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RENEWABLE RESOURCES FOR BIOSURFACTANT PRODUCTION BY *Yarrowia lipolytica*

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Abstract - In this work, the production of a biosurfactant synthesized by *Yarrowia lipolytica* using different renewable resources as carbon source was investigated. Crude glycerol, a biodiesel co-product, and clarified cashew apple juice (CCAJ), an agroindustrial residue, were applied as feedstocks for the microbial surfactant synthesis. The microorganism was able to grow and produce biosurfactant on CCAJ and crude glycerol, achieving maximum emulsification indexes of 68.0% and 70.2% and maximum variations in surface tension of 18.0 $mN.m^{-1}$ and 22.0 $mN.m^{-1}$, respectively. Different organic solvents (acetone, ethyl acetate and chloroform–methanol) were tested for biosurfactant extraction. Maximum biosurfactant recovery was obtained with chloroform–methanol (1:1), reaching 6.9 $g.L^{-1}$ for experiments using CCAJ and 7.9 $g.L^{-1}$ for media containing crude glycerol as carbon source. The results herein obtained indicate that CCAJ and the co-product of biodiesel production are appropriate raw materials for biosurfactant production by *Y. lipolytica*.
Keywords: Biosurfactant; Yeast; Surface tension; Glycerol; Cashew apple juice.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by living organisms, mostly on microbial cell surfaces or excreted extracellularly, and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension at the surface and interface, respectively (Muthusamy *et al*., 2008). Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceutical, biomedical and food processing industries (Mulligan, 2004).

Most of the surface active compounds currently in use are synthesized chemically. However, microbially produced surfactants offer several advantages over their chemical counterparts, such as biodegradability, low toxicity and production from renewable substrates (Mercade and Mansera, 1994). Although biosurfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs. One possible strategy for reducing costs is the utilization of alternative substrates such as agroindustrial wastes (Maneerat, 2005). The choice of inexpensive raw materials is important to the overall economy of the process because they account for 50% of the final product cost (Makkar and Cameotra, 1997).

In the literature, different renewable resources and agro industrial residues have been used for biosurfactant production. Molasses (Makkar and Cameotra, 1997), cassava wastewater (Nitschke and Pastore, 2002), olive oil mill effluent (Mercade and Mansera, 1994), animal fat (Desphande and Daniels, 1995), weathered diesel oil (Mariano *et al*., 2008 and Sousa *et al*., 2012), waste frying oils (Vedaraman

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and Venkatesh, 2011), distillery residues and whey (Dubey and Juwarkar, 2001) have been studied as low cost substrates for microbial growth and biosurfactant production.

Cashew apple is a tropical pseudofruit in which the real fruit is a nut, a well-known product around the world (Campos *et al*., 2002). Internal and external market consumption of cashew nuts, in the year of 2004, was about 232,000 t. However, only 12% of the total peduncle is processed. In the northeast of Brazil, the cashew agroindustry has an outstanding role in the local economy. However, only a small part of the pseudofruit produced is used industrially and the amount wasted, approximately 94.0%, presents high potential as a fermentation medium, since it is rich in carbohydrate, fibers, vitamins and minerals salts (Rocha *et al*., 2007).

The use of cashew apple juice was evaluated by Honorato *et al*. (2007) to produce high added value products such as dextran, lactic acid, mannitol and oligosaccharides. Rocha *et al*. (2006) studied cashew apple juice (CAJ) as a fermentation medium for biosurfactant production by *Acinetobacter calcoaceticus.* The microorganism was able to grow and produce biosurfactant on CAJ, reducing the surface tension of the medium from 72 to 62 $mN.m^{-1}$. The biosurfactant also achieved a maximum emulsion index of 58.8% for kerosene. Rocha *et al*. (2009) developed a growth medium made of mineral medium and yeast extract containing clarified cashew apple juice as carbon source and employed it to cultivate an isolate of *Bacillus subtilis* LAMI008 and produce surfactin. The biosurfactant produced (3.5 $mg.l^{-1}$) was capable of emulsifying kerosene, achieving an emulsification index of 65% and the surface tension decreased from 58.95 to 38.10 $mN.m^{-1}$.

Another potential renewable source for microbial production of surfactant is glycerol from biodiesel production. Because biodiesel production is increasing exponentially, the crude glycerol generated by the transesterification of vegetable oils is also being produced in large quantity. The current annual amount of glycerin arising from this biodiesel production amounts to some 1.9 Mton and will continue to rise proportionally. Nowadays, the world market for pure glycerol of high quality for industrial applications (chemical and pharmaceutical) only amounts to some 0.9 -1.0 Mton per year (2007) (Hoogendoorn *et al*., 2007). Therefore, either new applications for glycerin need to be developed and/or the existing pathways need to be expanded. The use of crude glycerol as feedstock to obtain biotechnology products, such as biosurfactants, can alleviate many industrial process waste management problems. Glycerol has been successfully used as a water soluble carbon source for different microbial productions (Amaral *et al*., 2009).

