CHEMICAL ENGINEERING TRANSACTIONS
VOL. 43, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-34-1; **ISSN** 2283-9216
DOI: 10.3303/CET1543047

Biosurfactant Production by *Bacillus subtilis* Using the Residue from Processing of Pineapple, Enriched with Glycerol, as Substrate

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The growing commitment to environmental issues, along with new legislations have made the use of synthetic surfactants become unviable by the chemical industry. Thus, the development of new technologies to produce biosurfactants increased considerably. Biosurfactants are groups of chemical compounds produced by bacteria, fungi and yeasts by biodegradation of renewable raw materials. The use of natural surfactant has become a very attractive alternative to replace synthetic surfactants due to their biodegradability, structural diversity, low toxicity and because they generate less environmental impact. Most of studies related to this topic is focused on identifying potential surfactants, in the evaluation of their properties and optimization of fermentation processes for their production by microorganisms. This study aimed to the production of biosurfactants by *Bacillus subtilis* using the residue from processing of pineapple enriched with glycerol as substrate, at 37 °C. Three different concentrations of glycerol, 3 %, 5 % and 10 % were used and performed in the fermentation process. It was observed that the most significant results in reducing the surface tension and hence in higher production of biosurfactants, was performed in substrate containing 3% glycerol, which was obtained 23 % of surface tension reduction during fermentation for 24 hours and increased emulsifying activity up to 62.5 %.

1. Introduction

Surfactants are widely used in various industrial aspects, mainly in the chemical, petroleum, pharmaceutical and food industries. They are chemical compounds consisting of two portions, one hydrophobic and one hydrophilic, allowing them to act at the interfaces between aqueous and non aqueous components. The hydrophobic portion is usually a hydrocarbon chain, while the hydrophilic portion can be ionic, nonionic or amphoteric (Nitschke and Pastore, 2002). The wide use in different industrial branches is also because the surfactants possess the tendency to form aggregates called micelles. Micelles are formed at low concentrations of water and there is a minimum concentration necessary for their formation, called Critical Micelle Concentration (CMC) (Barros et al., 2007).

Biosurfactants are groups of chemical compounds produced by bacteria, fungi or yeasts, that also have hydrophobic and hydrophilic portions which through their accumulation at the immiscible fluid interfaces can reduce surface tension, increasing the surface area of such compounds with different degrees of polarity and allowing increased mobility, bioavailability and biodegradation (Banat et al., 2000). Natural surfactants exhibit the same properties of chemical surfactants, such as emulsification, foaming ability, lubricity, detergency, solubilization and dispersion phases (Desai and Banat, 1997).

Currently, biosurfactants are not yet able to compete economically in the market with chemically synthesized compounds, due to the high cost of production. This is a result of inefficient methods of bioprocessing, low productivity of microbial strains, and especially the need for substrates that have a very high cost (Haba et al., 2000).

Please cite this article as: Ehrhardt D., Secato J., Tambourgi E.B., 2015, Biosurfactant production by bacillus subtilis using the residue from processing of pineapple, enriched with glycerol, as substrate, Chemical Engineering Transactions, 43, 277-282 DOI: 10.3303/CET1543047

The biosurfactants are gaining notoriety for having some advantages over synthetic surfactants, such as biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable sources with low cost (Nitschke and Pastore, 2006).
Thus, the use of substrates of low economic value is a way to reduce the final cost of production of microbial metabolites. An alternative that has been extensively researched in recent years is the use of agricultural residues as substrates for the production of various compounds of microbial origin, such as biosurfactants. Residues rich in carbohydrates and lipids and with high concentrations of micronutrients to the microbial metabolism are those characterized as the best substrates for microbial growth, and also for the production of biosurfactants (Barros et al., 2008).
Several factors affect the production of biosurfactants, such as the nature of the sources of carbon and nitrogen used as well as the presence of phosphorus, iron, manganese and magnesium in the middle of production. In addition, other factors such as pH, temperature, agitation, and manner of conducting the process are extremely important in the quantity and quality of biosurfactant produced (Banat, 1995).In general, the microorganisms are capable of producing various products with excellent surface-active properties. Their use in certain applications depends on the cost of production and purification for specific activities. Thus, there is a tendency to perform work aimed at identifying potential surfactants, the evaluation of their properties and the optimization of fermentation processes for their production (Kronemberger, 2007).
Therefore, the aim of this study was the biosurfactant production by Bacillus subtilis through the fermentation of the residue from processing of pineapple enriched with glycerol as a carbon source. The bacterium Bacillus subtilis is a gram positive bacillus soil, nonpathogenic (Paccez, 2007), widely used in industrial production mainly because of the easy and low cost of their culture (Westers, 2004). The application of the pineapple residue as a substrate renewable source ensures low cost production, since the pineapple is extensively cultivated in Brazil and these residues are discarded.

