Vol.57, n.2: pp. 295-301, March-April 2014
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Crude Glycerol from Biodiesel Industry as Substrate for Biosurfactant Production by *Bacillus subtilis* ATCC 6633

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ABSTRACT

Glycerol, a co-product of the biodiesel industry, may be a suitable raw material for the production of high added-value compounds by the microorganisms. This study aimed to use the glycerol obtained from the biodiesel production process as the main carbon source for biosurfactant production by Bacillus subtilis *ATCC 6633. Results indicated that the strain lowered the surface tension of the cell-free fermented broth to 31.5 ± 1.6 mN/m, indicating the production of biosurfactant. The critical micelle concentration (CMC = 33.6 mN/m) obtained was similar to the previously reported for biossurfactants isolated from other* Bacillus*. The produced biosurfactant was able to emulsify n-hexadecane and soybean oil.*

Key words: Biosurfactant, Biodiesel, Glycerol and Surfactin

INTRODUCTION

Biosurfactants are biological surface-active compounds released by the microorganisms that can have some influence on the interfaces (Salihu et al. 2009). They are proteins with detergent, emulsifier, and surfactant power to lower the surface tension of water and other solvents, and have potential applications in environmental uses such as organic pollutants treatment and oil recovery (Neves et al. 2007). Microbial surfactants are produced primarily by the bacteria. However, they are also produced by the yeasts and fungi (Fiechter 1992). The production of biosurfactants is often related to the consumption of hydrocarbons, including oily residues, and occurs during the exponential cellular growth (Neves et al. 2007). But they can also be produced from the sugars (sucrose, glucose and lactose), glycerol, vegetable oils, or starch as carbon sources (Cirigliano and Carman 1984; Guerra-Santos et al. 1984; Makkar and Cameotra 2002; Reis et al. 2004; Amaral et al. 2006; Giro et al. 2009). Those amphiphilic compounds are capable to reduce surface and interfacial tension between the two liquids (Bicca et al. 1999) and they have important characteristics when compared to synthetic surfactants, such as high biodegradability, low toxicity, high surface activity, solubility in alkaline water, thermal stability, resistance to high saline concentrations and stability against variations of pH (Kim et al. 2002). Another advantage is that biosurfactants can be synthesized from the renewable substrates. They also have high chemical diversity, allowing specific applications for each particular case (Desai and Banat 1997).

The largest market for the biosurfactants is in the oil industry, where they are used in the production of oil, or incorporated into the formulations of

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lubricating oils (Van Dyke et al. 1991). Other applications include bioremediation and dispersion of oil spills, removal and mobilization of residual oil in storage tanks, as well as in enhanced oil recoveries (Nitschke and Pastore 2002).

The microbial surfactants are not yet capable for competing economically with the chemical surfactants in the market, mainly due to its high costs (Pruthi 1997; Davis et al. 1999). The production of biosurfactants is a great challenge in biotechnological processes in the present economy. The success of its production depends on the development of an industrial bioprocess based on low cost raw materials, which may not exceed 10 to 30% of the cost of the final product (Cameotra and Makkar 1998). Thus, the use of a co-product from the biodiesel production (glycerol) as a carbon source could be a good alternative for the synthesis of biosurfactants by fermentation, since glycerol has a low market value due to the presence of various impurities in it (Thompson et al. 2006). Furthermore, the expansion of biodiesel production has made the price of glycerol to fall gradually, which may help to reduce the cost of biosurfactants production.

The purpose of the present work was to use the glycerol obtained from the biodiesel production process as the main carbon source for biosurfactant synthesis by *Bacillus subtilis* ATCC 6633, with the goal of lowering the production costs associated with surfactin production. This stain has been reported for the production of biosurfactants from different carbon sources (Reis et al. 2004).

MATERIALS AND METHODS

Microorganism

B. subtilis ATCC6633, obtained from André Tosello Tropical Foundation – Campinas, SP, Brazil, was maintained on APGE medium, consisting of (g/L) 5.0 peptone, 5.0 glucose, 2.5 yeast extract and 15.0 agar at 4°C and sub-cultured monthly.

