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# Bioemulsifier Production in Batch Culture Using Glucose as Carbon Source by *Candida lipolytica*

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## Abstract

The yeast *Candida lipolytica* IA 1055 produced an inducible extracellular emulsification activity while utilizing glucose at different concentrations as carbon source during batch fermentation at 27°C. In all glucose concentrations studied, maximum production of emulsification activity was detected in the stationary phase of growth, after pH reached minimal values. The bioemulsifier isolated was a complex biopolymer constituting proteins, carbohydrates, and lipids. The results obtained in this work show that the biosynthesis of a bioemulsifier is not simply a prerequisite for the degradation of extracellular hydrocarbon.

**Index Entries:** *Candida lipolytica*; biosurfactant; bioemulsifier; glucose fermentation.

## Introduction

Many prokaryotic and eukaryotic microorganisms satisfy their carbon and energy requirements by using water-immiscible substrates such as alkanes and oils. The growth on immiscible substrates is often associated

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with the production of surface-active compounds. Several different microbial products that exhibit surface-active properties have been identified in the past. These biosurfactants are produced by certain bacteria, yeast, and filamentous fungi. They include a wide variety of chemical structures such as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polysaccharide-protein complexes, and polysaccharide–protein–fatty acid complexes *(1)*.

Biosurfactants offer several advantages over chemical surfactants such as lower toxicity, biodegradability, and a wide range of properties for industrial applications. In addition, they might be able to replace, in some instances, the traditional synthetic surfactants *(2–5)*.

As described, water-immiscible substrates are commonly used for the production of surface-active components. Although less qualified for spontaneous formation, the production of biosurfactants from carbohydrates has been reported with *Arthrobacter* sp., *Bacillus subtilis*, *Torulopsis bombicola*, and *Pseudomonas aeruginosa*, among others *(6)*.

Although *Candida lipolytica* is known to produce an extracellular emulsification activity when grown with a number of water-immiscible carbon substrates *(7–9)*, we report here a batch process for bioemulsifier production by *C. lipolytica* using glucose as the carbon source that could potentially be used in food systems.

## Materials and Methods

### Microorganism

*C. lipolytica* IA 1055 was obtained from the culture collection of the Departamento de Antibióticos da Universidade Federal de Pernambuco. The microorganism was maintained at 4°C on yeast mold agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), glucose (1%), and agar (5%). Transfers were made to fresh agar slants each month to maintain viability.

### Production Media and Culture Conditions

Cultures were grown on a medium containing 0.6% (w/v) yeast nitrogen base (YNB) supplemented with 0.5, 1, and 1.5% (w/v) glucose. The YNB was sterilized by filtration (0.45-µm filter; Millipore, Bedford, MA).

*C. lipolytica* was grown in solid medium at 27°C for 48–72 h. Then, a loopful of the cream-colored culture was transferred to 250-mL Erlenmeyer flasks containing 50 mL of the liquid medium, yeast mold broth (CYM), and incubated aerobically for 1 d at 27°C on a rotary shaker (150 rpm).

The production of the emulsifiers was carried out in Erlenmeyer flasks with a 1000-mL capacity containing 300 mL of YNB medium supplemented with glucose (0.5, 1, and 1.5%) inoculated with $10^2$ CFU/mL of the 24-h culture grown on CYM and shaking at 150 rpm for 144 h at 27°C. The pH of the medium was not adjusted during cultivation.

Fermentations were monitored by aseptically removing samples (4 mL) every 24 h during the experiment. Microbial growth was determined by viable cell count. Samples were used for analytical measurements. All the assays were carried out in triplicate.

### *Isolation of Bioemulsifier*

The 144-h culture was filtered on a Whatman no. 1 paper and then filtered through a 0.45-µm membrane. The cell-free filtrate was transferred to dialysis tubing and concentrated to 50 mL by lyophilization. The concentrated filtrate was extracted with 500 mL of chloroform-methanol (2:1); a cream precipitate formed in the aqueous phase after the second extraction. The precipitate was collected on Whatman no. 1 filter paper and air-dried *(7,8)*.