2. Materials and Methods

2.1 Cultivation of microorganism, inoculum preparation and fermentation

Strains of Bacillus subtilis, kindly provided by André Tosello Foundation, were used.
For growth of Bacillus a nutrient medium was prepared with 5 % glucose, 0.46 % peptone and 0.06 % sodium chloride. Initially, preparation of the pre-inoculum was performed in a 50 mL conical flask containing 15 mL of the nutrient medium described above. To this medium was added the Bacillus subtilis and incubated in a shaker with constant agitation and at 37 °C for 6 h to adaptation of the microorganism. Subsequently, 10 mL of the pre-inoculum was transferred to a 150 mL Erlenmeyer flask containing 100 mL of the same nutrient medium with the same concentrations of glucose, peptone and sodium chloride, and left in a shaker under constant agitation and 37 °C, for 14 h, ensuring the growth of the microorganism.
The standardization of the inoculum was performed using a nutrient medium (the same composition and following proportion described above) adjusted in a spectrophotometer at a wavelength of 625 nm to absorbance range of 0.08 to 0.1.

2.2 Biosurfactant production

The production of the biosurfactant was performed using the fermentation process of 6 Erlenmeyer flasks containing 80 mL of the residue of the processing of pineapple enriched with glycerol as substrate and added 8 mL of the inoculum. The flasks were placed in a shaker under stirring and at 37 °C for 24 h. During this period, six samples were taken to assess the effectiveness of biosurfactant production by Bacillus subtilis under conditions of agitation and temperature imposed. Such effectiveness was evaluated by determining the surface tension and the emulsification index of each sample.
The enriched with glycerol was evaluated by performing three fermentations, as described above, each one with a different concentration of glycerol (3 %, 5 % and 10 %).

2.3 Surface tension

For determination of surface tension, the samples were centrifuged withdrawn 3,500 rpm for 10 min. The surface tension test was conducted according to the weight drop method described by Behring (2004) in which 10 drops of biosurfactant are weighted and their surface tension converted in mass. All tests were performed in duplicate.

2.4 Emulsificantion index

Emulsification was measured by the ratio oil / biosurfactant according to the methodology proposed by Cooper and Goldenberg (1987). Two different types of oils (soybean oil and motor oil) was used , and analyses were

performed in duplicate.
In graduated tubes were added 1 mL of the oil and then 1 mL of the biosurfactant produced, previously centrifuged at 3,500 rpm for 10 minutes. The tubes were stirred by vortexing for one minute and left to stand for 24 h. After 24 h the index analysis was done by measuring the height of the emulsion layer formed and the total height. The emulsification index was calculated by Eq (1):

$$\% = \frac{height\ of\ the\ emulsion\ layer\ \times 100}{total\ height} \quad (1)$$

3. Results

Production of biosurfactants using the residue from processing of pineapple enriched with glycerol as substrate was determined by reducing the surface tension and rate of emulsion.
It was observed that the best results in reducing the surface tension are for samples containing 3 % glycerol, while the tension reduction with 10 % glycerol was ineffective, confirming that such high concentration does not allow a good stability of the microorganism and consequently does not generate a satisfactory biosurfactant production.
The surface tension reduction in production with 3 % glycerol reached 23 % (Figure 1). The fermentation substrate contact with 5 % achieved a 21 % reduction in surface tension (Figure 2). However, this reduction decreases to only 7 % when the amount of glycerol increases to 10 % (Figure 3), which shows that the excess glycerol as carbon source contributes to inhibit the microorganism growth and thus the production of biosurfactant.