Raw material

Glycerol used in this work resulted from the transesterification of soybean oil by methanol in alkaline medium (NaOH). Pre-treated glycerol (neutralized with H_2SO_4 and methanol evaporated) was provided by Empresa Brasileira de Bioenergia Ind. Com Ltd. (EBB, Ceará, Brazil).

Culture medium and cultivation conditions

Inoculum preparation

The microorganism was inoculated onto APGE plates and incubated at 30°C for 24 h. From this, three colonies were transferred to 250-mL Erlenmeyer flasks, containing 50 mL of medium (PGE), composed of (g/L) 5.0 peptone, 5.0 glucose, 2.5 yeast extract, sterilized at 121°C for 15 minutes in the autoclave. The flasks were incubated in a rotary shaker (Tecnal – TE240, São Paulo, Brazil) at 180 rpm and 30°C for 24 h. Afterwards, the optical density (OD) of this culture was adjusted to 0.1–0.2 at 600 nm.

Culture medium

A defined mineral medium was firstly prepared according to Sar and Rosemberg (1983) with the following composition (g/L): K_2HPO_4 13.99, KH_2PO_4 6.0, $MgSO_4.7H_2O$ 0.2, $(NH_4)_2.SO_4$ 4.0, and 0.04% yeast extract. The mineral medium was sterilized at 110°C for 10 minutes. Then 0.1% (v/v) of a micronutrient solution, consisting of (g/L) EDTA 2.5g, $ZnSO_4.7H_2O$ 10.95, $FeSO_4.7H_2O$ 5.0, $MnSO_4.H_2O$ 1.54, $CuSO.5H_2O$ 0.392, $Co(NO_3)_2.6H_2O$ 0.25 and $Na_2B_4O_7.10H_2O$ 0.177, previously sterilized by filtration was added (Morán et al. 2000). Finally, 2% (v / v) of glycerol, previously sterilized at 121 ºC for 15 min, was added to the medium.

Batch fermentation

All the assays were conducted in Erlenmeyer flasks of 250 mL with 50 mL of culture medium on a rotary shaker (TE240 - Tecnal, São Paulo, Brazil) at 180 rpm and 30°C. The medium was inoculated (1%, v/v) and incubated for 72h in isothermal conditions. Samples (50 mL) were collected at pre-defined intervals of time and analysed for biomass, substrate and surfactin concentrations, emulsification index (E_{24}), surface tension and critical micelle concentration (CMC).

Analytical methods

Emulsification index (E_{24})

Emulsification index was determined according to Cooper and Goldenberg (1987), with slight modifications: 2.0 mL of cell-free supernatant was added to 2.0 mL of different hydrophobic sources (soy oil, kerosene, N-hexadecane) and the mixture was homogenized in a vortex-type stirrer for 2

min. After 24 h, the height of the emulsion layer was measured. The emulsifying activity (E_{24}) was calculated using the equation 1 (Desai and Banat, 1997).

$$E_{24}(\%) = \frac{H_{EL}}{H_S} * 100 \qquad (1)$$

Where H_{EL} is the height of the emulsion layer and H_S is the height of total solution.

Surface tension

Surface tension was determined in the cell-free fermented broth using a tensiometer (Krüss K6) at 25°C, according to the De Nöuy ring method (Zajic and Seffens 1984).

Emulsifying activity

Emulsification activity was determined in accordance with the methodology described by Cirigliano and Carman (1985), with slight modifications (Giro et al. 2009). The samples were filtered through a Millipore 0.45 µm membrane and the filtrate (1.0 mL) was placed in glass tubes (15 by 125 mm) and diluted with 1.0 mL of 0.1 M sodium acetate buffer (pH 3.6). Then, 0.5 mL of kerosene was added to the tube, which was homogenized in a vortex-type stirrer. The resulting emulsion remained at rest for 10 minutes, and then the aqueous phase absorbance was measured in spectrophotometer (Spectronic ® 20 Genesys) at 540 nm. One unit of emulsifying activity was defined as the amount of biosurfactant that resulted in an absorbance of 1.0 at 540nm (A_{540}) under the conditions described above.

