2+} is the ion that has the greatest effect on increase surfactin

productivity [24]. On the other hand, potassium ion stimulates surfactin secretion and may increase total surfactin production as well as *B. subtilis* mobility [25].

In this work, we aimed to produce biosurfactant utilizing a low-cost substrate (Trub) and evaluate the impact of nitrogen sources and the supplementation of the culture medium with metallic solutions on the production. The biosurfactant production in a bench-top fermenter was investigated as well. This work provides evidence of the improvement of biosurfactant production due to culture medium optimization, contributing to the viability of biosurfactant production and the sustainable development of the beer industry.

2. Materials and methods

2.1. Brewery waste

Trub (pH 5.73) was kindly provided by Kairós Craft Beer Factory (Florianópolis, SC, Brazil). Trub composition (Table 1) was determined by elemental analysis (Total Organic Carbon Analyzer, SHIMADZU, TOC-V_{CPH}, Japan), enzymatic-colorimetric method (Total Nitrogen, TN, Brazil), and by Atomic Absorption Spectrometry (Absorption Spectrophotometer, SHIMADZU, AA-6300, Japan) for quantification of metals (Fe, K, Mg, and Mn).

2.2. Microorganism and pre-culture

Bacillus subtilis ATCC 6051, purchased from the Culture Collection of the Tropical Foundation for Research and Technology André Tosello (Campinas, SP, Brazil), was stored in Nutrient Broth (NB) with glycerol (20%) at $-80°$C. The pre-culture (inoculum) was prepared by adding 2 mL from frozen stocks into 50 mL of NB, being subsequently incubated at 30°C and 160 rpm. The inoculum was standardized by adjusting its absorbance at 600 nm to 0.85 (UV-VIS Spectrophotometer, FEMTO, Cirrus 80, Brazil).

Biomass concentration was expressed in dry mass $\left(\mathrm{g\,L^{-1}}\right)$ after determination of the absorbance value (600 nm) and its correlation with the dry mass by a calibration curve (R^2 = 0.9981). At the end of the batch, broth aliquots had their absorbance

value measured and, when necessary, the broth was diluted to fit the calibration curve interval.

2.3. Biosurfactant production

The biosurfactant production was performed in 250 mL erlenmeyer flasks containing 100 mL of culture medium. The flasks were inoculated with the standardized culture at a concentration of 5% (v/v). The medium was composed by $\left(\mathrm{g\ L^{-1}}\right)$: $CaCl_2$ (0.1), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5), NaCl (0.1), and Trub (2%, v/v). Moreover, peptone and yeast extract were added to the medium at different concentrations, according to the Full Factorial Design (2^2), where two factors were evaluated: Yeast Extract (YE) and Peptone (PB). The levels were selected based on Maass et al. [26]. The levels of factors were, in g L^{-1}: 2.0 (-1) and 12.0 (+1) for YE, 0.4 (-1) and 1.4 (+1) for PB. Surface Tension (ST) was adopted as a response variable and two central points (CP) were also incorporated into the design. All experiments were performed under aseptic conditions during 72 h at 30°C, in duplicates. The stirring rate was maintained at 200 rpm and the initial pH was adjusted to 7.0.

At the end of assays, the final pH was measured and the culture broth was centrifuged (9000 rpm) for 20 min to remove cells and solid particles from Trub. The supernatant had its pH adjusted to 2.0 by adding HCl solution (4.0 M) and left overnight under refrigeration (4°C) for precipitate formation. The precipitated biosurfactant was centrifuged (9000 rpm, 20 min), washed twice with acidified water (pH 2.0) and resuspended in Milli-Q® water (Millipore, USA) [26].

The biosurfactant present in the foam collected was recovered in the same way as previously described, where the content from the foam collector coalesced, and the remaining liquid was centrifuged, followed by acid precipitation.