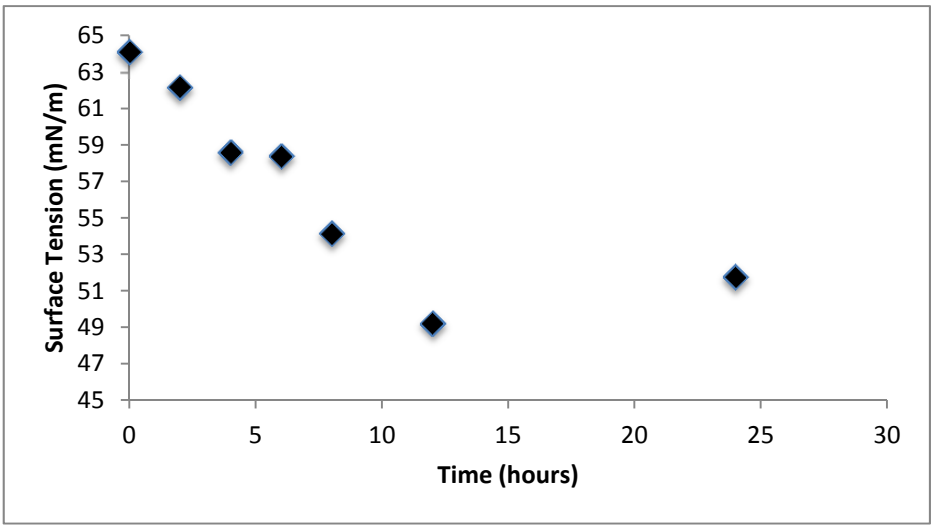

Figure 1: Analysis of surface tension over time with substrate supplemented with 3 % Glycerol

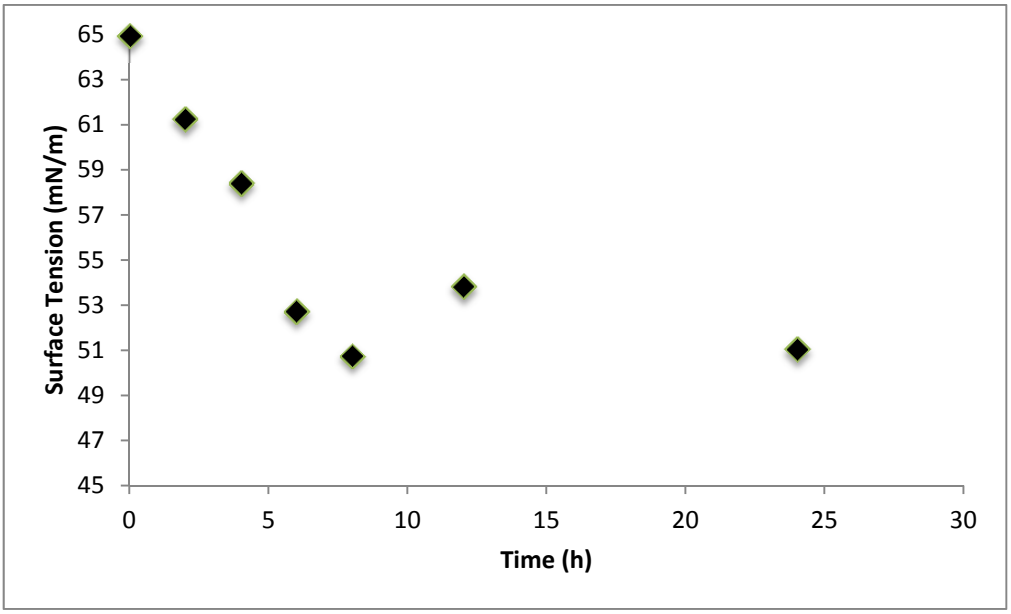

Figure 2: Analysis of surface tension over time with substrate enriched with 5 % Glycerol