Morita *et al*. (2007) evaluated glycerol microbial conversion into biosurfactant (glycolipids) by *Pseudozyma antarctica* JCM 10317, observing a glycolipid production of 16.3 $g.L^{-1}$ in a fed batch operation with glycerol as carbon source. Ciapina *et al*. (2007) evaluated the use of pure glycerol as carbon source for biosurfactant production by *Rhodococcus erythropolis*; after 51 cultivation hours, 1.7 $g.L^{-1}$ of biosurfactant were obtained.

Ashby *et al*. (2005) compared pure glycerol with biodiesel production residue as carbon sources for sophorolipid production by *Candida bombicola*. Fermentations with pure glycerol resulted in 9.0 $g.l^{-1}$ of sophorolipids, while fermentation with biodiesel residue provided a biosurfactant production of 6.0 $g.l^{-1}$.

Many microorganisms are able to metabolize glycerol, like the yeast *Y. lipolytica* (Fontes *et al*., 2010) and different strains of the bacterium *Pseudomonas aeruginosa* (Wu *et al*., 2008; Wei *et al*., 2005). Studies have shown that *Y. lipolytica* is capable of producing some interesting compounds from raw glycerol, such as biosurfactants and organic acids (Fontes *et al*., 2010; Papanikolaou and Aggelis, 2003).

This paper aims to contribute to the use of industrial byproducts as carbon and energy sources for cultivation of *Y. lipolytica* and production of biosurfactant. Crude glycerol, a biodiesel by-product, and clarified cashew apple juice (CCAJ), an agroindustrial residue, were applied as feedstocks for microorganism growth and microbial synthesis of the surfactant.

MATERIALS AND METHODS

Microorganism and Inoculum

A wild type strain of *Yarrowia lipolytica* (IMUFRJ 50682) was employed (Haegler and Mendonça-Haegler, 1981) and kept at 4 ºC on YPD-agar medium (w/p: yeast extract (Oxoid) 1.0%, peptone (Oxoid), 0.6%; glucose (Reagen), 2.0%, agar (Reagen), 2.5%). For inoculum conditions, cells were cultivated at 28 ºC in a rotary shaker at 160 rpm, in 500 ml shake flasks containing 200 ml of YPD medium. After 48 h of cultivation, these cells were used in sufficient amount to inoculate 1 mg of cells per ml of biosurfactant production media.

Culture Medium and Culture Conditions

Clarified Cashew Apple Juice (CCAJ)

CCAJ was obtained through a mechanical process and clarified as described by Honorato *et al*. (2007). The pH of CCAJ was adjusted to 7.0 with 0.5M NaOH and then filtered through a 0.45-µm membrane (Millipore Corp.) and exposed to ultraviolet radiation (6.000 µW·s/cm^2) for 1 h for sterilization in order to avoid loss of heat-labile components. Table 1 shows the CCAJ composition (Rocha *et al*., 2007) and Table 2 shows the different media evaluated in this study. The CCAJ was diluted one or ten times with distilled water and ammonium sulfate was used as nitrogen source. Biosurfactant production was carried out in 1000 ml shake flasks, containing 500 ml of the culture medium, in a rotary shaker at 28 ºC and 250 rpm for 96 h. Samples were collected at time-defined intervals of 24 hours and submitted to analysis for determination of reducing sugars, cell concentration, emulsification index and surface tension.

Glycerin

Glycerol P.A (Vetec Química, S/A) and the glycerin phase obtained from the transesterification of castor oil by methanol in alkaline medium (NaOH) were used for biosurfactant production by *Y. lipolytica*. The experiments were performed with crude and hydrolyzed glycerin. The glycerin used in this work was the co-product of biodiesel production, resulting from the transesterification of castor bean oil by methanol in alkaline medium (NaOH). Both materials from biodiesel production were prepared following the procedures described below.

Three percent (v/v) of pure glycerol, or crude or hydrolyzed glycerin were added to the production medium with 10.0 g.L^{-1} of ammonium sulfate and 0.5 g.L^{-1} of yeast extract as nitrogen sources. The production conditions used were the same as in the cashew apple juice experiments. Samples were collected at defined intervals of 24 hours and submitted to analysis for determination of glycerol, cell concentration, emulsification index and surface tension.

Crude Glycerin

The pH of crude glycerin was adjusted to 7.0 with 0.8M H_2SO_4 in order to eliminate free alkalinity. After that, it was heated (120 °C) for 1 h under agitation for methanol elimination. The sodium sulfate produced from the neutralization was separated by decantation.

Table 1: Composition of CCAJ utilized as raw material for biosurfactant production by *Y. lipolytica* (Rocha *et al*., 2007).