Biomass

Cell growth was determined by measuring the optical density of samples, using a UV-visible spectrophotometer (20 Genesis, BR) at 600 nm. Cell concentration was determined using a calibration curve that related the values of optical density and dry weight (Makkar and Cameotra 1998).

Surfactin concentration

Surfactin concentration was determined using a Waters high-performance-liquid chromatographer equipped with a UV detector (Model 2487, Waters), at 205 nm, and a Symmetry C_{18} column (150 x 4.6 mm, 5 µm, Waters, Ireland). The mobile phase consisted of 20% (v/v) trifluoroacetic acid (3.8 mM) and 80% (v/v) acetonitrile. The elution rate was 1.0 mL/min at 30°C and the sample size was 20 µL. The identity of the purified surfactin was obtained by using the commercially available 95% pure surfactin (Sigma-Aldrich) as the reference compound (Yeh et al. 2005).

Glycerol concentration

Glycerol concentration was determined using a Waters high-performance-liquid chromatography equipped with a refractive index detector and a Supelcogel C610H column (30 cm x 7.8 mm). H_3PO_4 (0.1%) in ultra-pure water (MiliQ) was used as mobile phase with the flow rate of 0.5 mL/min at 30°C and volume of injection was 20 µL (Sousa et al. 2012).

Determination of the critical micellar concentration

Different concentrations of the produced biosurfactant were obtained by performing several dilutions of the cell-free fermented broth, containing surfactin produced after 48 h of fermentation (Santa Anna et al. 2002). Superficial tension of the resulting solutions was measured at room temperature, as described above. The CMC was determined by plotting the surface tensions as a function of the logarithm of surfactin concentration and it was found at the point of intersection between the two lines that best fit the pre- and post-CMC data (Gudina et al. 2010).

RESULTS AND DISCUSSION

Biosurfactant production by *B. subtilis* ATCC6633 cultivated in mineral media supplemented with Glycerol.

The effect of a semi-defined medium, mineral medium using glycerol as carbon source on surfactin production by *B. subtilis* ATCC6633 was examined in this work by monitoring the changes in cell growth, glycerol consumption and surfactin concentration. The results as shown in Figure 1 revealed that the strain was able grow and to produce surfactin from glycerol. After 48 h of cultivation, there was an increased in the production of surfactin, reaching a concentration of 158.14 mg/L at 72 h of fermentation. At the same time, an increase in cell concentration was observed, reaching 1.69 g/L, with a glycerol conversion of 97%.

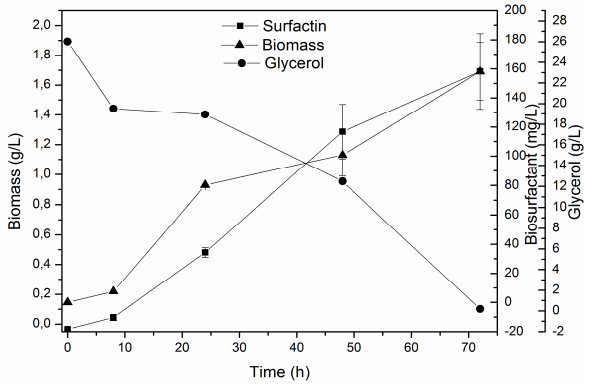

Figure 1 - Concentrations of biosurfactant, biomass and glycerol consumption by *B. subtilis* ATCC 6633 cultivated at 30°C and 180 rpm in mineral media supplemented with Glycerol.

Surface activity of the biosurfactant produced by B. subtilis ATCC 6633 cultivated in mineral media supplemented with Glycerol.

The surface activity of the biosurfactant was determined by measuring the surface tension, emulsifying activity and emulsion index of the supernatant from the batch culture of *B. subtilis* ATCC 6633. Figure 2 showed the results of surface tension of the cell-free fermented broth, which reached 31.5 ± 1.6 mN/m after 72 h of fermentation. These results indicated that the microorganism tested demonstrated ability to produce biosurfactant since the effectiveness of a surfactant was determined by its ability to reduce the surface and interfacial tensions. According to Gudina et al. (2010), a good surfactant reduces the surface tension of water from 72.0 to 35.0 mN/m, and the interfacial tension between water and hexadecane from 40.0 to 1.0 mN/m.

