J Ind Microbiol Biotechnol (2008) 35:907–914
DOI 10.1007/s10295-008-0364-3

ORIGINAL PAPER

Experimental design for the production of tensio-active agent by *Candida lipolytica*

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Received: 16 October 2007 / Accepted: 10 April 2008 / Published online: 18 June 2008

Abstract The strategy of optimization using sequential factorial design was employed to enhance the tensio-active emulsifying agent produced by *Candida lipolytica* using soybean oil refinery residue as substrate. A full factorial design was used to evaluate the impact of three fermentation factors—amounts of refinery residue, glutamic acid and yeast extract. This allowed exclusion of the yeast extract. Full factorials designs were then sequentially used to optimize the levels of the residue and glutamic acid. The surface tension value was finally reduced to 25.29 mN/m. The maximum emulsifier activity using different substrates was within 40 h of cultivation. The surface tension of the cell-free broth containing the biosurfactant remained very stable during exposure to a wide range of pH (2–12), temperatures (0–120°C) and salinity (2–10% NaCl). The combination of an industrial waste and a cheap substrate therefore seems to be very promising for the low-cost production of potent biosurfactant.

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Keywords Medium optimization · *Candida lipolytica* · Biosurfactant · Factorial design · Refinery residue

Introduction

Surfactants, amphiphilic molecules consisting of a hydrophilic head and a hydrophobic tail, are the active ingredients found in soaps and detergents. Due to their ability to concentrate at the air-water interface, they are commonly used to separate oily materials from a given medium. Surfactants increase the aqueous solubility of hydrophilic molecules by reducing their surface/interfacial tension at air–water and water–oil interfaces [24]. As the interfacial tension is reduced and the aqueous surfactant concentration is increased, the monomers aggregate to form micelles. The concentration at which micelles first begin to form is known as the critical micelle concentration (CMC). This concentration corresponds to the point where the surfactant first shows a stable low surface tension value [12].

Almost all surfactants being currently produced are chemically derived from petroleum. However, these synthetic surfactants are usually toxic themselves and hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in recent years, draw much attention to the microbial production of surfactants (biosurfactants) [30].

Biosurfactants are derived from living organisms, mainly microorganisms, and have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, biodegradability, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and

pharmaceutical industries. Some biosurfactants, moreover, are known to have therapeutic applications as antibiotics and antifungal or antiviral compounds. They can also be used in the bioremediation of soil or sand or in the cleanup of hydrocarbon contamination in groundwater [28, 32].

The types of biosurfactants include lipopeptides synthesized by many bacilli and other species, glycolipids synthesized by *Pseudomonas* and *Candida* species, phospholipids synthesized by *Thiobacillus thiooxidans*, and polysaccharide-lipid complexes synthesized by *Acinetobacter* species, or even the microbial cell surface itself [31, 34].

Even though interest in biosurfactants is steadily increasing, these compounds still do not compete economically with synthetic surfactants. To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering process, and the use of cost free or cost-credit feedstock for microorganism growth and surfactant production [17].

Agrorefinery residues with high contents of carbohydrates or lipids could in principle be used as substrates for biosurfactant production [15]. However, few attempts at using wastes for biosurfactants production and only few types of biosurfactants produced from wastes have been reported so far. Possible substrates for biosurfactant accumulation include peat hydrolysate, olive oil mill effluent, lactic whey, soybean curd residue, potato process effluent, molasses and cassava flour wastewater [17, 29].

To develop a process for maximum surfactant production, standardization of the medium and fermentation conditions is crucial. Medium improvement by the classical one-factor-at-a-time method in which one variable is changed while the others remain at fixed levels is laborious and time-consuming, especially when the number of variables is large. It can also be misleading, because possibly significant variable interaction effects are not accounted for. An alternative and more efficient approach is the use of statistically designed methods in which the levels of all variables are simultaneously changed [4]. Indeed, most recent optimization efforts have relied on statistical experimental design and response surface analysis [18] and, to a lesser extent, on artificial intelligence techniques such as genetic algorithms [13, 35]. Statistical designs constitute a powerful tool to evaluate the main as well as interaction effects of the fermentation parameters on process performance. They are an efficient way to generate useful information from limited experimentation, thereby cutting process development time and cost [4, 21]. In this study, a sequential strategy based on two-level factorial designs was employed to find optimal levels of substrate concentrations (soybean oil refinery residue and glutamic acid) for the production of a biosurfactant by *C. lipolytica*.

