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Biosurfactant Production Using *Bacillus Subtilis* and Industrial Waste as Substrate

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The worldwide growing environmental concern and existing control regulations led researchers to study alternative ways to process industrial waste and the use of natural surfactants, or biosurfactants, is among one of the most promising methods due the fact that such compounds are metabolic products of fungi, bacteria and certain strains of yeast. Biosurfactants are amphiphilic molecules with low toxicity, high biodegradability and structural diversity that are capable to reduce interfacial tension of mixtures of water and hydrocarbons and thus an excellent alternative to replace synthetic surfactants. Besides, several different microorganisms were proven able to produce biosurfactants by digesting renewable-based culture medium. This paper describes the biosurfactant production by *Bacillus subtilis*, Gram positive and non-pathogenic bacteria, using as substrate industrial waste containing high levels of glucose. Were tested several percentages of inoculum (5, 7.5 and 10%) and the produced biosurfactant was evaluated according to the emulsification index (using soybean oil or engine oil in proportion 1:1 with the broth), surface tension analysis and glucose content. The tests were carried out in an orbital-stirred shaker and temperature kept at 37°C. The biosurfactant produced using 10% of inoculum showed a surface tension reduction of 36%, and emulsification index of 75% and 83% for soy oil and motor oil, respectively.

1. Introduction

Biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms. All biosurfactants are amphiphilic molecules, meaning its structure consist of two parts: a polar (hydrophilic) and a nonpolar (hydrophobic) group. The hydrophilic group can be constituted by mono, oligo or polysaccharides; peptides or proteins and the hydrophobic site usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Lang, 2002).

A surfactant molecule is capable of forming stable emulsions and is present in formulations of food, pharmaceutical products, petroleum compounds, cosmetic, water and soil remediation, textile and some other processes (Schramm *et al*., 2003).

Due to their amphiphilic nature, biosurfactants increase the surface area of hydrophobic water-insoluble substances, increasing its bioavailability, and also change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents Desai and Banat (1997).

Due to their interesting properties such as lower toxicity, higher degree of biodegradability, higher foaming capacity and optimal activity at extreme conditions of temperatures, pH levels and salinity, they have been increasingly attracting the attention of the scientific and industrial community (Banat *et al*., 2010). The examples of biosurfactant applications are listed in many review papers (Muthusamy *et al*., 2008; Soberón *et al*., 2011). Several factors affect production of biosurfactants, such as the nature of carbon and nitrogen sources used, as well as the presence of phosphorus, iron, manganese and magnesium. In addition, other factors such as pH, temperature, agitation and operation mode are extremely important to quantity and quality of produced biosurfactant (Banat, 1995).

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The biotechnological processes underlying microbial surfactants production should be based on the utilization of cheap substrates, in order to make commercialization possible. In addition to the cost of the substrates, the operating cost for the production of microbial surfactants is also a major concern in the commercial viability of biosurfactants. The use of biosurfactant is not widely encouraged yet, due to high cost involved in production and purification. Many researches choose using waste like medium of fermentation to biosurfactant production. Daverey *et al.* (2011) used wastewater from a local dairy industry that was simultaneously utilized and treated for the production of sophorolipids (SLs), a glycolipids type of biosurfactant, by *Candida bombicola*. Fontes *et al.* (2012) used glycerine and clarified cashew apple juice as feedstocks for the microbial surfactant synthesis by *Yarrowia lipolytica*. Luna *et al.* (2012) studied two wastewater systems: corn steep liquor and ground–nut oil refinery as low cost nutrients for the production of a biosurfactant by *Candida sphaerica* (UCP 0995). Cassava flour wastewater was used as culture media for biosurfactant production by *Bacillus sp.* and results showed a surface tension of 59 to 26 mN/m (Nitschke and Pastore, 2004).
This study aims to investigate biosurfactant production by *Bacillus subtilis* using an industrial wastewater containing high glucose content as substrate, so that the process is economically viable.

2. Material and Methods

2.1 Microorganism and Inoculum

The microorganism *Bacillus subtilis* was used, strain obtained from the Brazilian Collection of Microorganisms from Environment and Industry - CBMAI, located in CPQBA / UNICAMP, (CBMAI 369). The microorganism was peaked in Nutrient Agar and kept refrigerated until further use. The medium used for inoculum preparation was Nutrient Broth (1 % glucose, 0.46 % peptone, and 0.06 % sodium chloride).15 mL of pre-inoculum was prepared (same composition the medium) in a 50 mL Erlenmeyer flask.
To this medium was added the *Bacillus subtilis* and incubated in a shaker with constant agitation and at 37°C for 6 hours to adaptation of the microorganism. Subsequently 10 mL of the pre-inoculum was transferred to a 250 mL Erlenmeyer flask containing 100 mL of the same nutrient medium and incubated in shaker for another 12 h at 37°C. The standardization of the inoculum was performed using a nutrient broth (same composition used above), adjusted in a spectrophotometer (625 nm wavelength), AJX-1900 to a range of 0.08 to 0.1 of absorbance.

