

## NEW BIOEMULSIFIERS PRODUCED BY *CANDIDA LIPOLYTICA* USING D-GLUCOSE AND BABASSU OIL AS CARBON SOURCES

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Submitted: June 20, 2001; Returned to authors for corrections: April 17, 2001; Approved: May 06, 2003

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### ABSTRACT

*Candida lipolytica* IA 1055 produced extracellular biosurfactants with emulsification activity by fermentation using babassu oil and D-glucose as carbon sources. Natural seawater diluted at 50% supplemented with urea, ammonium sulfate, and phosphate was used as economic basal medium. The best results were achieved with the **YSW-B<sub>2</sub>** medium, which contained urea, ammonium sulfate, and babassu oil and with **YSW-B<sub>3</sub>** medium, which contained urea, ammonium sulfate, phosphate, and babassu oil, kept under fed batch fermentation for 60 hours with 5% of babassu oil. For the two media, the maximum specific growth rates were 0.02 h<sup>-1</sup> and 0.04 h<sup>-1</sup>; the generation times were 34.6 h<sup>-1</sup> and 17.3 h<sup>-1</sup>, and the emulsification activities were 0.666 and 0.158 units, respectively. The molecules of these new bioemulsifiers were constituted of carbohydrates, proteins and lipids.

**Keywords:** Bioemulsifier, *Candida lipolytica*, seawater, babassu oil

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Biosurfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic domains. These compounds are capable of reducing surface and interfacial tension between liquids, solids and gases, allowing them to mix or disperse readily as emulsions in water or other liquids. Microbial compounds that are capable to exhibit particularly high surface activity and emulsifying activity are classified as biosurfactants (1). A number of microorganisms are excellent sources of potentially useful amphipatic biopolymers, for instance *Acinetobacter radioresistens* (5); *Candida bombicola* (9,13), and *Yarrowia lipolytica* (14).

Bioemulsifiers are biodegradable and can be produced from renewable and cheap substrates. Native vegetable oils have been used as carbon source and seawater as media component for production of biosurfactants by *Candida lipolytica*. This paper reports the results attained on bioemulsifier production through the use of economic substrates in seawater media supplemented with babassu oil, and D-glucose as control.

*Candida lipolytica* IA 1055 was obtained from the culture collection of the Departamento of Antibióticos of Universidade Federal de Pernambuco. Stock cultures of the organism were maintained at 4°C on Yeast Mold Agar slants containing yeast extract (0.3%); malt extract (0.3%); D-glucose (1.0%); tryptone (0.5%); and agar (1.5%).

Carbon sources utilized throughout the study were 5% babassu oil and 1% D-glucose, added to a basal medium called Yeast Salt Water (YSW). Growth media were prepared as follows: **YSW-G** – ammonium sulfate 0.1g l<sup>-1</sup>, D-glucose 1g l<sup>-1</sup>; **YSW-G<sub>1</sub>** – ammonium sulfate 0.1g l<sup>-1</sup>, urea 0.1g l<sup>-1</sup>, D-glucose 1g l<sup>-1</sup>; **YSW-B<sub>2</sub>** – urea 0.25g l<sup>-1</sup>; acid phosphate of potassium 1.36g l<sup>-1</sup>, babassu oil 5 ml<sup>-1</sup>; and **YSW-B<sub>3</sub>** – ammonium sulfate 0.1g l<sup>-1</sup>, urea 0.25g l<sup>-1</sup>, and acid phosphate of potassium 1.36g l<sup>-1</sup>, and babassu oil 5 ml<sup>-1</sup>.

The media were diluted 1:1 (v/v) in distilled water and natural seawater (NaCl final concentration 13%); the pH was set to 5.3±0.2, followed by sterilization at 121°C for 15 minutes. The seawater used as basal medium was collected in Boa Viagem

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beach, and the salinity was determined by the Mohr-Knudsen method as described in Strickland and Parsons (11).