Parameters	CCAJ
Glucose (g.l^{-1})	43.67 ± 0.30
Fructose (g.l^{-1})	42.43 ± 0.10
Soluble proteins (mg.ml^{-1})	0.10 ± 0.00
Total protein (mg.ml^{-1})	5.19 ± 0.00
Phosphorous (g.l^{-1})	1.21 ± 0.00
Potassium (g.l^{-1})	13.13 ± 0.90
Calcium (g.l^{-1})	< DL*
Magnesium (g.l^{-1})	1.17 ± 0.10
Sodium (g.l^{-1})	0.09 ± 0.00
Sulfur (g.l^{-1})	0.81 ± 0.00
Copper (mg.l^{-1})	< DL*
Iron (mg.l^{-1})	6.97 ± 2.70
Zinc (mg.l^{-1})	11.20 ± 4.30
Manganese (mg.l^{-1})	6.40 ± 0.40

pH = 4.32 ± 0.01. * DL = detection limit

Table 2: Composition of CCAJ media for biosurfactant production by *Y. lipolytica*

Experiments	Carbon source	Nitrogen source
1	CCAJ 1:1[a]	---
2	CCAJ 1:1[a]	10.0 g.l^{-1} $(NH_4)_2SO_4$
3	CCAJ 1:10[b]	---
4	CCAJ 1:10[b]	10.0 g.l^{-1} $(NH_4)_2SO_4$

[a]CCAJ was diluted one time with distilled water. [b]CCAJ was diluted ten-fold with distilled water.

Hydrolyzed Glycerin

Hydrolyzed glycerin was prepared by acid hydrolysis of crude glycerin as described in Sousa *et al*. (2011). The glycerin hydrolysis was conducted at room temperature with concentrated H_2SO_4. The volume of H_2SO_4 needed was determined by the total alkalinity of glycerin (Eq. (1)). Then, water was added to the system in the ratio of 1:3 with respect to the mass of glycerin. The hydrolysis was conducted in a separation funnel for 24 h to allow phase settling to occur (glycerin and fatty acid). The resulting hydrolyzed glycerin was used as the carbon source in batch experiments.

$$V_{H_2SO_4} = \frac{AT \text{ x } m}{N} \quad (1)$$

where V_{H2SO4} is the volume of H_2SO_4 (L), AT is the total alkalinity ($mg.L^{-1}$), m is the mass of glycerin (mg) and N is the normality of H_2SO_4.

Biosurfactant Extraction

Biosurfactant extraction was adapted from the method described by Amaral *et al*. (2006). The culture medium was centrifuged (26,000 x *g*) at 25 °C for 20 min and then filtered through a 0.45-µm membrane (Millipore Corp.). Approximately 150 mL of the cell-free filtrate was transferred to a 3.6-ft (110-cm) length of dialysis tubing (diameter, 4 cm; molecular cut-off, 12,000 Da) and concentrated to 25 mL by pervaporation. The concentrated crude extract (25 mL) was used for surfactant extraction in a 500 ml separatory funnel at 25 °C. Three different solvent systems were used: concentrated crude extract/chloroform/methanol (1:1:1, 1:2:2, 1:4:4, 1:6:6 and 1:8:8, v/v), concentrated crude extract/ acetone (1:3, v/v), concentrated crude extract/ethyl acetate (1:2, v/v). The concentrated crude extract and the solvents were shaken for five minutes and the white precipitate formed was centrifuged (32,000 x *g*) at 25 °C for 30 min, re-suspended in water and lyophilized.

Surface Tension (ST)

The surface tension was determined on cell-free broth, obtained by centrifugation at 1000 x *g* for 10 min, with a Tensiometer K 100 (Kruss) using the ring method at room temperature (25 ± 2 °C). Surface tension was evaluated as a variation (ΔST) between the initial surface tension (cell-free culture medium at the beginning of the experiment) and the final surface tension.

Emulsification Index (EI)

The emulsification index was determined by using a modification of the method described by Iqbal *et al*. (1995). The EI of cell-free samples was determined by adding 1 mL of hexadecane to the same amount of sample, vortex-mixing this mixture for 2 min and leaving it to stand for 24 hours. The EI is given as the percentage of height of the emulsified layer (cm) divided by the total height of the liquid column (cm).

Cell Determination

Cell concentration was followed by optical density measurements at 570 nm and the values were converted to mg/ml using a factor previously determined (Amaral *et al*., 2006).

Determination of Reducing Sugars

Total reducing sugars were determined colorimetrically by the dinitrosalicylic acid (DNS) method (Miller, 1959).

Glycerol Concentration

Glycerol concentration was analyzed by enzymatic-colorimetric assay using a triglycerides kit (GPO/POD – CELM/Brazil).

Determination of Total Carbon and Nitrogen

The total carbon contents of the crude and hydrolyzed glycerin were measured by means of a dry combustion method (Shimadzu, TOC-V CPH/CPN). For total nitrogen determinations, the samples were digested with potassium persulfate and analysed by the method described for nitrate (Grasshoff, 1999).