2.2 Substrate

The was obtained from a candy producer company from Campinas region. The waste was collected immediately after the process output without undergoing any treatment. This residue was kept frozen until its use.

2.3 Fermentation

The fermentations were conducted in Erlenmeyer Flasks of 250 ml capacity containing 80 mL of industrial waste containing high rate of glucose that were inoculated with 5, 7,5 and 10% inoculum. The flasks were autoclaved, inoculated and brought to an orbital shaker under stirring and at 37 °C for 18 h. The measured parameters were glucose, surface tension and emulsification index.

2.4 Surface tension

The surface tension measurements were determined according to Behring (2004) methodology. The samples were centrifuged at 3,500 rpm for 10 minutes. The burette was calibrated with sodium dodecyl sulfate solutions at various concentrations and tested with pure water before each fermentation. All tests were performed in triplicate.

2.5 Emulsification Index (E_{24})

The emulsification index was determined according to the methodology proposed by Cooper and Goldenberg (1987). The fermentation medium was centrifuged (10,000 rpm, 15 minutes) to obtain the cell-free supernatant. A 1 ml aliquot was collected from the supernatant to mix with 1 ml of oil tested in test tubes. In this study were used soybean oil and motor oil. The mixture was stirred by vortex for 1 min and then allowed to stand for 24 h. The emulsification index was calculated by Equation (1):

$$\% = \frac{Height_{emulsion}}{Total\ Height_{mixture}} \quad (1)$$

2.6 Glucose Analysis

For glucose analysis was used the kit Laborlab, the Glucose GOD-PAP Liquid Stable. Three test tubes were prepared containing the blank, the standard and the sample solutions in specific volumes. After mixing and incubation for 5 min at 37 °C, the absorbance was measured. The reading was performed in a spectrophotometer (505 nm wavelength), AJX-1900. All tests were made in triplicate.

3. Results

The results obtained for the decrease in surface tension during the fermentation as a function of the percentage of inoculum, ae shown in Figures 1-3.

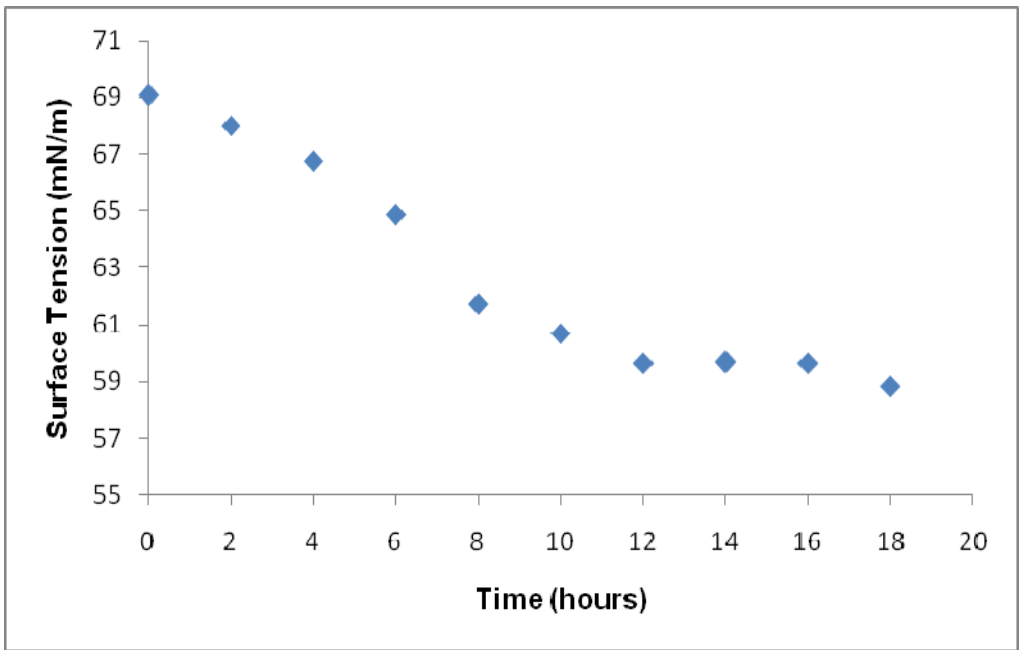

Figure 1: Analysis of surface tension with 5% inoculum

The decrease in of superficial tension is the primary means one can elect microorganisms capable to produce biosurfactants, although emulsifying and dispersing agents will not necessarily decrease the superficial tension (Youssef *et al*., 2004; Shepherd *et al.*, 1995). Yet, a decrease in surface tension is a strong indication of biosurfactant production, being confirmed by its emulsion index.