Cells grown in Yeast Mold Broth were used as inoculum (1%, v/v). The fermentation process was carried out in 1000ml-Erlenmeyer flasks, containing 300ml of medium. The flasks were incubated at 28°C on an orbital shaker at 150 rpm for 240 hours. After 60 hours of fermentation, 5% of babassu oil was added to the medium.

Aliquots of 7 ml of the culture were collected every 24 hours for pH measurement using a potentiometer and for counts of viable cells, determined by spreading 0.1ml of the appropriate dilution onto Yeast Mold Agar. Colony counts were performed after incubation at 28°C for 48 hours.

The specific growth rate was measured according to Pirt (8).

In order to determine the emulsification activity, aliquots of 2 ml of the cultures submitted to filtration through a 0.22 µm Millipore membrane filter were mixed with 2ml of 0.1M sodium acetate buffer (pH 3.0) and 1ml of n-hexadecane in a screw-capped tube. The mixture was then homogenized in a vortex for 2 minutes at 25°C. After 10 minutes, the absorbance was measured at 540nm in a spectrophotometer. The blank consisted in 2ml of sterile YSW medium. One unit of emulsification activity was defined as the amount of emulsifier that resulted in an absorbance of 1.0 at 540nm (2).

For partial purification of the bioemulsifier, yeasts cells were removed from bioemulsifier-containing media, by filtration through a Whatman n° 1 paper and then through a 0.22 µm Millipore membrane filter.

The cell-free filtrate was dialyzed against deionized water, (five changes) and then concentrated by lyophilization. The concentrate was treated twice with chloroform-methanol (2:1, v/v) at 25°C. The solvents were removed by evaporation. The white precipitate formed in the aqueous phase was collected in Whatman no. 42 filter paper and dried. The dry material was redissolved in ultra pure water for analytical determinations.

The bioemulsifiers produced by *C. lipolytica* were submitted to determination of the total amount of carbohydrates, proteins and lipids, using colorimetric methods (LABTEST Diagnostic®-Brazil).

After 41 hours, the cell concentration in YSW-G medium was  $1.0 \times 10^6$  CFU/ml. After 50 hours, a diauxic behavior was observed. The number of cells after 89 hours decreased to  $3.2 \times 10^2$  CFU/ml and the specific growth rate was  $\mu_{\max} = 0.04 \text{ h}^{-1}$ . During fermentation course, the pH dropped from 4.8 after 17 hours to 2.7 at the end of the growth, suggesting that fermentation is controlled by pH (Fig. A). The cell concentration in YSW-G<sub>1</sub> medium, which corresponds to YSW-G medium plus 1% urea, after 23 hours of cultivation was  $1.1 \times 10^6$  CFU/ml. The peak was obtained after 168 hours of fermentation, with  $6.0 \times 10^6$  CFU/ml. There was a growth decrease after 192 hours. The kinetics parameters were a maximum specific growth rate of  $\mu_{\max} = 0.05 \text{ h}^{-1}$  and generation time of 13.8 hours. After 23 hours of fermentation the pH was 8.04, and 7.04 in the end of the

process, and the emulsification activities were 0.030, and 1.717 units, respectively (Fig. 1B).

The growth decrease of *C. lipolytica* was probably due to the addition of urea, which increased the pH of the medium. Cooper and Paddock (3) observed that *Torulopsis bombicola* growth decreased when urea was used instead of yeast extract in media with glucose and vegetable oils. Other microbial emulsifiers (sophorose lipids) have been synthesized by degradation of insoluble substrates such as vegetable oils or soluble substrates, like carbohydrates, as carbon sources, utilizing inexpensive media (6,12,13).

In this study, the YSW-G medium was the most effective for growth of *Candida lipolytica*. This result with the medium YSW supplemented with glucose without urea is corroborated by Cooper and Paddock (3,10).