RESULTS AND DISCUSSION

Clarified Cashew Apple Juice (CCAJ) as Biosurfactant Production Medium

Initially, biosurfactant production with CCAJ diluted with water (1:1) was evaluated without nitrogen source addition (exp.1) and with ammonium sulfate addition (1% w/v) (exp.2) in sufficient amounts to obtain a molar carbon to nitrogen ratio (C/N) of 11.65, which was previously shown to promote higher biosurfactant production (Fontes *et al*., 2010). Process parameters (cell concentration

and reducing sugar concentration) were monitored during the experiment and the results are presented in Figure 1(a).

It was verified that, in the culture medium with CCAJ supplemented with ammonium sulfate, the cell growth level was higher, reaching 8.27 g dry weight of biomass per litter, whereas in the culture medium without nitrogen source addition biomass production was 6.21 g dry weight of biomass per litter. Similar results were found by Rocha *et al*. (2007), who evaluated cashew apple juice as raw material for *Pseudomonas aeruginosa* in submerse cultivation and observed a higher cell growth with the nitrogen source supplement because the juice presents low total and soluble protein concentration.

Concerning the reducing sugar consumption, a higher consumption rate was found in exp.2 due to the availability of a nitrogen source. The reducing sugar was not totally consumed during the process, leaving approximately 13.0 $g.L^{-1}$ (exp.2) and 20.0 $g.L^{-1}$ (exp.1) after 96 hours of cultivation, which can possibly be attributed to the lack of nitrogen source at this point.

It was also verified that the pH in both experiments slowly decreased to 3.8 (exp.1) or 3.2 (exp.2) at 48 hours of the bioprocess, being practically constant beyond this point (data not shown).

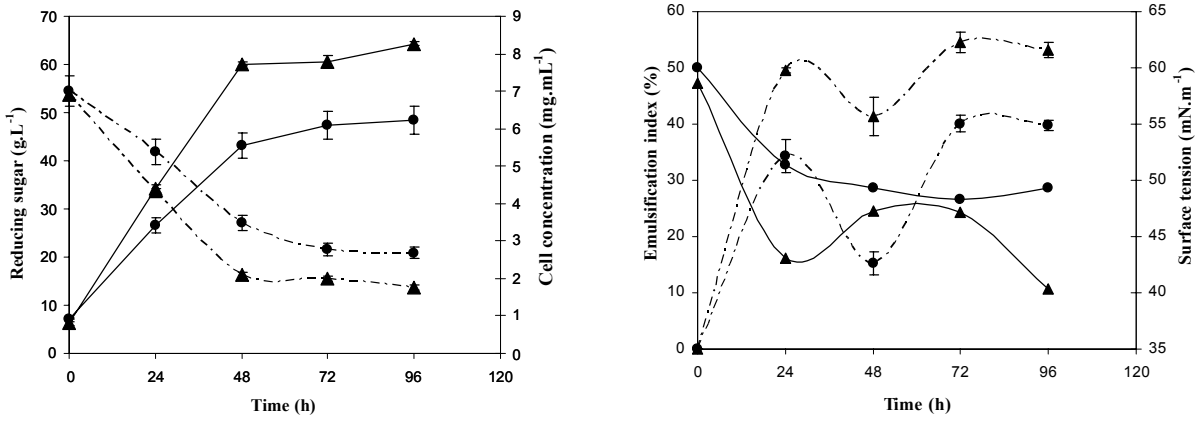
Surface tension and emulsification index evolution throughout the experiments are presented in Figure 1(b), where it is possible to observe that *Y. lipolytica* was able to produce biosurfactant in culture medium containing CCAJ as raw material. The highest biosurfactant production was detected in the culture medium supplemented with ammonium sulfate, reaching an EI of 53.2% and a ΔST of 18.3 $mN.m^{-1}$.

There was no significant surface tension reduction in the medium without nitrogen source addition, with a ΔST of only 11.7 $mN.m^{-1}$ and an EI of 40.1%. According to Rocha *et al*. (2007), the biosurfactant production by *P. aeruginosa* occurred with ten-fold diluted cashew apple juice as carbon source supplemented with nitrogen source. In order to test this condition, ten-fold diluted CCAJ without nitrogen source addition (exp.3) and with ammonium sulfate 1% w/v (exp.4) was used for biosurfactant production by *Y. lipolytica*, like Rocha *et al*. (2007).

Figure 2(a) presents the results for the cell growth profile and the total reducing sugar consumption. The cell growth profile was different from that obtained with 1:1 diluted cashew apple juice. In 48 h of cultivation the cells were still in the exponential phase of growth, entering the stationary phase only after 72 h of the process. On the other hand, Rocha *et al*. (2007) observed a stationary phase of growth for *P. aeruginosa* within 30 and 25 h, respectively. The maximum biomass concentration obtained was slightly lower than that achieved with 1:1 diluted medium, as well as the specific growth rate, which can be seen in Table 3.