Materials and methods

Microorganism

The microorganism *C. lipolytica* UCP 0988 was kindly supplied from the Culture Collection of Nucleous of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil. The microorganism was maintained as the anamorph state at 5°C on yeast mold agar (YMA) slants containing (w/v): 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar. Transfers were made to fresh agar slants each month to maintain viability.

Growth conditions

The production medium used for the experiments consisted of the following: 0.1% NH_4NO_3, 0.02% KH_2PO_4 and 0.02% $MgSO_4{\cdot}7H_2O$. The medium was supplemented with soybean oil refinery residue, glutamic acid and yeast extract. The refinery residue was obtained from ASA Indústria e Comércio LTDA (Recife-PE, Brazil). The composition of the refinery residue was previously described [22].

The inoculum was prepared in an Erlenmeyer flask with a capacity of 250 ml containing 50 ml of yeast mold broth (YMB) and was inoculated using a microbial loop, incubated in an orbital shaker at 150 rpm and 28°C for 24 h. The pH of the culture medium was adjusted to 5.7 by addition 1 M NaOH solution or 1 M HCl solution. All fermentations were conducted in 250 ml Erlenmeyer flasks containing 50 ml of the production medium. Immediately after inoculation of 5% of 10^8 cells/ml, the flasks were incubated for 72 h at 28°C in an orbital shaker at 150 rpm. The composition of the culture medium was varied according to the experimental designs described below. All experiments were performed in duplicate.

Experimental factorial designs

A sequential strategy based on two-level full factorial designs was employed to optimize biosurfactant production. In the first step, a full 2^3 factorial design, augmented with a center point run in quadruplicate, was used to evaluate the impact of three independent factors—the amounts of soybean oil refinery residue, glutamic acid and yeast extract—on the medium's surface tension, using the levels shown in Table 1. After the results of this first design were analyzed, it became clear that the amount of yeast extract could advantageously remain fixed at its lowest level. The subsequent designs, therefore, were based only on the remaining two factors, the amounts of residue and glutamic acid. Four full two-level designs on these two variables were successively carried out, progressively exploring the

Table 1 Levels of the full 2^3 factorial used as a starting point to assess the effects of the medium constituents on biosurfactant production by *Candida lipolytica*

Factors	Coding		
	−1	0	+1
a—Residue (%)	1	2.5	4
b—Yeast extract (%)	0	0.1	0.2
c—Glutamic acid (%)	0	0.5	1

response surface with the goal of finding the combination of residue and glutamic acid levels yielding the lowest surface tension values.

Determination of the dry weight of the culture

For cell growth determination, 50 ml samples were centrifuged at 4,400 rpm at 28°C during 15 min. After washing with demineralised water, the cell pellet was dried in an oven at 105°C for 24 h, cooled in a desiccator and weighed. They were then repeatedly re-weighed until a constant dry weight was obtained.

Assay of emulsification activity

Emulsification activity was determined using the method described by [6]. Samples from shake flask cultures were centrifuged at 10,000×*g* for 15 min. The cell-free broth (2 ml) obtained after centrifugation was diluted with 2 ml of 0.1 M sodium acetate buffer (pH 3.0) and 1 ml of one of the following substrates: *n*-hexadecane, corn oil, cotton seed oil and canola oil. The mixture was placed in a screw-capped tube and shaken for 2 min at 27°C. The resulting uniform emulsion was allowed to sit for 10 min, after which its absorbance was measured at 540 nm. The blank used contained 2 ml of sterile production medium. One unit of emulsification activity (UEA) was defined as the amount of emulsifier that affected an emulsion with an absorbance at 540 nm of 1.0.

For estimation of the emulsification index, 2 ml of *n*-hexadecane or motor oil or corn was added to 2 ml of the cell-free culture broth in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The emulsification index was calculated by measuring the emulsion layer thus formed [8].