2.4. Supplementation of culture medium with metals solutions

The influence of metals on biosurfactant production was evaluated by the addition of iron, potassium, magnesium, and manganese solutions into the culture medium. Three different concentrations (mM) were evaluated for each metal, as follows: $FeSO_4$ (0.008, 1.2 and 4.0), KH_2PO_4 (5.0, 10.0 and 30.0), $MnSO_4$ (0.01, 0.1 and 0.3), and

$MgSO_4$ (0.04, 0.6 and 2.4). The experiments were carried out in triplicate. First, the concentrations of each metal were evaluated by their addition to the culture medium, one at a time. After the definition of the best concentration for each metal, the culture medium was supplemented with all metals at their best condition aiming to evaluated synergetic effects on biosurfactant production. The concentrations were defined according to the works done by Cooper et al. [27], Wei and Chu [28], Wei et al. [25], Gudiña et al. [29], and Huang et al. [24].

2.5. The production of biosurfactant in stirred tank bioreactor

Biosurfactant production was performed in a 5 L stirred tank bioreactor (Fermenter, BIO-TEC-FLEX, Tecnal, Brazil) equipped with pH and pO_2 electrodes (Mettler-Toledo International Inc., USA) and 2 L of working volume. The airflow rate was adjusted 1.0 vvm and the temperature and stirring rate were maintained at 30°C and 225 rpm, respectively. These conditions were based on works done by Yeh et al. [30], Amani et al. [31], and Joshi et al. [32]. The pH was maintained at 7.0 by the addition of HCl and NaOH solutions, both at 1 M. The inoculum (OD_{600} 0.85, 10% v/v) was added to the culture medium and the process lasted 24 h. The excessive foam was channelled through an upper reactor outlet, being collected in a sterile foam collector coupled to the fermenter.

2.6. Determination of Surface Tension (ST)

The ST of the cell-free supernatant was measured using a digital Tensiometer (Tensiometer, KSV, Sigma 702, Finland) by the Wilhelmy platinum plate method. All measurements were performed in triplicate at 25 $\pm$ 2°C.

2.7. Determination of substrate concentration (Trub)

The substrate concentration was determined by the Phenol-Sulfuric calorimetric method [33], aiming to quantify sugar content in trub. Glucose was used for the preparation of the standard curve (R^2 = 0.9975), and the absorbance of the samples was measured in a spectrophotometer (UV-VIS Spectrophotometer, FEMTO, Cirrus 80, Brazil) using a wavelength of 490 nm.

2.8. Determination of biosurfactant concentration

The determination of the biosurfactant concentration (Bs, mg L^{-1}) was carried out through the relationship between Bs and ST. A calibration curve [34] for standard surfactin (Sigma-Aldrich, 98%) provides the following equations: $ST = 100.85e^{-0.345[Bs]}$ (R^2 = 0.989), for ST values between 72.73 and 42.73 mN m^{-1}, and $ST = -1.455\,[Bs] + 45.2$ (R^2 = 0.953), for values up to 31.05 mN m^{-1}.

2.9. Statistical analysis

Statistical analysis was performed using STATISTICA® 13.5 software. Analysis of variance (ANOVA) and lack of fit test were employed to verify the adequacy of the empirical regression model. In addition, the results were analysed by Tukey's test at a significance level of 5%.

3. Results and discussion

The biosurfactant produced in this study was identified, by Maldi-ToF-MS, in our previous work [35] as lipopeptide with a mixture of structural analogs from surfactin class. Nazareth et al. [35] evaluated the ability of the biosurfactant, produced in this work, as antimicrobial and antibiofilm agents.

3.1. Effect of nitrogen sources on the biosurfactant production using Trub as a carbon source

Biosurfactant production is affected by culture medium, in which carbon, nitrogen and trace element sources are essential to promote the production [36, 37]. Hence, the evaluation and optimization of the culture medium for biosurfactant production has been the target of investigations in the literature [20, 25, 38].

In this work, we evaluated the influence of nitrogen sources in biosurfactant production by *B. subtilis* using Trub as an alternative carbon source. A Full Factorial design (2^2) was elaborated having yeast extract (YE) and bacteriological peptone (PB) as independent variables in the design. The levels of each variable, as well as the results for all assays, are presented in Table 2.