### *Assay of Emulsification Activity*

Samples (3 mL) from shake-flask cultures were filtered by using a Millipore 0.22-µm membrane filter. The filtrate (2 mL) was placed in a screw-capped tube and diluted with 2 mL of 0.1 *M* sodium acetate solution (pH 3.0); one milliliter of hexadecane was added, the tube was capped, and the mixture was shaken for 2 min at 25°C. The resulting uniform emulsion was allowed to sit for 10 min, after which its absorbancy was measured at 540 nm. The blank used contained 2 mL of sterile YNB medium. One unit of emulsification activity was defined as the amount of emulsifier that affected an emulsion with an absorbancy at 540 nm of 1.0 *(7,8)*.

### *Analytical Methods*

Glucose was determined enzymatically in the metabolic liquid with a glucose analyzer (Bioclin-Quibasa, Brazil).

Characterization of biopolymers by high-performance liquid chromatography (HPLC) was performed at room temperature on a Shimadzu UV-VIS detector. Cell-free filtrates were injected (20 µL). The biopolymers were separated by one ULTRON ODS $C_{18}$ size-exclusion column. The mobile phase was methanol-water (1:1) at a flow rate of 1.0 mL/min. Column effluent was monitored at 225 nm. Dextran (100 µg/mL) was used as standard.

Protein was determined by the micro-Kjeldhal method *(10)*. Carbohydrate was determined by the phenol–sulfuric acid procedure *(11)*, using glucose as the standard. Lipids were determined by the colorimetric method of total lipids (Bioclin-Quibasa, Brazil).

## Results and Discussion

*C. lipolytica* has been shown to produce bioemulsifiers when it was grown with a number of water-immiscible carbon substrates. However, previous reports did not show the capacity of the yeast in producing an emulsification activity using glucose as the carbon source *(1,2)*.

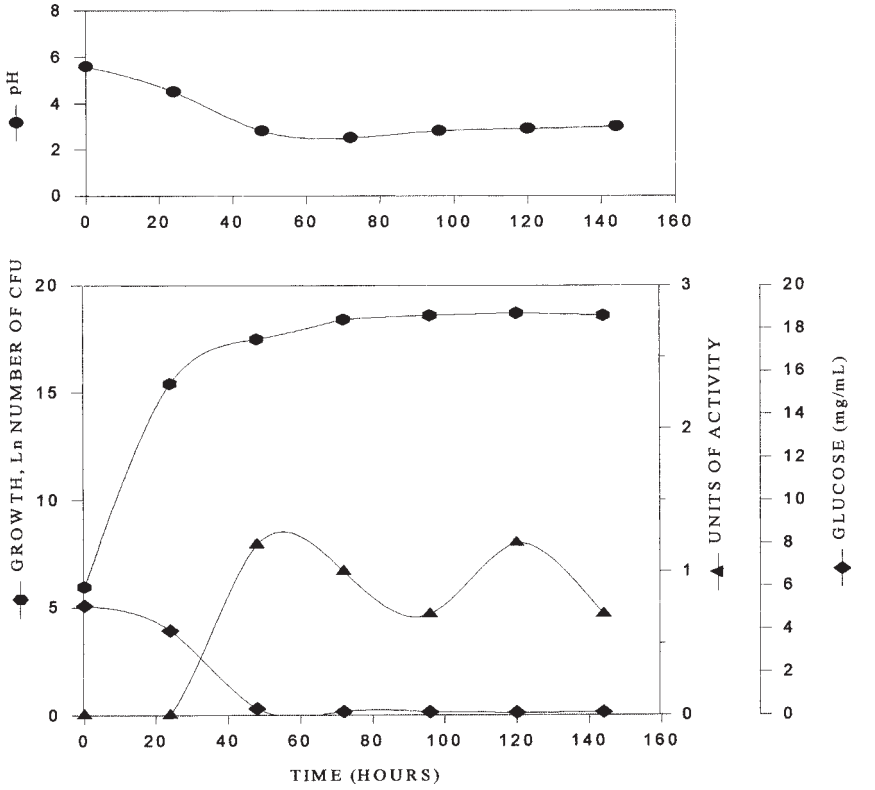


Fig. 1. Bioemulsifier production by *C. lipolytica* grown in YNB medium (0.6%) supplemented with 0.5% glucose. The conditions were as indicated in Materials and Methods: biomass produced, emulsification activity, glucose consumption, and pH.