Surface activity

Surface tension was determined on cell-free broth obtained by centrifuging the cultures at 10,000×*g* for 15 min with a Tensiometer model Sigma 70 (KSV Instruments Ltd., Finland) using the Du Nouy ring method at room temperature.

Stability studies

Stability studies were done using the cell-free broth obtained centrifuging the cultures at 10,000×*g* for 15 min. Four milliliter of the culture broth free of cells were heated at 70, 100 and 120°C during 1 h, and cooled to room temperature, after which the emulsification activity was measured. The emulsification capacity of culture broth free of cells was also determined after exposure at lower temperature (0–5°C). To study the pH stability of the cell-free broth, the pH of the cell-free broth was adjusted to different pH values (2–12) and the emulsification activity was measured. The culture liquid pH was adjusted with 1 M NaOH. The effect of NaCl concentrations (2–10%) on the emulsification capacity of the culture broth free of cells was also determined [1].

Statistical analysis

All data analyses and graphs were made with the Statistica 6.1 software package [27]. The statistical significance of the results was tested at the $P < 0.05$ level.

Results and discussion

Optimization of medium constituent levels

In the first design, three factors that in principle could be highly influential on biosurfactant production were considered: the concentrations of soybean oil refinery residue, yeast extract and glutamic acid, set at all combinations of the extreme levels given in Table 1. The surface tension results of the eight experimental runs of the initial, full 2^3 design, plus the four replicates run at the central point are given in Table 2. The mineral constituents of the medium were kept the same in all experimental runs, and the surface tension values were determined after 72 h of cultivation.

The effects calculated from the data in Table 2 are given in Table 3. Only the residue main effect (**a**) and its two-way interaction with glutamic acid (**a**, **c**) are statistically significant at the 95% confidence level. Since both values are negative, this means that raising the amount of residue will tend to lower the surface tension, and more so if the glutamic acid concentration is raised at the same time. This is easily visualized on the 3D plot shown in Fig. 1. Although the other effects are not significant, it should be noticed that the yeast extract main effect on the surface tension is positive, indicating that to obtain lower tension values it is preferable to keep this factor at its lower level. The best result, 26.32 mN/m, is that of run no. 6, closely followed by that of run no. 8 (29.22 mN/m), which only differs from it by the level of yeast extract.

Table 2 Surface tension values obtained in the 2^3 factorial design. Factors **1**, **2** and **3** are the concentrations of residue, yeast extract and glutamic acid, respectively. Levels are coded as in Table 1

Run	Factor level			Surface tension (mN m^{-1})
	a	**b**	**c**	
1	−1	−1	−1	38.99
2	+1	−1	−1	34.25
3	−1	+1	−1	44.08
4	+1	+1	−1	37.16
5	−1	−1	+1	41.77
6	+1	−1	+1	26.32
7	−1	+1	+1	43.41
8	+1	+1	+1	29.22
9	0	0	0	40.97, 38.72, 41.71, 42.49

Table 3 Main and interaction effects calculated from the data in Table 2. Statistically significant values (at the 95% confidence level) are shown in boldface

Effect	Numerical value
a—residue	**−10.33**
b—Yeast extract	3.13
c—Glutamic acid	−3.44
ab	−0.23
ac	**−4.50**
bc	−0.86
abc	0.86