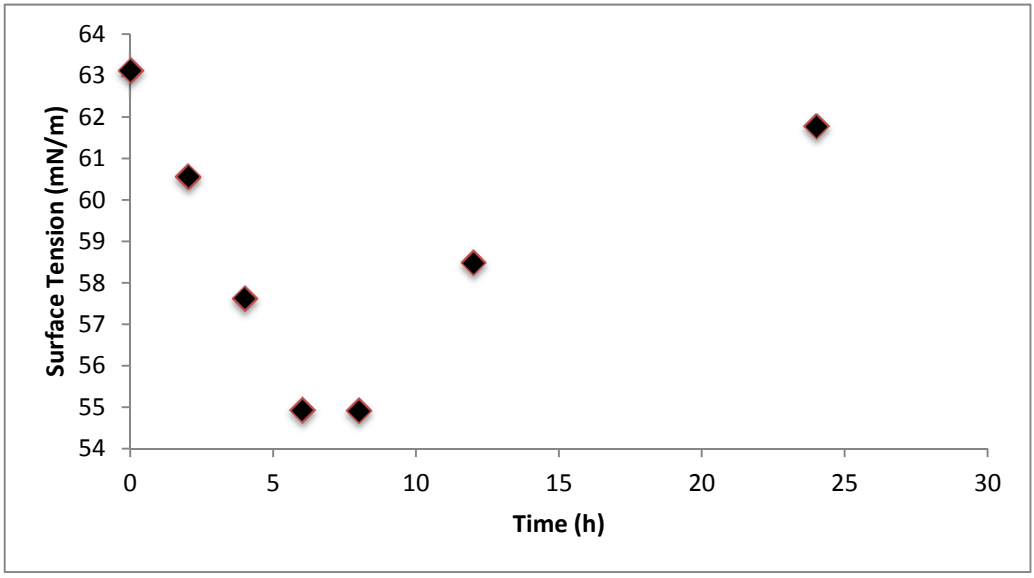

Figure 3: Analysis of surface tension over time with substrate enriched with 10 % Glycerol

Silva et. al (2010) studied the influence of different concentrations of glycerol as a substrate in biosurfactant production by Pseudomonas aeruginosa. The surface tension was analysed with 2 %, 3 %, 5 % and 10 % of glycerol. The best results with greater reductions in surface tension were obtained with 3 % of glycerol, also confirming that the excess of glycerol isn't favorable for the growth of the microrganism and subsequent production of biosurfactant.

The emulsificantion index also evaluates biosurfactant production. Tests were made with soy oil and motor oil and can be observed that a more effective emulsion was obtained with motor oil. Despite the considerable emulsification with three different concentrations of glycerol, it was observed that the best result, 62.5 % of emulsification was obtained with the substrate containing 3 % glycerol in engine oil. The index values resulting emulsion are recorded in the following tables.

Table 1: Emulsification Index according to the ratio oil / biosurfactant from the substrate supplemented with 3 % Glycerol

Samples	Time (h)	Índex obtained after 24 h	
		Soy Oil	Motor Oil
1	2	31.0 %	62.5 %
2	4	40.0 %	53.6 %
3	6	43.3 %	60.0 %
4	8	46.9 %	58.1 %
5	12	46.7 %	58.3 %
6	24	40.6 %	46.7 %

Table 2: Emulsification Index according to the ratio oil / biosurfactant from the substrate supplemented with 5 % Glycerol

Samples	Time (h)	Índex obtained after 24 h	
		Soy Oil	Motor Oil
1	2	58.1 %	48.3 %
2	4	53.1 %	45.2 %
3	6	45.2 %	56.2 %
4	8	40.6 %	50.0 %
5	12	46.9 %	58.6 %
6	24	48.4 %	51.7 %

Table 3: Emulsification Index according to the ratio oil / biosurfactant from the substrate supplemented with 10 % Glycerol

Samples	Time (h)	Índex obtained after 24 h	
		Soy Oil	Motor Oil
1	2	51.4 %	44.8 %
2	4	48.6 %	41.4 %
3	6	55.8 %	46.9 %
4	8	50.0 %	53.3 %
5	12	51.4 %	57.1 %
6	24	54.3 %	56.2 %

In studies presented by Braga et. al (2009), the enrichment with glycerol in biosurfactant production wasn't favorable. The study evaluated the emulsification index obtained in biosurfactant production by Chromobacterium Violaceum with substrates enriched with 1% and 10% of glycerol for 48 h. It was observed lower indexes with 10% of glycerol in all samples collected. Furthermore, other studies made by Braga et al. (2009), as analysis of biomass and emulsification activity, didn't show favorable results with higher concentrations of glycerol either.

4. Conclusion

The biosurfactant production by Bacillus subtilis using the residue from processing of pineapple enriched with glycerol as substrate showed greater efficacy with lower concentration of glycerol (3 %) fermentation at 23 % reduced surface tension.

However, it is observed that the increase of glycerol was unfavorable for the growth of the microorganism and thus the biosurfactant production. High amounts of glycerol resulted in a greater difficulty to the adequacy of Bacillus subtilis, reflecting a lower surface tension reduction.

Is important to note that the studies reported here were performed only with commercial glycerol and would need future studies with other types of glycerol to measure if they behave the way as observed in this study. Nevertheless, it is noted the viability of Bacillus produce biosurfactants using pineapple residue as a substrate, a renewable source that ensures a reduction in the cost of production of natural surfactants, which can minimize the economic problem for the production and become financially feasible to replace biosurfactants by

synthetic surfactants. There is still, however, a need to improve and expand such production process with the development of new technologies for application and suitability production conditions.

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