The highest biosurfactant concentration obtained was 96.56 mg L^{-1} in assay 4, and similar values were obtained for runs 2, 7, and 9 (Table 2). Note that yeast extract concentration was at the positive level for the mentioned tests, suggesting that YE improved biosurfactant production. Studies in the literature also shown an enhancement of biosurfactant production by *B. subtilis* when cultivated in medium containing organic nitrogen source. Ponte Rocha et al. [39] evaluated biosurfactant production by *B. subtilis* LAMI008 and clarified cashew juice as a carbon source. The authors studied the influence of YE supplementation on cell growth and surfactin production. The reduction of ST was more effective when using YE in the medium, ranging from 58.95 to 38.10 mN m^{-1}. Alvarez et al. [40] also investigated surfactin production by *Bacillus subtilis* in Trypticase Soy Broth (TSB) at 32°C and 170 rpm. The strain produced 99.6 mg L^{-1} of surfactin.

Sharma and Pandey [41] evaluated the influence of different nitrogen sources (yeast extract, iron nitrate, ammonium sulfate, ammonium nitrate, and urea) on cell growth and biosurfactant production in a medium composed of waste crude oil as carbon source. The authors observed that YE (1.5% w/v) promoted the highest cell growth of *B. subtilis* RSL2 and a reduction of ST to 40 mN m^{-1}. In a similar investigation, Parthipan et al. [42] evaluated the influence of nitrogen sources (ammonium nitrate, ammonium phosphate, ammonium sulfate, ammonium chloride, peptone, potassium nitrate, yeast extract, and urea) on biosurfactant production by *B. subtilis* A1 using sucrose (2% w/v) as a carbon source. Optimum growth and lipopeptide production were achieved when YE acted as a nitrogen source (3% w/v).

The highest biomass concentration was achieved at the central point (Table 2), suggesting this is the best condition for microbial growth. Nitrogen sources, such as YE, increase cell growth by 25% due to the presence of amino acids and other cell growth-stimulating compounds. In addition, the presence of complex B vitamins is necessary to synthesize enzymes and coenzymes, also contributing to cell growth [39].

According to Wacker et al. [43], bacteria can optimize their metabolism through regulatory systems that allow the sequential use of available carbon and nitrogen sources. In metabolism, glycolysis is induced by glucose. However, a complete induction occurs only in the presence of available amino acids, present in nitrogen sources. Amino

acids are precursors in the synthesis of the surfactin heptapeptide group, making nitrogen metabolism essential in the production of the lipopeptide.

The effect of factors on the response variable was analyzed in the Pareto chart (Figure 1), showing that only YE had a significant effect on the ST. This fact was also confirmed by analysis of variance (ANOVA) since the *p-value* is below 0.05 (Table 3). The interaction between both independent variables was also statistically significant, indicating that the synergistic effect between the factors may improve biosurfactant production.

The experimental data were adjusted to the linear regression model and validated by ANOVA, as shown in Table 3. The model presented a coefficient of determination (R^2) of 0.93345 and adjusted coefficient of determination (R^2Adj) of 0.90018, *i.e.* 93% of the variations are explained by the model. Moreover, the *lack of fit* test showed insignificant result, indicating agreement between the experimental values and those predicted by the model. Thus, the model is adequate to describe the response variable as a function of the independent variables and is represented by Equation 1:

$$\mathrm{ST} = 29.14155 - 0.07540\,(\mathrm{YE}) + 0.93800\,(\mathrm{PB}) - 0.09650\,(\mathrm{YE} \times \mathrm{PB}) \qquad (1)$$

From the linear regression model, it was possible to plot the response surface and the contour lines as a function of YE and PB. In the surface graph (Figure 2a), it was observed that smaller values of ST were obtained for YE above 9 g L^{-1}. Figure 2b shows the response tendency of the dependent variable. When these lines do not present curvature, there is no interaction effect between the independent variables [44]. Clearly, there was an interaction between the factors due to the curvature in contours (Figure 2b), in which the region that has the lowest ST values corresponds to YE between 8 and 14 g L^{-1}, for any corresponding PB concentration.

According to the analyses presented for the Full Factorial Design (2^2), a kinetic study (Figure 3) of biosurfactant production was evaluated using Central Point (CP) conditions. The medium adopted in this condition will be called M-I from now. The M-I was composed by (g L^{-1}): $CaCl_2$ (0.1), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5), NaCl

(0.1), Yeast extract - YE (7.0), Peptone - PB (0.9), and Trub (2%, v/v). The conditions of the central point (CP) were adopted since considerably lower reagent concentrations would be required to achieve the surfactin concentration close to the obtained in test 4, which corresponds to the maximum value (Test 4: 96.56 mg L^{-1} of surfactin; CP: 90.31 mg L^{-1} of surfactin).