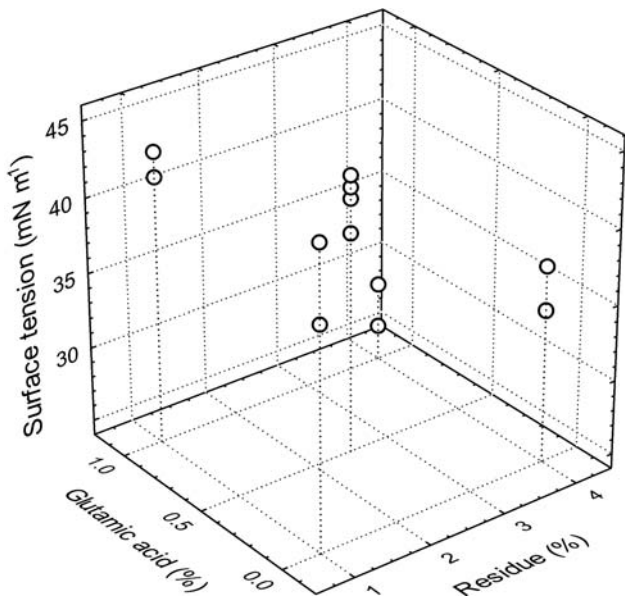

Fig. 1 Three-dimensional plot of the surface tension values against the residue and glutamic acid concentrations, for the first design. The smallest values occur when both of these factors are at their higher levels

Factorial design studies conducted with *Serratia* sp. SVGG16 showed a reduction of the surface tension of the medium from 67.8 to 34.4 mN/m after 96 h of cultivation [10]. Mulligan [16], observed that the biosurfactants produced by *B. subtilis* reduced the surface tension of the culture medium to 27 mN/m. Recently, Sarubbo et al. [24] observed that the biosurfactant produced by *C. glabrata* UCP 1002 reduced the surface tension from 68 mN/m to 31 mN/m, while the biopolymer produced by *C. bombicola* ATTC 22214 reduced the surface tension of the water from 72 to 38.9 mN/m [19].

Table 4 Surface tension values obtained in the follow-up 2^2 factorial designs. The designs are presented down the table in the order in which they were carried out

Run	Residue (%)	Glutamic acid (%)	Surface tension (mN m^{-1})
1	4	1	28.78
2	8	1	26.68
3	4	2	26.96
4	8	2	26.62
5	6	1.5	26.63, 26.44, 26.59
1′	5	1.25	33.43
2′	7	1.25	25.76
3′	5	1.75	27.07
4′	7	1.75	26.42
5′	6	1.5	26.82, 26.44, 26.60
1″	6	1	25.29
2″	8	1	26.71
3″	6	1.5	26.60
4″	8	1.5	25.99
5″	7	1.25	25.76, 25.68, 25.70
1‴	5	0.5	26.34
2‴	6	0.5	25.65
3‴	5	1	26.58
4‴	6	1	25.29
5‴	5.5	0.75	26.70, 26.35, 26.50

The use of soybean oil refinery residue [23] was used in this work as carbon source considering chemical composition (fatty acids 60% and carbohydrate 35%). In addition, the use of the residue and glutamic acid in substitution of yeast extract permitted us to select a low-cost medium.

To find out if it was possible to further reduce the medium surface tension values, this first study was followed by four others in which only the levels of soybean oil refinery residue and glutamic acid were varied, taking as the starting point the best conditions of Table 2. All these subsequent designs were full two-level factorials, augmented with a replicated central point. The results thus obtained are collected in Table 4. The range spanned by them is remarkably small, indicating that the surface response at this experimental region is an almost horizontal plateau. Fig. 2 shows a quadratic function fitted to the points of the three last designs. It is clear that the most favorable region, represented by the dark gray area,

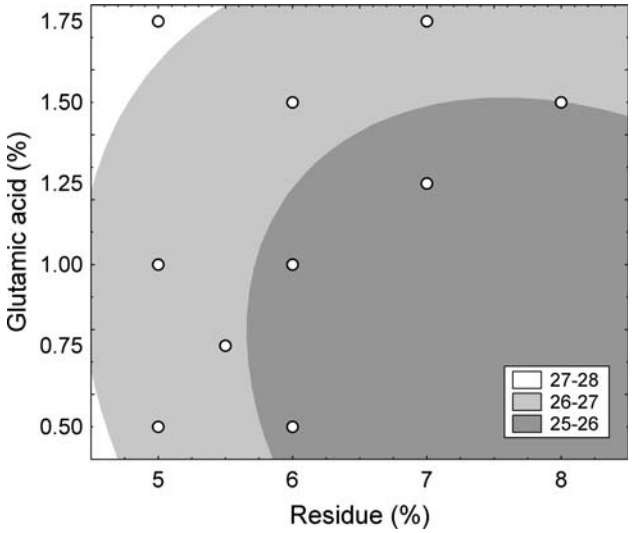

Fig. 2 Quadratic surface function fit to the data of the three last designs. Its curvature is very slight. The best (lowest) surface tension values are obtained in the region represented by the *dark gray* area, that is, with smaller glutamic acid and larger residue concentrations

