Improvement of biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358 and its potential application in bioremediation

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HIGHLIGHTS

- *Wickerhamomyces anomalus* CCMA 0358 produced a promising biosurfactant.
- Biosurfactant production was optimized through response surface methodology.
- The surface tension was reduced in 24 h from 49 mN/m up to 29 mN/m in bioreactor.
- The biosurfactant was stable at extreme conditions of salinity and temperature.
- The biosurfactant facilitated the recovery of 20% of oil from contaminated sand.

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ABSTRACT

In this work, biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358 was increased through the development of an optimized culture medium using response surface methodology. The optimized culture medium contained yeast extract (4.64 g/L), ammonium sulfate (4.22 g/L), glucose (1.39 g/L) and olive oil (10 g/L). Biosurfactant production using this medium was validated both in flasks and bioreactor, and the surface tension was reduced from 49.0 mN/m up to 31.4 mN/m and 29.3 mN/m, respectively. In both cases, the highest biosurfactant production was achieved after 24 h of growth. *W. anomalus* CCMA 0358 demonstrated to be a fast biosurfactant producer (24 h) as compared to other yeast strains previously reported (144–240 h). The produced biosurfactant remained stable at high temperature (121 °C), NaCl concentrations as high as 300 g/L, and pH values between 6 and 12. The crude biosurfactant allowed the recovery of 20% of crude oil from contaminated sand, being a promising candidate for application in bioremediation or in the petroleum industry.

1. Introduction

Surface-active compounds (surfactants) exhibit a wide variety of applications and are present in nearly every product and aspect of our daily life. They are included as active ingredients in the formulation of detergents, cleaning and personal care products and cosmetics, and are also used in bioremediation, as well as in agriculture, food, paper, textile and petroleum industries, among others [1–7]. Due to their structure, surfactants reduce the surface and interfacial tensions, which confers them properties such as detergency, emulsifying activity, foaming, and ability to solubilize and disperse hydrophobic compounds in aqueous phases [1,8,9]. The current worldwide production of surfactants is estimated to be 15 million tons per year [3]. Most of the traditional chemical surfactants commonly used are obtained from petrochemical resources, and exhibit high toxicity and low biodegradability [9,10]. Given the increasing concern regarding the environmental hazard of synthetic surfactants, and in order to address the growing demand for eco-friendly alternatives, research focusing on the production of bio-based surfactants has increased in the past few years [11]. The market for these "green" alternatives to the traditional chemical surfactants is expected to increase up to USD 2800 million by 2023, with a worldwide production around 462 kilo tons per year [12].

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Among the bio-based surfactants, biosurfactants, a heterogeneous group of surface-active compounds synthesized by different microorganisms, have emerged as promising alternatives to the synthetic surfactants. In the past 10 years, biosurfactants have received pronounced attention owing to their excellent interfacial activities, low toxicity, high biodegradability, and stability under extreme conditions of temperature, pH and salinity [4,8,11,13]. As a result of these properties, biosurfactants could replace chemical surfactants in many industrial applications [14–18]. However, their large-scale industrial production and commercialization are still limited by the relatively low efficiency of their production and recovery processes, as well as the high price of the culture media used for their synthesis, which results in high production costs. Extensive efforts have been made to produce biosurfactants at a competitive cost through the use of agro-industrial wastes and by-products as substrates, and the development of effective recovery processes [3,9,19–26]. Furthermore, the application of statistical experimental designs such as response surface methodology (RSM) has been successfully used to optimize the composition of the culture medium and the culture conditions to maximize biosurfactant production by different microorganisms [23–25].

In this work, the composition of the culture medium was optimized through RSM to increase the biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358. The results obtained were validated in shaken flasks and bioreactor. The stability of the biosurfactant produced by this yeast at different environmental conditions, as well as its potential applicability in bioremediation was also studied.

2. Experimental

2.1. Strain and culture conditions

The yeast strain *W. anomalus* CCMA 0358 (isolated from coffee processing by-products) was obtained from the Culture Collection of Agricultural Microbiology, CCMA (Department of Biology, Federal University of Lavras, Brazil). This isolate was identified as a promising biosurfactant producer in our previous work [27]. The yeast was grown in YEPG medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose; pH 6.5) at 28 °C and 200 rpm.