Pareilleux *(9)* isolated surface-active compounds from *C. lipolytica* when grown on *n*-alkanes, but when the yeast was cultivated on glucose such surface-active materials were never produced. Later, Cirigliano and Carman *(7,8)* also isolated and characterized a bioemulsifier from the same yeast when it was grown with a number of water-immiscible carbon substrates, and they only observed the production of a negligible emulsification activity when *C. lipolytica* was grown in YNB supplemented with 1% glucose.

*C. lipolytica* was studied for the production of bioemulsifiers under batch fermentations with glucose (0.5, 1, and 1.5%) as the carbon source. Figures 1–3 show the growth, emulsification activity, pH determination, and glucose consumption.

The results show that *C. lipolytica*, in all glucose concentrations studied, demonstrated an exponential phase up to 48 h. For the maximum growth rate ($\mu_{max}$), similar values were observed for the different glucose concentrations of 0.24 $h^{-1}$ for 0.5 and 1% and 0.26 $h^{-1}$ for 1.5%, with generation times of 2.8 and 2.6 h, respectively.

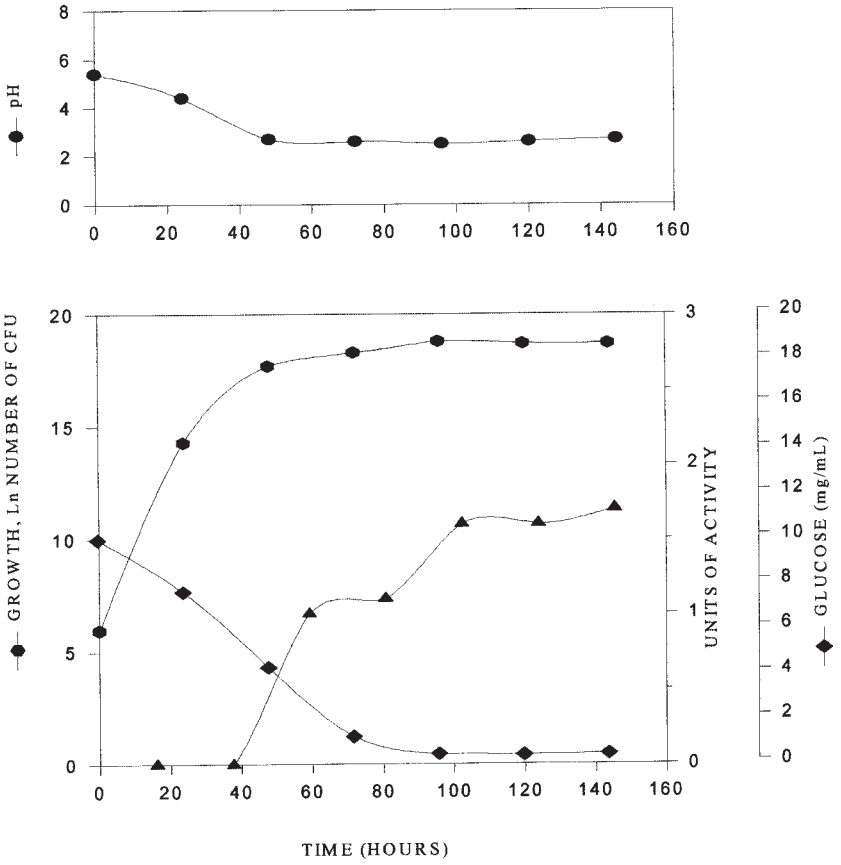


Fig. 2. Bioemulsifier production by *C. lipolytica* grown in YNB medium (0.6%) supplemented with 1% glucose. The conditions were as indicated in Materials and Methods: biomass produced, emulsification activity, glucose consumption, and pH.

For the three conditions studied (0.5, 1, and 1.5% glucose), glucose uptake began in the early phase of the culture, largely supported yeast growth, and was consumed according to a constant ratio. The glucose consumption rate for cultivation with glucose concentration of 0.5 and 1% decreased when the growth rate attained the stationary phase of growth, whereas for the third concentration (1.5%), the glucose consumption maintained a constant ratio until the end of cultivation, probably owing to the presence of residual glucose because of the carbon substrate concentration in the medium. However, regarding glucose consumption and biomass production, the concentration of carbon source did not affect the growth level (maximum), as demonstrated in Figs. 1–3. This behavior is probably owing to the utilization of carbon source for secondary metabolite production (bioemulsifier).