Emulsifying properties of biosurfactants produced by *B. subtilis* ATCC 6633 cultivated in mineral media supplemented with glycerol were determined by measuring the emulsifying activity, which determined the ability of biosurfactant in forming oil-water emulsion, and emulsifying index, which determined the capacity of surfactant in forming emulsions on different hydrophobic substrates (soybean oil, kerosene and n-hexadecane). Results of Figure 3 showed that higher and more stable emulsification activities, between 2 and 3 units, were detected when soilbean oil was used as hydrophobic compoud. These results were superior to those described for the synthetic commercial surfactants tested by Amaral et al. (2006). They were also superior to those described to other biosurfactants from *Yarrowia lipolytica* (Amaral et al. 2006), *C. lipolytica* IA (Sarubbo et al. 2001) and *Nocardia sp.* L-417 (Kim et al. 2000). Regarding emulsifying index, best result of E24 was obtained by using n-hexadecane (53.3%), followed by soybean oil (43.3%). No measurable emulsifying index was obtained when kerosene was used.

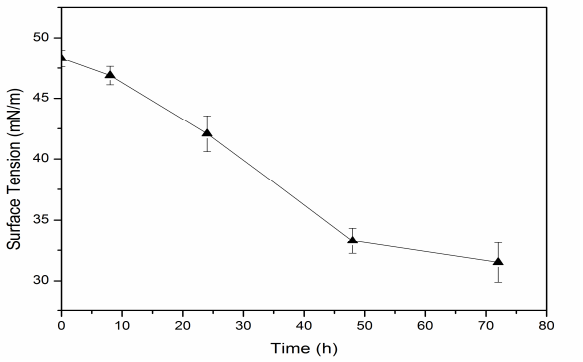

Figure 2 - Surface tension of the cell-free fermented broth during cultivation of *B. subtilis* ATCC 6633 at 30°C and 180 rpm in mineral media supplemented with Glycerol.

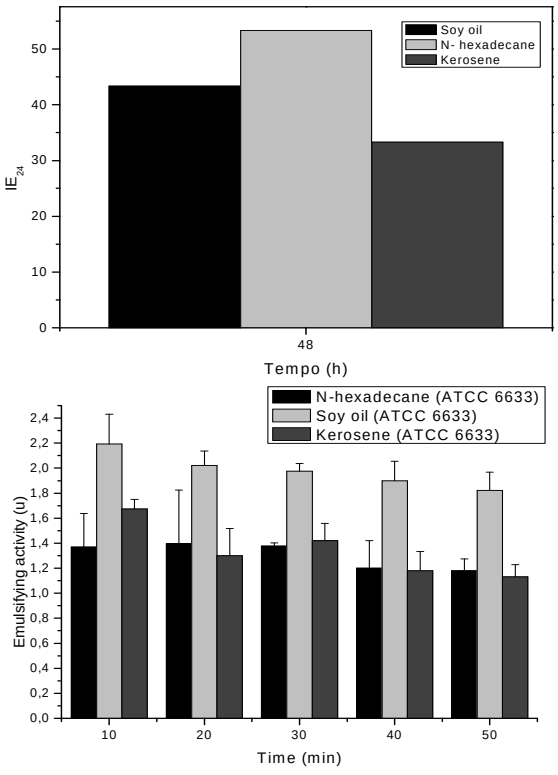

Figure 3 - Emulsifying activity and emulsification index (I_{E24}) of the biosurfactant produced by *Bacillus subtilis* ATCC 6633, after 48 h of fermentation at 30 ºC and 180 rpm, against N-Hexadecane, soy bean oil and kerosene.

Critical micelle concentration and minimum surface tension

In order to determine the critical micelle concentration of the crude surfactant produced by *B. subtilis* ATCC 6633 grown in a mineral media containing glycerol as carbon source, the relationship between the surface tension and surfactin concentration was determined (Fig. 4A). There was a progressive decrease in surface tension with increasing concentrations of surfactin. For biosurfactant concentrations higher than 30 mg/L, the surface tension became stable and no appreciable reduction was observed even for the highest concentrations tested. A semi-logarithm plot of surface tension versus surfactin concentration allowed determining the CMC value as 33.6 mN/m. A minimum surface tension value of 32.3 mN/m was obtained at a surfactin concentration of 71.9 mg/L.