Figure 3a shows a significant increase in biosurfactant concentration (121.20 ± 1.98 mg L^{-1}) after 40 h of cultivation, reaching a maximum concentration of 121.80 ± 2.26 mg L^{-1}. In addition, a production profile associated with cell growth was observed since biosurfactant production was directly proportional to microbial growth. In *Bacillus* ssp., the expression of the surfactin genes occurs mostly in the transition from exponential to stationary growth phase and is associated with increased cell densities [45]. From Figure 3b, it is noted that the critical micelle concentration was reached between 28 and 40 h of cultivation, and the pH remained close to 8.0 after 40 h.

A similar result was also reported by Vedaraman and Venkatesh [46], in which critical micelle concentration was achieved after 48 h. The authors evaluated biosurfactant production by *B. subtilis* MTCC 2423 in a medium composed of mineral salts and glucose (50 g L^{-1}), producing 983 mg L^{-1} of surfactin. This value is quite higher than this work, probably due to the high glucose concentration when compared to our work. de Sousa et al. [47] also reported a similar surfactin production to our work, achieving approximately 120 mg L^{-1} after 48 h. The authors aimed to evaluated biosurfactant production by *B. subtilis* ATCC 6633 using glycerol (2% v/v), obtained from the biodiesel production process, as the main carbon source.

3.2. Effect of culture medium supplementation with metals solutions for biosurfactant production

The influence of metals supplementation (Mg^{2+}, K^{+}, Mn^{2+}, and Fe^{2+}) to improve biosurfactant production was evaluated in this work. Solutions of $FeSO_4$, KH_2PO_4, $MnSO_4$, and $MgSO_4$ were added individually to the culture medium and, for each metal, three different concentrations were evaluated.

Low ST values were obtained for all solutions tested, even considering the dilution (1:10, ST^{-1}) (Figure 4). The addition of $FeSO_4$ solution (1.2 mM) resulted in the

lowest ST. A similar effect was reported by Wei et al. [48], in which an improvement in surfactin production by *B. subtilis* ATCC 21332 was achieved when used iron-enriched medium. The authors reported that the addition of Fe^{2+} (4.0 mM) led to an 8-fold increase in cell concentration and 10-fold in surfactin yield when compared to the assay without Fe^{2+} supplementation. According to Wei et al. [48], there are two possible mechanisms for the iron role in surfactin production by *B. subtilis*. Surfactin has a chelation effect in iron ions, which may severely decrease the bioavailability of Fe^{2+}. Thus, a high Fe^{2+} supplementation needs to be done for regular operation of cellular function. On the other hand, *B. subtilis* might presents defective in the Fe^{2+} transport system, which demand high concentrations of Fe^{2+} to ensure its efficient uptake.

A new approach, involving the use of iron nanoparticles to increase surfactin production, has been reported in the recent literature. Yang et al. [49] reported that Fe nanoparticles improved the expression of genes involved in surfactin biosynthesis and increased the permeability of cell membranes, allowing the secretion of surfactin more efficiently. Their results showed an increase in surfactin production from 4.93 to 7.15 g L^{-1} in shake flask, and from 5.94 to 9.18 g L^{-1} in a bioreactor by adding 5 g L^{-1} Fe nanoparticle. Modabber et al. [50] evaluated surfactin production using three different media (medium containing starch-coated Fe^0 nanoparticles, starch-coated Fe^{3+} nanoparticles, and culture medium without nanoparticles). The study revealed that the culture medium containing Fe^{3+} nanoparticles (1 mg L^{-1}) had higher biomass and surfactin production and lower surface tension compared to the culture medium without Fe^{3+} nanoparticles.

For a more accurate study, analysis of variance (ANOVA) was performed to verify if the ST^{-1} values for each metal assay differed statistically from the ST^{-1} values of the control sample (without metals addition, M-I medium), as shown in Table 4. Note that all evaluated metals can be considered different from the control sample since the concentrations studied for each metal had a significant effect.