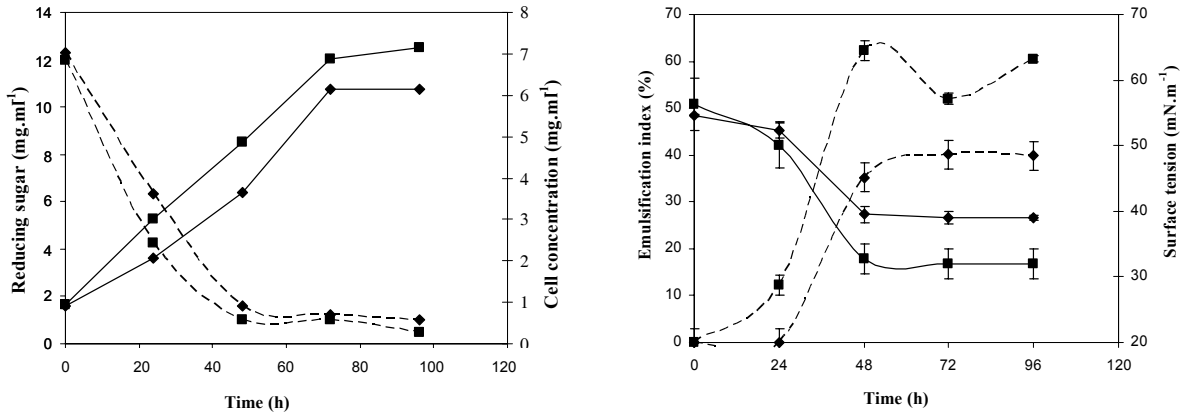
The reducing sugar was practically all consumed in 48 h on both experiments. A higher sugar consumption rate was detected in comparison to the medium diluted 1:1 (Table 3).

Figure (2b) presents the results for biosurfactant production by *Y. lipolytica* with ten-fold diluted CCAJ. Both culture media evaluated seem to favor biosurfactant production, which was estimated by the EI and the surface tension reduction. The highest ΔST (24.3 $mN.m^{-1}$) and EI (65.0%) were obtained in exp. 4, which contained 1.0% ammonium sulfate.



(a) (b)

Figure 1: (a) Reducing sugar consumption (---) and cell growth profiles (—) of *Y. lipolytica*. (b) Emulsification index (---) and surface tension (—) of the cell-free broth of *Y. lipolytica* grown in CCAJ diluted 1:1 without nitrogen source addition (exp.1 ●) and with ammonium sulfate addition (1.0% w/v) (exp.2 ▲).



(a) (b)

Figure 2: (a) Reducing sugar consumption (---) and (—) cell growth profiles of *Y. lipolytica*. (b) Emulsification index (---) and surface tension (—) of biosurfactant produced by *Y. lipolytica* grown in CCAJ diluted 1:10 without nitrogen source (exp.3♦) and supplemented with ammonium sulfate (1.0% w/v) (exp.4■).

Table 3: Parameters for biosurfactant production by *Y. lipolytica* with CCAJ.

Parameter	CCAJ 1:1 diluted		CCAJ 1:10 diluted	
	Exp.1	Exp.2	Exp.3	Exp.4
μ (h^{-1})	0.07	0.05	0.03	0.03
-dS/dt ($g.(l.h)^{-1}$)	0.58	0.78	0.24	0.22
ΔST ($mN.m^{-1}$)	11.68	18.28	12.55	24.33
EI (%)	40.10	53.18	40.11	65.00
X_f ($mg.ml^{-1}$)	6.21	8.27	6.15	7.15

μ: specific growth rate
-dS/dt: sugar consumption rate
ΔST: surface tension variation
EI: Emulsification index
X_f: final cell concentration

The culture medium without nitrogen source addition failed to provide a significant surface tension reduction (12.5 $mN.m^{-1}$) or a good value for EI (40.1%), very similar to exp.1 (Table 3). According to Willumsen and Karlson (1996), a biosurfactant with good emulsifier properties presents an EI over 50.0%. These results show the importance of nitrogen source supplement for biosurfactant production in CCAJ medium. Although the majority of microorganisms produce biosurfactant under limiting nitrogen conditions (Willumsen and Karlson, 1996), a sufficient initial amount of nitrogen is required and the type and quantity of this component vary according to the microorganism used.

According to Rocha *et al*. (2007), *P. aeruginosa* was able to reduce the surface tension of cashew apple juice medium from 50 to 29.5 $mN.m^{-1}$ using peptone (5.0 $g.L^{-1}$) as nitrogen source. When the authors used ammonium sulfate (5.0 $g.L^{-1}$) and $NaNO_3$ (5.0 $g.L^{-1}$), biosurfactant production was not favored.

Biosurfactant production by *Acinetobacter calcoaceticus*, using natural cashew apple juice and a mineral complex medium was evaluated by Rocha *et al*. (2006). The authors observed that the maximum EI value reached was 58.8%, lower then the one obtained in the present work. The surface tension reduction was not significant (from 70.0 to 63.0 $mN.m^{-1}$) during the experiments. Contrary to what was observed in this work, biosurfactant production occurred only during the stationary phase growth, as a typical secondary metabolite.