Table 1 shows the CMC of SDS and several biosurfactant isolated from different *B. subtilis* strains. As observed these compounds could reduce the surface tension of water to values around 30-35 mN/M and the CMC of the biosurfactants ranged from 1-2000 mg/L (Sobrinho 2007). By comparison, the results obtained in this work were in agreement with those obtained from the literature, which showed the potential of using glycerol as a carbon source for surfactin production *by B. subtilis* strains.

Table 1 - Minimal surface tensions and critical micelle concentration (CMC) obtained for several surface active compounds.

Surfactant	Surface Tension (mN/M)	CMC (mg/L)	Reference
SDS (Synthetic)	33.7	270.0	Hirata et al. 2009
Surfactin	27.5	150.0	Fox and Bala (2000)
Surfactin	28.7	78.6	Reis et al. (2004)
Surfactin/fengycin	27.0	40.0	Sivapathasekaran et al. 2010
Crude lipopeptide	29.1	140.0	Mukherjee et al. 2005

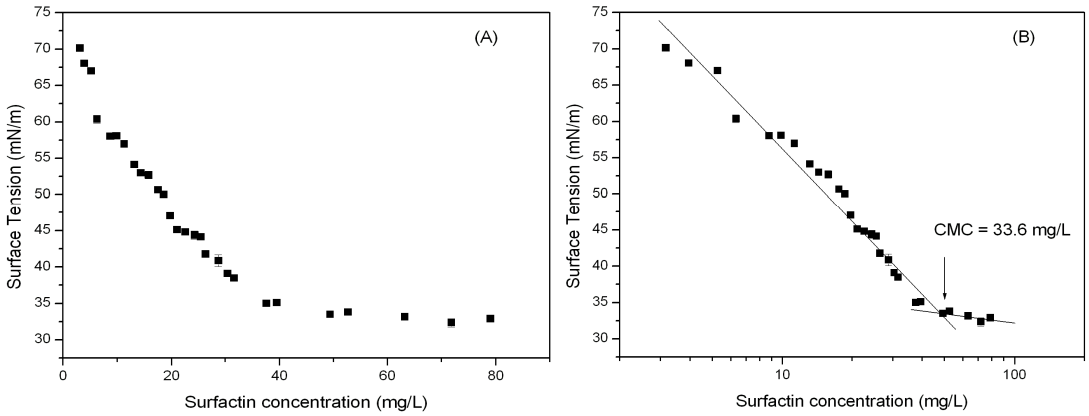

Figure 4 - Influence of surfactin concentration on the surface tension of the cell-free fermented broth. (A) Surface tensin of the crude surfactin produced by B. subtilis ATCC 6633 after 48 hours of fermentation at 30°C and 180 rpm. (B) Surface tension as a function of the logarithm of surfactin concentration. The reference surface tension value was 72 mM/m.

CONCLUSIONS

Bacillus subtilis ATCC 6633 was able to grow and produce surfactin in the medium formulated with crude glycerol, reaching 158.14 mg/L of biosurfactant and lowering the surface tension of the cell-free fermented broth to 31.5 ± 1.6 mM/m after 72 h of fermentation. Bacterial growth in mineral medium supplemented with residual glycerol yielded a biosurfactant with an E24 of n-hexadecane (53.3%), followed by soybean oil (43.3%). The emulsifying activity was high and stable when soybean oil was used as hydrophobic compound. The minimum surface tension and the critical micelle concentration of the biosurfactant produced by *B. subtilis* ATCC 6633 in this work were similar to the results previously reported for biosurfactants produced by the same strain using different substrates. The results indicated that glycerol could be a suitable substrate for the development of a cost-effective bioprocess for

biosurfactant production. Nevertheless, in order to achieve a commercially competitive bioproduct, it is still necessary to increase the production yield, which may be achieved by optimizing some operational parameters, such as pH, temperature and aeration rate.

ACKNOWLEDGMENTS

The authors thank CNPq and CAPES for the financial support that made this work possible.

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Received: January 15, 2013;
Accepted: October 10, 2013.