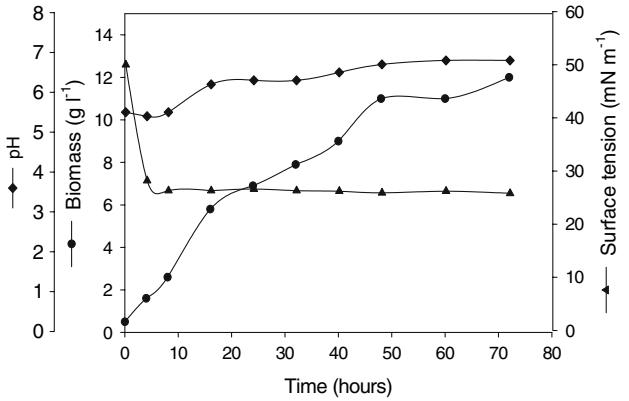

Fig. 3 Growth, surface activity and pH profiles of *Candida lipolytica* UCP0988 grown in the optimized medium (6% refinery residue, 1% glutamic acid, 28°C)

includes smaller glutamic acid concentrations and intermediate to higher residue concentrations.

In the factorial design experiments with *C. lipolytica* showed the tendency for increasing the catabolism of carbon source utilizing the industrial residue, and the maintenance of the glutamic acid, as nitrogen source. However, in many fermentative processes, the relation carbon/nitrogen is a very sensible parameter that influences metabolites accumulation in the medium. In this case, the preferred condition was residue at 6 and 1% of glutamic acid sources for high level of biosurfactant production. The regulation mechanism by the nitrogen is not completely understood. In this paper we have shown the high level of residue and low level of glutamic acid (proportion 6:1) could lead to enhanced growth and the production of biosurfactant. Glutamic acid was found to be the best nitrogen source. On the other hand, the excess of the nitrogen source displace the substrate for the biosynthesis of cellular material, limiting the accumulation of other metabolites. Thus, the results obtained in this work are in accordance with the described in the literature once the increase of the carbon source leaded to the reduction of the surface tension [26].

The reduction of surface or interfacial tension has often been used as the primary criterion for screening microorganisms for bioemulsifier production. According to Pruthi and Cameotra [20] the reduction of surface tension of the medium is a rapid method for assay of maximum biosurfactant formation prior to their actual isolation. Furthermore, some microbial emulsifiers such as the sophorolipids from *Torulopsis bombicola* [9], actually *C. bombicola* have been shown to reduce surface and interfacial tension but not to be good emulsifiers [33]. Taking these facts into account, the ability to form stable emulsions of the biosurfactants produced by *C. lipolytica* cultivated in the refinery residue was evaluated in the first factorial design with different substrates. The cell-free broth containing the biosurfactant produced by *C. lipolytica* emulsified 79% of the motor oil, but corn oil and *n*-hexadecane were not effectively emulsified (data not shown). These findings suggest that the emulsifier's activity depends on its affinity for hydrocarbon substrates, which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium. The results obtained in this work show the ability of the biosurfactants produced to act as potent surface-active compounds and as bioemulsifiers. The liposan from *C. lipolytica* ATCC8662 has been shown not to reduce the surface tension of water and yet has successfully emulsified commercial edible oils [7].

Fermentation kinetics of the biosurfactant produced under optimized condition

Biosurfactant production by *C. lipolytica* was carried out in the optimized medium containing 6% soybean oil refinery residue and 1% glutamic acid. As shown in Fig. 3, growth started without a lag time and stopped after about 48 h, when biomass reached 11 g/l. The specific growth rate was 0.15 h^{-1}, with a generation time of 4.62 h. During the first 12 h, the pH practically did not vary, but after that point it started to rise, reaching 6.8 after 72 h. The surface tension of the culture broth (50 mN/m) dropped rapidly after inoculation, reaching its lowest value (25.29 mN/m) during the exponential phase after about 16 h, and remaining stable after that. This value compares favorably with that reported for the most powerful biological surfactant, surfactin [8], and Yansan [3] indicating excellent surface-active properties. Recently, the biosurfactant produced by *C. lipolytica*

in canola oil and glucose showed to reduce the surface tension to 30 mN/m during the exponential growth phase [23].