It is already known that the metabolism velocity of products is affected by the pH of the medium. In a fermentative process with no controlled pH, its value at anytime is the result of the microorganism metabolism in rela-

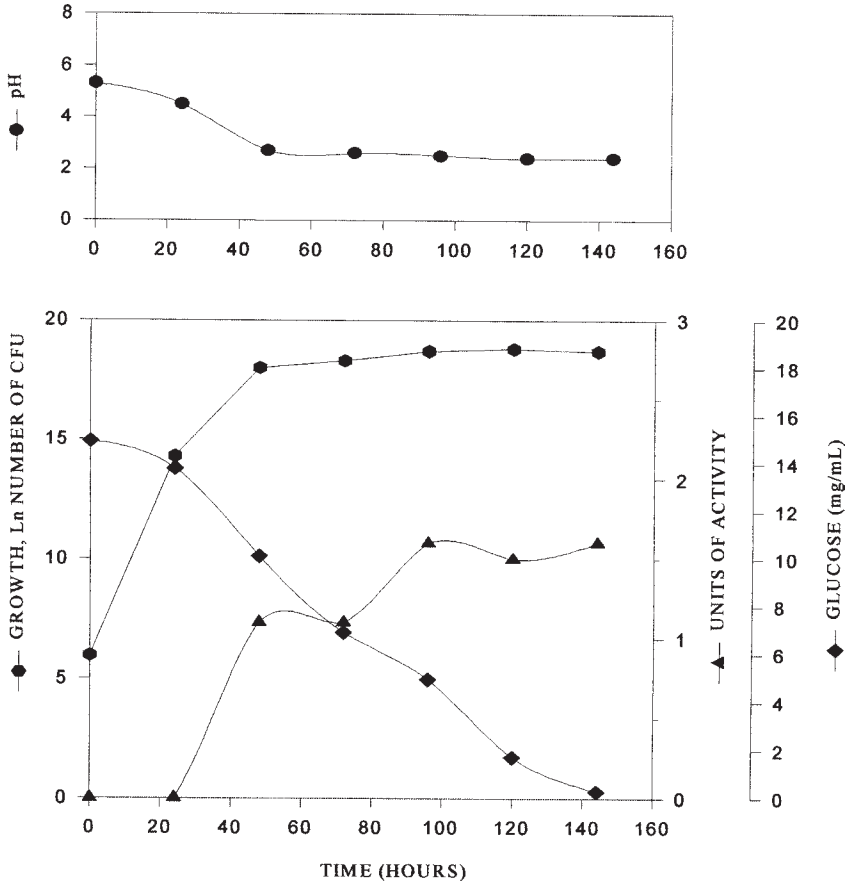


Fig. 3. Bioemulsifier production by *C. lipolytica* grown in YNB medium (0.6%) supplemented with 1.5% glucose. The conditions were as indicated in Materials and Methods: biomass produced, emulsification activity, glucose consumption, and pH.

tion to the initial pH and to the composition of the medium *(12)*. In our case, the initial pH of the cultures was approx 5.5 and the final was about 3.0, showing the medium's acidification. We can also observe from Figs. 1–3 that the pH decreased, and the maximum quantity of emulsifier activity was detected after the culture medium reached the minimal values, corresponding to the stationary phase of growth, until the last 144 h.

The production of emulsification activity was found not to be growth associated for the three conditions studied. It increased after the cells entered the stationary phase of growth. For the cultivation with 0.5% glucose concentration, Fig. 1 shows two peaks of activity, the first after 48 h of growth and the second after 120 h, demonstrating a diauxic profile. On the other hand, for the cultivation with a glucose concentration of 1% (Fig. 2), the emulsification activity was basically stable with high values until the end of fermentation, while for the third glucose concentration (1.5%), the activity curve presented two phases, the first between 48 and 72 h and