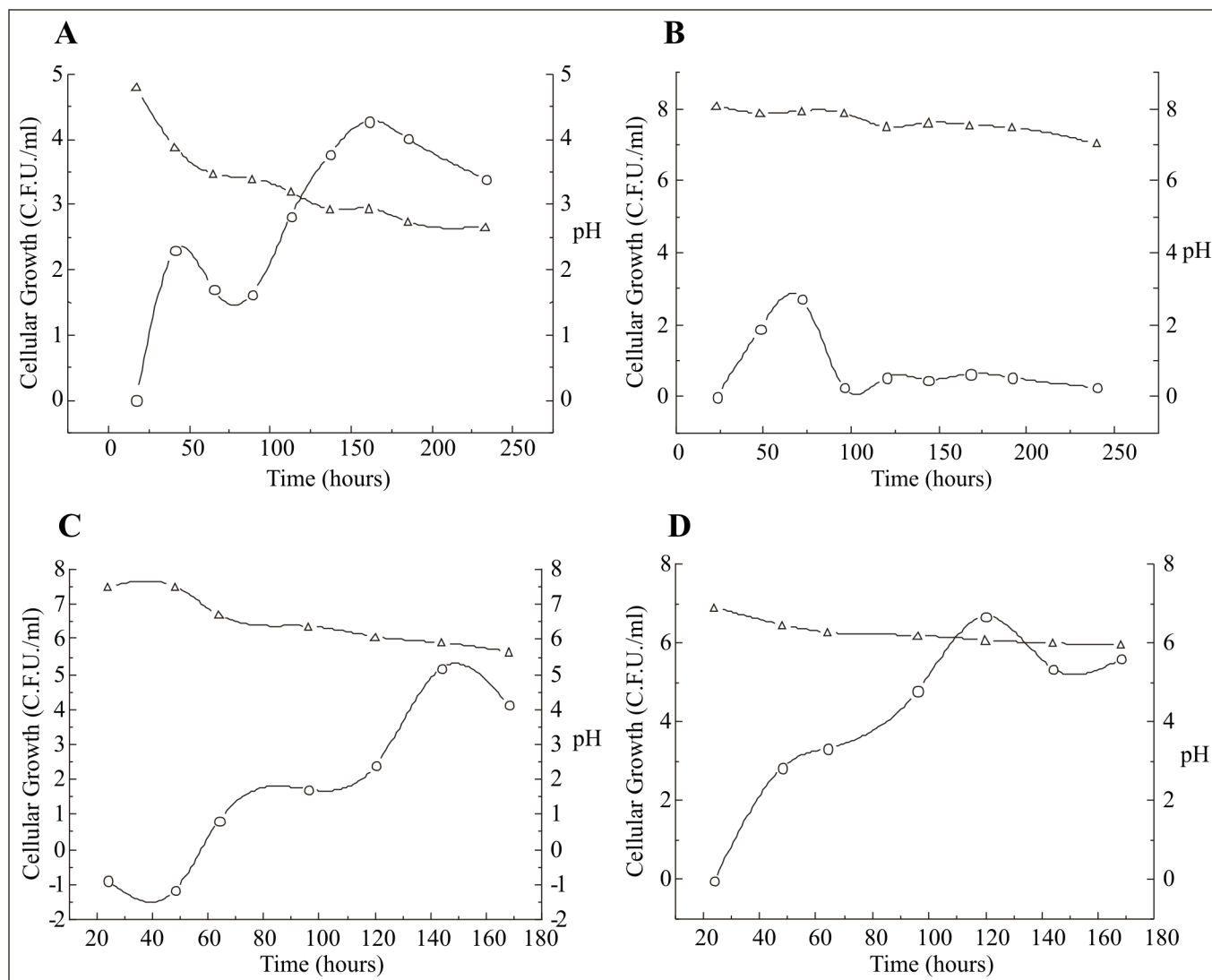
After 48 hours of cultivation,  $3.1 \times 10^2$  viable cells were observed in YSW-B<sub>2</sub> medium. The maximum number of cells was attained after 144 hours, with  $1.8 \times 10^7$  CFU/ml. The pH was 7.5 in the first stage (48 h) of the fermentation, and decreased to 5.6 in the end. The maximum specific growth rate was  $\mu_{\max} = 0.02 \text{ h}^{-1}$ , the generation time was  $34.6 \text{ h}^{-1}$ , and the emulsification activity was 0.666 units (Fig. 1C).