Figure 4 shows, for different substrates, the kinetics of the emulsifying activity of *C. lipolytica* UCP0988 grown in the optimized medium. Higher and more stable emulsification activities, between 4.8 and 5.5 UEA, were detected for all substrates after the microorganism had entered its stationary growth phase. *Yarrowia lipolytica* showed values around 2.0 UEA after 170 h of cultivation for Yansan, an emulsifier produced in a medium supplemented with glucose. Yansan was also tested for different aliphatic HCs, showing emulsification activities values between 1.0 and 3.0 [3]. Sarubbo et al. [25] detected values of 1.8 UEA for the biosurfactant produced by *C. lipolytica* IA 1,055 cultivated in glucose as carbon source, while the biosurfactant from *C. lipolytica* cultivated in corn oil showed values around 3.727 UEA after 127 h of fermentation [2]. Kim et al. [14] detected values of 2.51 UEA for the biosurfactant produced by *Nocardia* sp. L-417. The results found in this work for the biosurfactant produced by *C. lipolytica* were also superior to those described for the synthetic commercial surfactants tested by Amaral et al. [3].

Biosurfactant properties

The stability of the surface tension is an important factor for the utilization of biosurfactants under specific environmental conditions [16]. The influence of temperature over the surface tension of the cell-free broth produced by *C. lipolytica* in the optimized medium is shown in Fig. 5. The results obtained showed that the cell-free broth was thermally stable. Similar behaviors regarding stability were also observed for the biosurfactants produced by *Bacillus subtilis* [5] and by *Nocardia* sp. L-417 [14] when the cell-free broths were heated at 100°C. Liposan from *C. lipolytica* ATCC8662 found to be relatively stable between 30 and 90°C, but lost 60% of its activity after boiling for 1 h [6].

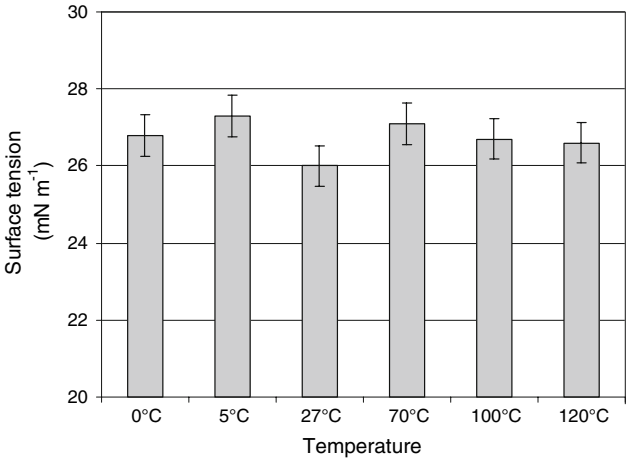

Fig. 5 Effect of temperature on the emulsifying activity of cell-free broth of *C. lipolytica* grown in the optimized medium. SD of 0.12

The cell-free broth of *C. lipolytica* was adjusted to various pH in the range 2–12 at room temperature, following which the surface activities were measured (Fig. 6). The surface tensions were maintained practically uniformly at all pHs, indicating that variation in pH had no appreciable effect on surface tension. The little change observed at pH 12 must be consequence of the desnaturation of proteinaceous compounds of the biosurfactant under extreme pHs, as suggested by Ghurye et al. [11].