The largest decrease in surface tension was obtained for the assay using a 10% inoculum level. The initial value was 70 mN/m, reaching 44 mN/m after 22 hours of fermentation (a 36% drop). For the 7.5% and 5% inoculum fraction showed a decline in surface tension of 14% and 20%, respectively. It may be noted thus, the higher the percentage of the inoculum, the greater the reduction in surface tension.

Studies conducted by Nitschke and Pastore (2006) using cassava as substrate and *Bacillus subtilis* in the synthesis of biosurfactant (surfactin) showed a reduction of surface tension by 37% relative to the starting point, reaching levels below 30 mN/m.

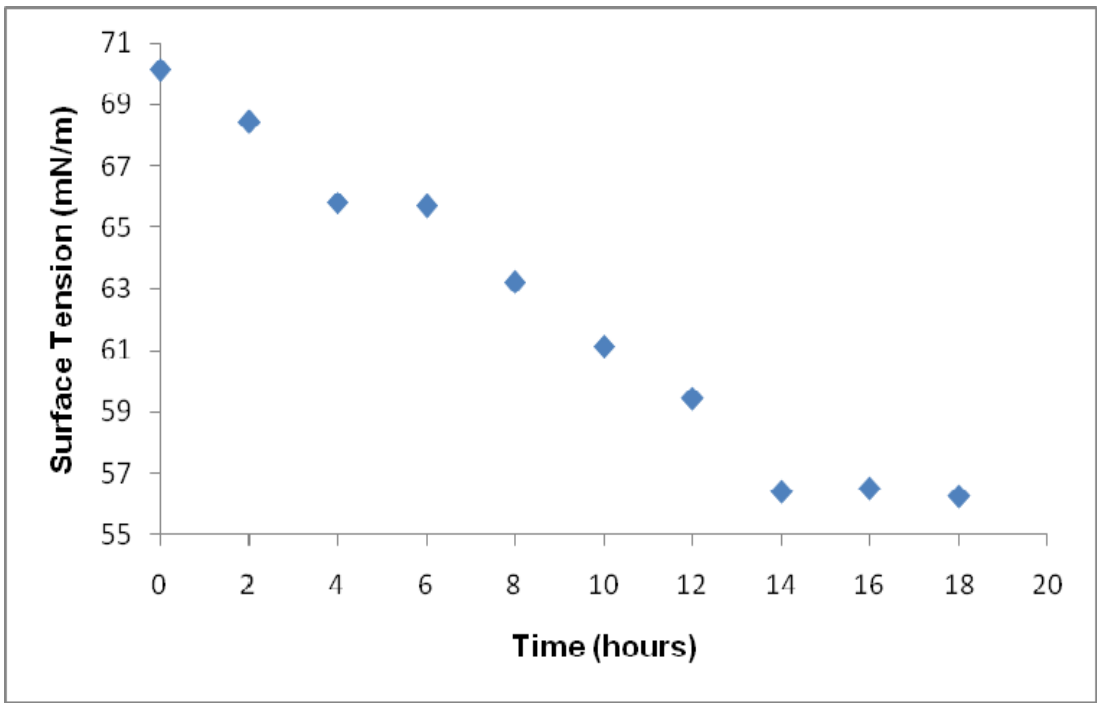

Figure 2: Analysis of surface tension with 7.5% inoculum

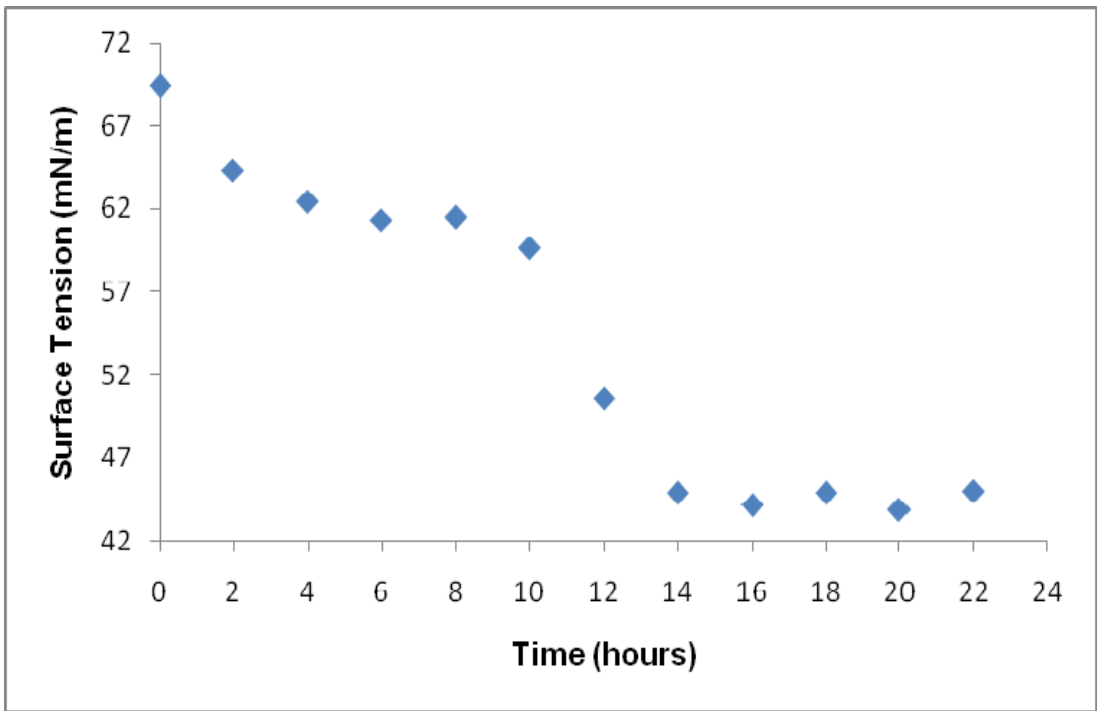

Figure 3: Analysis of surface tension with 10% inoculum

The results obtained in relation to emulsification index and glucose during the fermentation can be seen in Table 1, 2 and 3. The emulsification 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.

Table 1: Emulsification index and glucose content for the produced biosurfactant with 5% inoculum

Time (hours)	Glucose (mg/dl)	E_{24} (Soy Oil)	E_{24} (Motor Oil)
0	524	-	26.6
2	536	-	28.6
4	520	-	24.7
6	449	-	23.8
8	421	-	29.6
10	453	-	32.8
12	378	-	35.5
14	396	-	36.4
16	375	-	36.3
18	365	-	31.9
20	333	-	30
22	312	-	26.1

Table 2: Values of emulsification indexes and glucose of the produced biosurfactant with 7.5% inoculum

Time (hours)	Glucose (mg/dl)	E_{24} (Soy Oil)	E_{24} (Motor Oil)
0	452	31.8	37.5
2	441	38.5	36.5
4	539	34.2	36.3
6	385	33	42.8
8	374	34.1	45.9
10	321	36.9	48.2
12	411	48.9	52.7
14	317	50.2	53.9
16	347	51.3	50.2
18	361	51.1	50.2
20	374	46.9	50.1