On the other hand, a Tukey test was performed (data not shown) with a 95% confidence level to evaluate the impact of each concentration in ST. The results indicated that the concentrations of $MnSO_4$ solutions did not differ from each other. A similar fact could be observed for the concentrations of $MgSO_4$ and KH_2PO_4 solutions,

which suggests the use of lower concentration for these compounds to reduce the use of reagents. The concentrations of 0.008 and 1.2 mM of $FeSO_4$ solutions differ from each other in terms of impact on ST, suggesting the use of 1.2 mM since it presented a lower ST.

According to Wei et al. [25], the addition of metals individually to the culture medium does not always lead to maximum biosurfactant production, and the synergistic effect may be greater than the individual effect of the factors. The authors got this conclusion after evaluated surfactin production in MSM (Mineral Salt Medium) with glucose and trace elements (K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, and Mn^{2+}). Each trace element was removed from MMS one at a time. The authors found that without Mg^{2+} or K^+, the surfactin yield presented 25% of the value obtained from the control assay (Full MSM) and 33% in the absence of Mn^{2+} and Fe^{2+}. Without only Fe^{2+} or Mn^{2+}, the surfactin productivity was close to control, achieving 80% of the control value, suggesting the correlation between metals is important to increase surfactin yield.

In order to verify the presence of synergistic effects to improve biosurfactant production, the process behaviour was evaluated by adding solutions of all metals in the culture medium (Figure 5). Thus, based on the ANOVA (Table 4) and ST^{-1} values (Figure 4), a 76 h batch was performed, adding to the culture medium (M-I) metals solutions with the following concentrations: $FeSO_4$ (1.2 mM), KH_2PO_4 (5 mM), $MnSO_4$ (0.01 mM) and $MgSO_4$ (0.04 mM). The medium supplemented with metals was called M-II.

Figure 5a shows that a high biosurfactant concentration (210.11 mg L^{-1}) was obtained after 28 h of cultivation, and this value is almost two times higher than the maximum surfactin concentration (121.80 mg L^{-1}) obtained in the assay without the presence of metals in the medium (Figure 3) after 52 h.

Similarly to our work, Gudiña et al. [29] reported a positive effect on biosurfactant production when metals solutions (iron, manganese, and magnesium) were added to the culture medium containing Corn steep liquor (CSL) as a carbon source. The results showed an increase in biosurfactant production, being 3.6 times higher than the production in a culture medium without supplements.

Moreover, the critical micelle concentration - CMC (Figure 5b), characterized by

the formation of micelles, was achieved between 9 and 15 h of process, while between 28 and 40 h were required (Figure 3b) in the kinetics without the presence of metals in the medium (M-I medium). This result is quite interesting since there was a significant increase in biosurfactant concentration in a short time. This fact may have occurred due to the addition of Mn^{2+} to the culture medium. Huang et al. [24] evaluated the role of Mn^{2+} in promoting surfactin production by *B. subtilis* ATCC 21332, revealing that the yield of surfactin increased upon Mn^{2+} addition. The authors reported that the glutamate synthase activity was improved by Mn^{2+} addition, which is responsible for increasing nitrogen transformation and absorption, and also for shortening the cycle duration in continuous-phased growth of *B. subtilis*.

According to Sen [51], the concentrations of $MnSO_4$ and $FeSO_4$ have a direct relationship with surfactin production. In addition, through the Taguchi method, the author found out that the interaction between $MnSO_4$ and $FeSO_4$ is strongly significant. Makkar and Cameotra [52] state that metal supplementation significantly affects cell growth and biosurfactant production by *B. subtilis* MTCC 2423 and the effect of multiple use of metal cations on the medium is more effective in producing biosurfactant than using them individually.

Additional parameters were evaluated to provide important process information. Volumetric productivity for M-I medium was 2.98 mg L^{-1} h^{-1} and for M-II was 7.43 mg L^{-1} h^{-1}. Thus, the biosurfactant production in M-II medium was almost three times higher than to the process with M-I medium. This demonstrated that the supplementation of culture medium with metals is pertinent for biosurfactant production.