Diluted cashew apple juice was not an adequate substrate for the growth of a *Bacillus subtilis* strain, because no biomass or biosurfactant were produced when this microorganism was cultivated on this medium. Only when ammonium sulfate was added was cell growth observed, but no significant surface tension reduction (from 64.5 to 56.8 $mN.m^{-1}$) was

achieved (Rocha *et al*., 2006). Therefore, in the present work, a higher surface tension variation was obtained (24.3 $mN.m^{-1}$).

According to the results, diluted cashew apple juice (1:10) supplemented with ammonium sulfate (1.0%) provided a better biosurfactant production (Table 3) in relation to cashew apple juice diluted 1:1.

In order to recover the biosurfactant from the fermentation medium, three types of solvent systems were tested. Several organic solvents can be used and they are used either singly or in combination for biosurfactant extraction (Ashby *et al*., 2005). Based on the amount of biosurfactant recovered in the extract from cell-free medium, the results indicate that a mixture of chloroform and methanol in the ratio 1:8:8 (6.85 g of biosurfactant.L^{-1}) was the best system for the extraction compared to acetone (0.33 g of biosurfactant.L^{-1}) or ethyl acetate (0.09 g of biosurfactant.L^{1}). Mixtures of solvents are commonly used to facilitate adjustment of the polarity between the solvent or extraction agent and the biosurfactant to be extracted.

Glycerin as Fermentation Medium for Biosurfactant Production

The glycerin phase composition resulting from biodiesel production varies largely considering the transesterification process and the efficiency of biodiesel separation. Therefore, the crude and hydrolyzed glycerin used in this work were first characterized in terms of glycerol concentration, total carbon, total nitrogen and pH. Table 4 presents the main parameters evaluated for the crude and hydrolyzed glycerin samples.

Table 4: Glycerin composition

Parameter	Crude glycerin	Hydrolyzed glycerin
Glycerol concentration ($g.l^{-1}$)	729.5 ± 0.3	741.9 ± 0.2
Total carbon ($g.l^{-1}$)	310.3 ± 0.1	292.8 ± 0.1
Total nitrogen ($g.l^{-1}$)	ND*	ND*

*ND not detected, pH = 6.0 ± 0.1

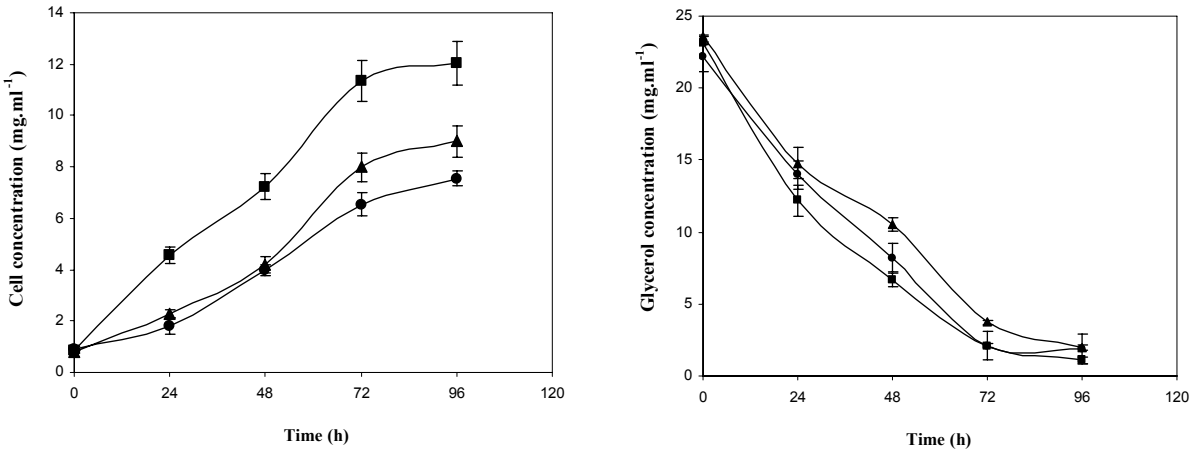
Both crude and hydrolyzed glycerin present high glycerol and total carbon concentrations, which are essential for biosurfactant production and cell growth. The pH of both samples after correction was 6.0; the pH of the glycerin phase without correction was 13.0, which is a very alkaline value for microbial growth. Total nitrogen was not detected in either sample, being lower than 1.4 μg of nitrogen.L^{-1} (limit for detection). Hence, because a nitrogen source is essential for cell growth and biosurfactant production, the medium with glycerin phase as substrate was supplemented with ammonium sulfate 10.0 $g.L^{-1}$ and yeast extract 0.5 $g.L^{-1}$. Pure, crude and hydrolyzed glycerol were all used at a concentration of 3.0% (v/v).