22	288	44.7	48.8

Table 3: Values of emulsification indexes and glucose of the produced biosurfactant with 10% inoculum

Time (hours)	Glucose (mg/dl)	E_{24} (Soy Oil)	E_{24} (Motor Oil)
0	339	36.8	38.5
2	332	37.2	40.8
4	259	33.9	42.7
6	278	36.2	36.7
8	241	41.7	40.4
10	253	41.2	40.1
12	321	40.8	75.4
14	458	68.9	80.2
16	428	68	82.9
18	321	75.6	83.8
20	358	74.5	79.0
22	307	74.1	79.3

The emulsification index expresses the ability of produced biosurfactants to emulsify hydrocarbons. According to Willumsen and Karlson (1997), one criteria considered to select a good emulsifier is the capability of forming the emulsion with a hydrocarbon and that it remains emulsified above 50% for 24 hours or more. Therefore, according to the results, the tests showed a good emulsifying power, achieving up to 75% of emulsification for soy oil and 93% for motor oil.

Barros *et al*. (2008) evaluated the emulsion obtained using the biosurfactant produced by *Bacillus subtilis*, cassava wastewater as substrate and several hydrocarbons and vegetable oil. Their results showed high emulsification index values for most of carbon sources used, including cyclic and aliphatic hydrocarbons, and vegetable oils with different fatty acids.

4. Conclusions

The results showed that the biosurfactant produced using glucose-rich industrial wastewater successfully emulsified engine oil samples and provided a significant decrease in surface tension when a culture medium containing 10% inoculum is used. Thus, the use of this low-cost substrate, whose treatment is expensive and costly, for biosurfactant production is feasible, since such compounds have a wide variety of applications in many industries.

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