After 28 h of fermentation, $Y_{P/S}$ for M-II was 0.125, and for M-I was 0.0032. This result suggests that the substrate was directed towards product formation more efficiently in the M-II medium. Nevertheless, $Y_{X/S}$ for M-II was 1.39, which indicates that the carbon source was preferably directed to biomass formation. The $Y_{X/S}$ for M-I was 0.55.

3.3. Biosurfactant production in stirred tank bioreactor

After the optimization of the culture medium for biosurfactant production in shake flasks, the potential scale-up in a stirred tank bioreactor with M-II medium was evalu-

ated. Culture conditions were maintained at 30°C and pH 7.0 by automatic control. In addition, this study sought a viable alternative to deal with foam formation in surfactin production through a sterile foam collector. A time-course of biosurfactant production with stirring rate and aeration rate of 225 rpm and 1.0 vvm, respectively, is shown in Figure 6, where the values presented refer to the data obtained from the culture broth inside the reactor, disregarding the contents of the foam collection vessel.

The sufficient supply of dissolved oxygen and mechanical agitation can improve lipopeptide production [21, 32]. Oxygen transfer in aerobic process is essential for cell maintenance, where an efficient agitation and aeration system is required for oxygen transfer in the gas phase to the liquid phase, allowing the oxygen to reach the cells in suspension [53]. The feasibility of surfactin production in bioreactors, as well as the adoption of strategies to combat excessive foaming during the process, were reported by Davis et al. [54], Chen et al. [55], and Willenbacher et al. [56].

In this work, the biosurfactant production was successfully performed on a larger scale, and the foam collecting was effective to provide an enriched surfactant solution (1118.20 mg L^{-1}), being 193 times higher than surfactin present into the fermenter broth (5.79 mg L^{-1}) after 24 h. This value was higher than reported by Chen et al. [55], in which surfactin concentration in the foam collected was 50 times higher than in the fermenter broth. In addition, considering the working volume, 137.54 mg L^{-1} of surfactin was achieved at the end of fermentation.

Davis et al. [54] reported that foam collecting is an efficient method for the recovery of surface-active compounds from culture broth since their results were promising in terms of enrichment and recovery of surfactin produced by *B. subtilis* ATCC 21332 in a medium containing 4% glucose. These systems can also be useful for processes under severe foaming conditions, avoiding the addition of costly antifoam agents, which may also inhibit cell growth [30]. Furthermore, the removal of biosurfactant by foam fractionation improves the production of biosurfactant, preventing product inhibition in the process [55].

The cell growth showed a similar profile to the process performed in shake flasks, presenting the highest concentration (2170.40 ± 19.22 mg L^{-1}) after 24 h of fermentation (Figure 6). The dissolved oxygen (pO_2) decreased from 100% to 23% after 9

h of fermentation (Figure 6), suggesting that the oxygen was being consumed at high rates due to the intense cell growth. The pO_2 increased back to nearly 78% at 15 h, remaining close to 90% at the end of the process.

4. Conclusions

Our finding reveals that Trub was suitable for surfactin production by *B. subtilis* ATCC 6051. Yeast extract had a significant effect on ST, suggesting that higher concentrations of YE are associated with an improvement in biosurfactant production. Furthermore, the addition of combined metals in the culture medium was effective in increasing surfactin production in a short time, in which volumetric productivity was 7.43 mg L^{-1} h^{-1} for a medium supplemented with metals, and for a medium without metals was 2.98 mg L^{-1} h^{-1}. The production in the bench-top fermenter was performed, providing a concentrated product.

Declaration of Competing Interest

None.

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Figure Captions

Figure 1. Pareto chart for ST according to the statistical analysis of the Full Factorial Design (2^2) carried out to evaluate the effect of YE and PB concentrations in culture medium for the biosurfactant production.

Figure 2. Response surface (a) and the corresponding contour lines (b) from a Full Factorial Design (2^2), considering surface tension (ST) as response variable, and yeast extract (YE) and peptone (PB) as factors.

Figure 3. Time-course of biosurfactant production in M-I medium at 30°C and initial pH 7.0. (a) Values as a function of surfactin concentration and (b) as a function of ST. S: substrate concentration (Trub), X: biomass concentration.