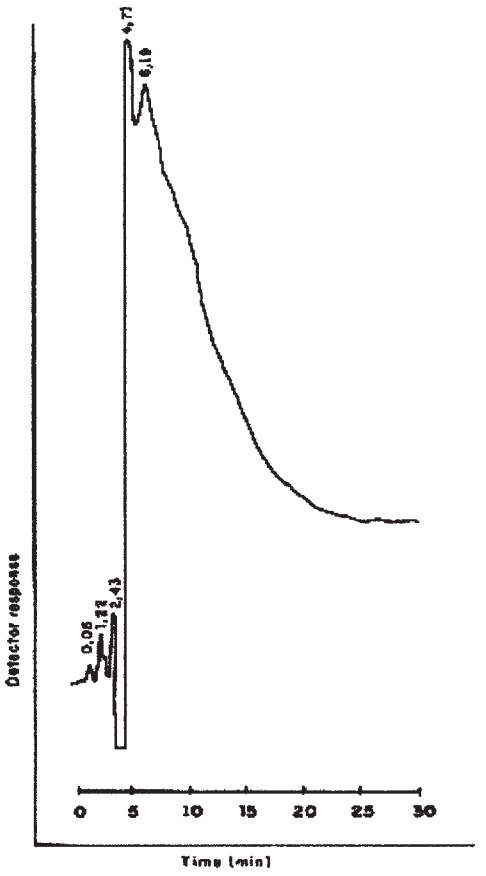


Fig. 4. Detector response for *C. lipolytica* cultivated in YNB medium (0.6%) supplemented with 1% glucose.

the second after 96 h, being practically linear until 144 h, with the higher values of activity (Fig. 3).

For partial characterization of biopolymers, one major peak for *C. lipolytica* cultivation in 1% glucose (Fig. 4) was detected at approx 5 min. Others were also identified in the beginning of the chromatogram at the same retention time of the peaks observed for the standard (Fig. 5), between approx 1 and 3 min, indicating the presence of carbohydrates in the bioemulsifier. However, for cultivation at glucose concentrations of 0.5 and 1.5%, we also observed small peaks at the same retention time for the standard and a main peak at the same retention time mentioned before. These major peaks observed in each one of the glucose concentrations studied are probably owing to the presence of other compounds in the bioemulsifiers obtained.

The precipitate analyzed from the cultivation of *C. lipolytica* at a glucose concentration of 1.5% contained about 47% protein, 45% carbohydrate (in accordance with the chromatograms), and 5% lipids.

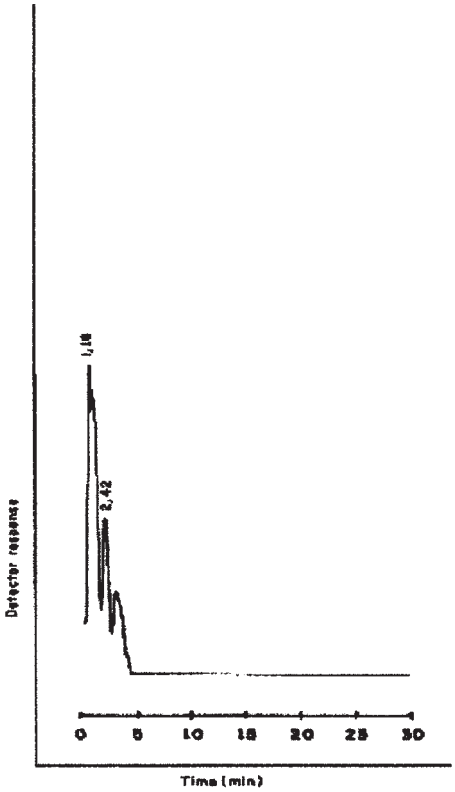


Fig. 5. Detector response for the standard (Dextran).

Our results are in accordance with those of Johnson et al. *(13)*, who showed the production of a growth-associated extracellular emulsifying agent by an oleaginous strain of *Rhodotorula glutinis* while utilizing glucose during fed-batch fermentation. Hommel and Huse *(14)* also observed the production of large amounts of sophorose lipids by *Candida apicola* when growing on glucose, fructose, or sucrose during the stationary phase. By contrast, no sophorose lipid formation was observed with galactose or maltose independently whether hydrocarbons were present or not. Another recent report demonstrated the necessity of glucose in culture media on the production of biosurfactants by two strains of biosurfactant-producing bacteria *P. aeruginosa*. Although the addition of crude oil at the stationary phase of growth resulted in an increase of biosurfactant production in cultures lacking glucose with or without crude oil, only a basal level of biosurfactant production was achieved *(15)*.

Several reports have been published on surfactant production by *C. lipolytica*, but for the conditions studied herein we did not find reports in the literature on the production of higher extracellular emulsification activity when using glucose as the carbon source by *C. lipolytica*. From our data we can conclude that the biosynthesis of a bioemulsifier is not simply a prerequisite for the degradation of extracellular hydrocarbon.

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