Figure 3(a) presents the growth kinetics profiles of *Y. lipolytica* on the media tested. Cell growth was higher when crude glycerin was used as substrate. This result can be attributed to the fatty acid present in the crude glycerin phase as an additional carbon source, increasing cell growth. According to Ashby *et al*. (2005), the residue of biodiesel synthesis from soy oil contains 40.0% of glycerol, 34.0% of compounds soluble on hexane (made up of 92.0% free fatty acid/fatty acid methyl esters and 6.0% of monoacyl glycerol e diacyl glycerol) and 26.0% water. After 96 h the biomass produced in the experiments with crude, pure and hydrolyzed glycerin was 12.0; 9.0; and 7.5 $g.L^{-1}$, respectively. Similar results were obtained by Ashby *et al*. (2005), with a higher cell growth for *Candida bombicola* with biodiesel production byproduct (4.0%) than with pure glycerol (10.0% v/v).

In Figure 3(b), it is possible to observe that glycerol consumption by the yeast was similar for the three types of glycerin. At the end of the experiment (96 h), glycerol was practically all consumed in all experiments.

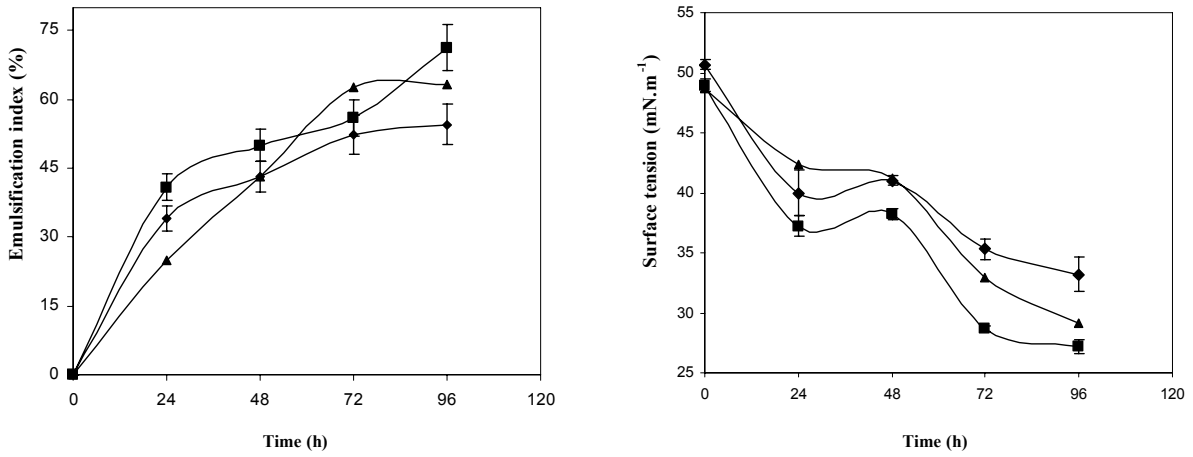
Glycerol is typically transported to the cell through the membrane by facilitated diffusion over a concentration gradient and then quickly converted to glycerol-3-phosphate by glycerol kinase and subsequently oxidized to dihydroxyacetone phosphate (DHAP), a common glycolytic pathway intermediate, by glycerol phosphate dehydrogenase. DHAP is enzymatically transformed to its isomer glyceraldehyde-3-phosphate (G3P) and both can be converted to glucose by gluconeogenesis and subsequently to the sugars present in the biosurfactant structure or can be directed to cellular growth. Alternatively, G3P can be converted to pyruvate for subsequent conversion to acetyl-CoA, the main metabolic precursor for fatty acid biosynthesis (Lehninger, 2006).

Figure 4 shows the evolution of the surface tension and the emulsification index for the experiments with glycerin. For both methods used to verify the presence of a biosurfactant being excreted into the culture medium, the higher production occurred in the culture media containing crude glycerin, reaching an EI of 70.2% and a ΔST of 22.0 $N.m^{-1}$ after 96 h.



(a) (b)

Figure 3: Time-course of cell growth (a) and glycerol consumption (b) by *Y. lipolytica* using pure (▲), crude (■) and hydrolyzed (●) glycerin as carbon source.



(a) (b)

Figure 4: Emulsification index (a) and surface tension (b) for biosurfactant produced by *Y. lipolytica* cultivated in pure (▲), crude (■) and hydrolyzed (●) glycerol as carbon source.

The fact that biosurfactant production is higher in the medium containing crude glycerin can be attributed to the presence of fatty acids. According to Weber *et al*. (1992), *Candida (Yarrowia)* species are able to incorporate fatty acid directly for biosurfactant production. While glycerol is mainly used for cell growth and production of the hydrophilic portion biosurfactant molecule production, fatty acids present on crude glycerin are used by the cell directly for biosurfactant production. This way, the biosurfactant lipidic portion can be influenced by the type of oil used for biodiesel production. It is important to point out that, during the experiments with crude glycerin the foam formation was less intense when compared to the culture medium containing pure or hydrolyzed glycerin.