Figure 4. Surface tension (ST^{-1}, 1:10) values for individual addition of metals solutions into the culture medium for biosurfactant production by *B. subtilis* ATCC 6051.

Figure 5. Time-course of biosurfactant production in shake flasks at 30°C and initial pH 7.0. The culture medium was supplemented with metals solutions (M-II medium). (a) Values as a function of biosurfactant concentration and (b) as a function of ST. S: substrate concentration (Trub), X: biomass concentration.

Figure 6. Time-course of biosurfactant production in bioreactor at 30°C and pH 7.0. (a) Values as a function of surfactin concentration and (b) as a function of ST. S: substrate concentration (Trub), X: biomass concentration.

Table List

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Table 1: Composition of Trub.

Component	**Concentration** $\left(\mathbf{mg\ L^{-1}}\right)$	**Method**
Fe	< 0.14	Atomic Absorption Spectrophotometer (AA 6300 – SHIMADZU, Japan)
Mn	0.3772	
K	426.72	
Mg	74.53	
TOC*	31.50	Total Organic Carbon Analyzer (TOC – SHIMADZU, Japan)
TN*	3.45	Enzymatic-Colorimetric (Kit Gold Analisa Diagnostics, Brazil)

*In g L^{-1}. TN: total nitrogen; TOC: total organic carbon [35].

Table 2: Full Factorial Design (2^2) for biosurfactant production with surface tension (ST) as response variable. The experiments were performed in duplicates with two central points.

Run	**YE**		**PB**		**ST**	**Biosurfactant**	**Biomass**	**pH_{final}**
	g L^{-1}	Level	g L^{-1}	Level	(mN m^{-1})	(mg L^{-1})	(mg L^{-1})	
1	2	(-1)	0.4	(-1)	29.61	18.43	1587.85	8.11
2	12	(+1)	0.4	(-1)	28.13	94.36	1916.22	8.00
3	2	(-1)	1.4	(+1)	30.01	20.01	1593.14	8.21
4	12	(+1)	1.4	(+1)	27.98	96.56	1895.04	7.91
5	7	(0)	0.9	(0)	28.66	85.98	2726.56	8.41
6	2	(-1)	0.4	(-1)	29.12	20.69	1662.00	8.16
7	12	(+1)	0.4	(-1)	28.32	92.71	1953.30	7.85
8	2	(-1)	1.4	(+1)	30.21	20.22	1614.33	8.09
9	12	(+1)	1.4	(+1)	28.03	92.03	1884.44	7.85
10	7	(0)	0.9	(0)	28.43	90.31	2112.19	8.19

YE: Yeast Extract; PB: Peptone.

Table 3: ANOVA for fitted model of the surface tension (ST) as a function of studied variables (Yeast Extract and Peptone) for biosurfactant production.

Source of variation	**SS**	***df***	**MS**	**F**	***p***
Yeast Extract (YE)	5.265013	1	5.265013	141.6849	0.000074
Peptone (PB)	0.137813	1	0.137813	3.7086	0.112095
YE x PB	0.4656	1	0.4656	12.5299	0.016567
Lack of fit	0.232562	1	0.232562	6.2584	0.054378
Pure error	0.185800	5	0.037160		
Total SS	6.286800	9			

Table 4: ANOVA for ST^{-1}. Assays with different metals concentrations in the culture medium for biosurfactant production.

Source of variation	**SS**	***df***	**MS**	**F**	***p-value***
Between treatments	11271.36	1	11271.36	21524.61	0.0000
[$FeSO_4$]	7.15	2	3.57	6.83	0.0011
Error	3.14	6	0.52		
Between treatments	10577.09	1	10577.09	12413.01	0.0000
[KH_2PO_4]	10.06	2	5.03	5.90	0.0045
Error	5.11	6	0.85		
Between treatments	10871.19	1	10871.19	45426.49	0.0000
[$MnSO_4$]	2.52	2	1.26	5.26	0.0013
Error	1.44	6	0.24		
Between treatments	11639.89	1	11639.89	35283.39	0.0000
[$MgSO_4$]	2.97	2	1.48	4.49	0.0043
Error	1.98	6	0.33		