Figure 7 shows the effect of sodium chloride addition on surface tension of the biosurfactant produced by *C. lipolytica* cultivated in the optimized medium. Little changes were observed with the addition of 10% sodium chloride. Similar effects were observed for other biosurfactants produced by bacteria [1]. The sophorolipids of *T. bombicola*,

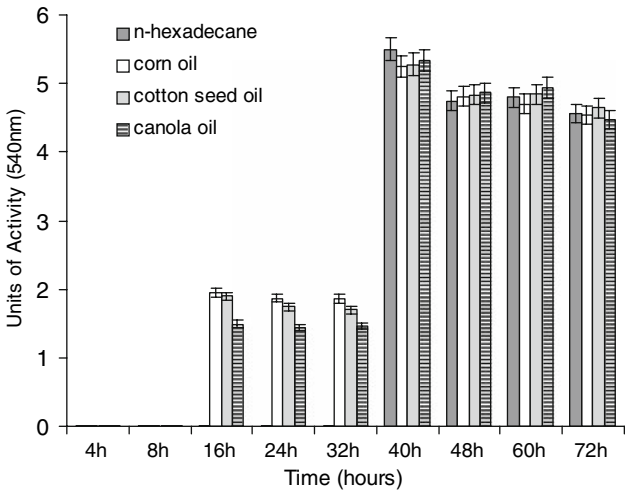

Fig. 4 Kinetics of the emulsifying activity of the biosurfactant produced by *C. lipolytica* grown in the optimized medium (*Bar* 1 SD)

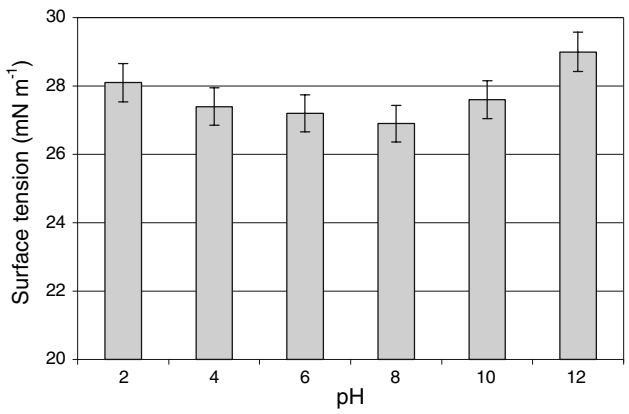

Fig. 6 Effect of pH on the emulsifying activity of cell-free broth of *C. lipolytica* grown in the optimized medium. SD of 0.10

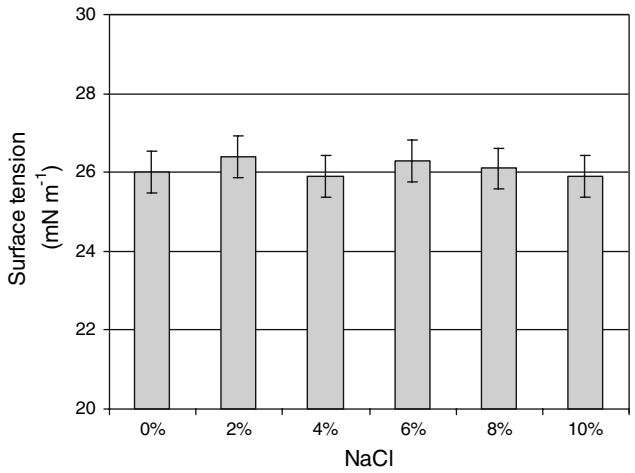

Fig. 7 Effect of different sodium chloride concentrations on the emulsifying activity of cell-free broth of *C. lipolytica* grown in the optimized medium. SD of 0.09

actually *C. bombicola,* showed consistent properties between pH values of 6–9, various salt concentrations and temperatures ranging from 20 to 90°C [9]. Sarubbo et al. [24] observed a reduction of approximately 20% of surfactant activity with the addition of up 10% (w/v) sodium chloride, showing a relative tolerance over these salt concentrations.

Conclusions

The new biosurfactant produced by *C. lipolytica*, besides being a good surfactant, has attractive properties as a tensoactive compound. The biosurfactant has several properties that are desirable for industrial processes. It is not affected by temperature, pH, and NaCl concentrations. So, the biosurfactant has shown several properties which could be attractive and potent surface-active compound useful in many fields of industry.

Acknowledgments We are grateful to the CNPq, CAPES, FINEP, and Nucleous of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil.

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