Souza *et al*. (2011) studied biosurfactant production by *P. aeruginosa* using the glycerin (6.0%) obtained from a biodiesel production process as carbon source and sodium nitrate as nitrogen source. The authors obtained 64.0% of EI and a reduction in surface tension of 45.7%. Higher EI values were obtained in the present work with *Y. lipolytica* and crude glycerin (71.2%) and a similar surface tension reduction.

The biosurfactant extraction was performed in culture media containing crude glycerol as carbon source because it showed better emulsification index values and a greater surface tension variation. The ability of solvents to extract the biosurfactant produced by *Y. lipolytica* was evaluated using different extraction systems: concentrated extract/chloroform/ methanol (1:8:8, v/v), concentrated extract/acetone (1:3, v/v), concentrated extract/ethyl acetate (1:2, v/v).

The results presented in Figure 5 show that chloroform/methanol was the best system for the extraction, as was observed for the extraction of the biosurfactant produced with CCAJ. This result can be attributed to the hydrophobic/ hydrophilic balance of the chloroform/methanol solvent system.

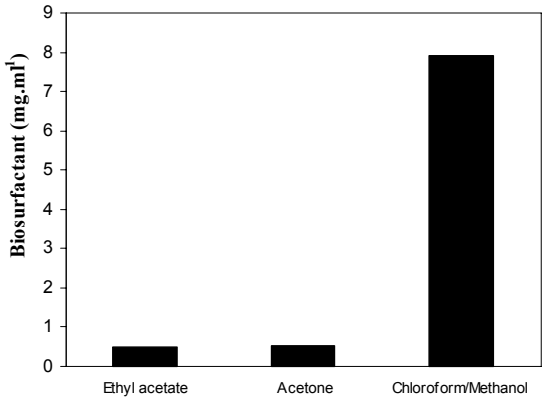


Figure 5: Biosurfactant extraction using different solvent systems.

In order to investigate the influence of solvent proportion on biosurfactant extraction from the concentrated extract, five different concentrated extract /chloroform/methanol ratios were used: 1:1:1; 1:2:2; 1:4:4; 1:5:5; 1:8:8. The results show that the best ratio was 1:8:8, which promoted an extraction of 7.90 g of biosurfactant per 1000 mL of concentrated cell-free medium (Figure 6). This is a significant value when compared to the amount obtained by Sheperd *et al*. (1995) for biosurfactant produced by *Candida utilis* in the presence of corn oil (0.9 $g.l^{-1}$). Table 5 show the amount of biosurfactant produced by different yeasts.

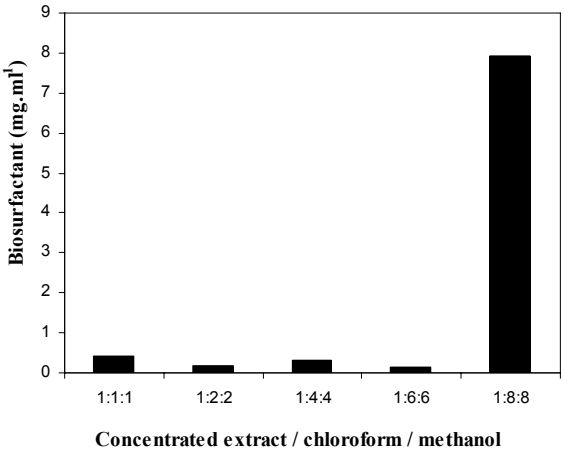


Figure 6: Biosurfactant extraction using chloroform/ methanol.

Table 5: Amount of biosurfactant produced by different yeasts.

Micoorganism	Carbon source	Biosurfactant ($g.l^{-1}$)	Reference
Yarrowia lipolytica	Crude Glycerin	7.9	Present work
Yarrowia lipolytica	Cashew apple juice	6.9	Present work
Candida lipolytica	Canola oil/ Glucose	8.0	Sarubbo *et al*. (2007)
Candida sphaerica	Ground-nut oil refinery residue	4.5	Sobrinho *et al*. (2008)
Candida lipolytica	Industrial waste	4.5	Rufino *et al*. (2007)
Candida glabrata	Cotton seed oil /glucose	10.0	Sarubbo *et al*. (2006)
Candida antarctica	soapstock	15.9	Bednarski *et al*. (2004)
Candida utilis	Corn oil	0.9	Sheperd *et al*. (1995)

CONCLUSIONS

The results indicate that *Y. lipolytica* was able to grow and produce biosurfactant when cultivated in all the media studied. This study shows that traditional carbon sources for biosurfactant production can be replaced by residual glycerol or CCAJ. The increasing biodiesel production generates large amounts of crude glycerin, which could be successfully used as a carbon source for biosurfactant production, as demonstrated by the results present in this manuscript. Similarly, the use of CCAJ as a culture medium would provide waste management alternative for the productive chain of cashew nut, an important industrial segment on the Northeast of Brazil.

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