After 64 hours of fermentation *C. lipolytica* cultivated in the medium YSW-B<sub>3</sub> showed  $2.8 \times 10^5$  CFU/ml. The maximum number of viable cells occurred after 168 of cultivation. At the beginning of the process (24 h), the pH was 6.4, decreasing to 5.9 after 168 hours. A maximum specific growth rate of  $\mu_{\max} = 0.04 \text{ h}^{-1}$ , generation time of  $17.3 \text{ h}^{-1}$ , and 0.158 units of emulsification activity were observed (Fig. 1D).

Johnson (4) reported that the maximum emulsification activity in *Rhodotorula glutinis* was achieved in the presence of KNO<sub>3</sub>, followed by ammonium salt and urea. Our study with *C. lipolytica* showed that urea as an additional nitrogen source in the two tested media did not cause any decrease in growth rate when batch fermentation at exponential phase was used. These results suggested a decrease in the interfacial tension of babassu oil-containing YSW-B<sub>2</sub> and YSW-B<sub>3</sub> media caused by *C. lipolytica*, presumably due to release of fatty acids in the initial stages of fermentation. The production of fatty acids declines after 60 h of fermentation, but the interfacial tension continues.

The bioemulsifiers obtained from YSW-G and YSW-B<sub>3</sub> media were composed by proteins (54.3 and 43.0% w/v respectively), carbohydrates (35.5 and 40.0% w/v respectively) and lipids (8.4 and 16.0% w/v respectively).

The high production of emulsifiers in both media was probably caused by the presence of potassium phosphate. Palejwala and Desai (7) observed the influence of phosphate anions in the medium on the production of emulsifiers. It should be noticed that emulsifiers were produced in the two examined media (YSW-B<sub>2</sub> and YSW-B<sub>3</sub>) using low-cost substrates (babassu oil and seawater), which can be used for bioemulsifier production by *Candida lipolytica*.



**Figure 1.** Growth (○) of *Candida lipolytica* and pH (△) of YSW-G (A), YSW-G<sub>1</sub> (B), YSW-B<sub>2</sub> (C) and YSW-B<sub>3</sub> (D) media incubated at 28°C for 240 hours, 150 rpm.

#### ACKNOWLEDGEMENTS

Study supported by grants from CNPq, PRONEX, FINEP/CETPETRO and UNICAP.

#### RESUMO

##### **Novos bioemulsificantes produzidos por *Candida lipolytica* usando D-glicose e óleo de babaçu como fontes de carbono**

*Candida lipolytica* IA 1055 produziu biosurfactantes extracelulares com atividade de emulsificação, através de fermentação utilizando óleo de babaçu e D-glicose como fontes

de carbono. A água do mar diluída a 50% suplementada com uréia, sulfato de amônio e fosfato foi usada como meio basal. Os melhores resultados foram atingidos com os meios YSW-B<sub>2</sub> (contendo uréia, sulfato de amônio e óleo de babaçu) e YSW-B<sub>3</sub> (contendo uréia, sulfato de amônio, fosfato e óleo de babaçu), através de fermentação em batelada alimentada com 5% de óleo de babaçu. A velocidade específica de crescimento foi de 0,02 h<sup>-1</sup> e 0,04 h<sup>-1</sup>; tempo de geração de 34,6 h<sup>-1</sup> e 17,3 h<sup>-1</sup> e atividade de emulsificação igual a 0,666 e 0,158 unidades, respectivamente. As moléculas dos novos bioemulsificantes demonstraram ser constituídas por carboidratos, proteínas e lipídeos.

**Palavras-chave:** Bioemulsificante; *Candida lipolytica*; óleo de babaçu, água do mar

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