2.2. Biosurfactant production

The culture medium used for the production of biosurfactant by *W. anomalus* CCMA 0358 consisted of: 4 g/L yeast extract; 2 g/L glucose; 4 g/L ammonium sulfate; and 20 g/L olive oil [27]. The experiments were performed in flasks (500 mL capacity) containing 200 mL of culture medium. Each flask was inoculated with a pre-culture of *W. anomalus* CCMA 0358 (grown overnight in YEPG medium at 28 °C and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. Subsequently, the flasks were incubated at 28 °C and 200 rpm for 24 h. Samples (4 mL) were taken along the fermentation to evaluate growth and biosurfactant production. Cell growth was determined according to the number of cells counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). Afterwards, the samples were centrifuged (2700 × g, 15 min) and the cell-free supernatants were used to assess biosurfactant production through surface tension measurement, as described below.

2.3. Surface tension measurement

The surface tension of the cell-free supernatants was measured according to the Ring method as described elsewhere [27]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9 cm

Table 1
Central composite design factors and corresponding variation ranges according to the experimental levels.

Factor	Levels				
	−1.68	−1	0	+1	+1.68
	Concentration (g/L)				
Yeast Extract (X_1)	0.60	2.0	4.0	6.0	7.4
Ammonium Sulfate (X_2)	0.60	2.0	4.0	6.0	7.4
Glucose (X_3)	0.32	1.0	2.0	3.0	3.68

Table 2
Matrix of the central composite design showing the concentration of the different factors for the experimental design and the results of the experimental runs (predicted and observed).

Experiment	Concentration (g/L)			ST (mN/m)	
	X_1	X_2	X_3	Predicted	Observed
1	2	2	1	34.4	33.2
2	6	2	1	31.8	30.9
3	2	6	1	34.2	33.5
4	6	6	1	31.9	30.9
5	2	2	3	34.3	34.3
6	6	2	3	32.1	31.9
7	2	6	3	35.3	35.3
8	6	6	3	33.3	33.5
9	0.6	4	2	38.8	39.5
10	7.4	4	2	35.0	35.7
11	4	0.6	2	30.0	30.9
12	4	7.4	2	30.9	31.4
13	4	4	0.32	32.2	34.0
14	4	4	3.68	33.3	32.9
15	4	4	2	31.9	32.0
16	4	4	2	31.9	31.8
17	4	4	2	31.9	31.6

De Noüy platinum ring was used. All the measurements were performed in triplicate at room temperature (25 °C).

2.4. Culture medium optimization through response surface methodology (RSM)

The composition of the culture medium described above was optimized in order to maximize biosurfactant production by *W. anomalus* CCMA 0358. The concentration of three medium components and the effect of the interactions between them on biosurfactant production were analyzed and optimized by RSM using a central composite design (CCD). The medium components (independent factors) selected to perform the optimization process were yeast extract (X_1), ammonium sulfate (X_2) and glucose (X_3). Each factor was studied at five coded levels (−1.68, −1, 0, +1, +1.68), as shown in Table 1. Statistica 8.0 software was used to generate a set of 17 experiments, which included 3 replicates at the central point (Table 2).

The experiments were performed in flasks (500 mL capacity) containing 200 mL of the different culture media (prepared according to the CCD (Table 2)); all the media were supplemented with 20 g/L of olive oil. The flasks were inoculated and incubated at the same conditions described above. Cell growth and biosurfactant production were evaluated as previously described. The surface tension values obtained for each experimental run were taken as the response variable, and the biosurfactant production was assessed as the surface tension reduction. All experiments were performed in triplicate.

The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order

polynomial equation to define the predicted responses in terms of the independent factors:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response value; β_0 is the intercept term; β_i are the linear coefficients; β_{ii} are the quadratic coefficients; β_{ij} are the interaction coefficients; and X_i-X_j are the independent factors.

Statistica 8.0 software was used for the analysis of the experimental designs and subsequent regression analysis of the experimental data obtained. The coefficient of determination (R^2) was used as a parameter of quality of the polynomial model equation. The statistical significance of the model equation and the model terms was evaluated through the F-test, whereas the t-test was used to evaluate the significance of the regression coefficients. The fitted polynomial equation was then expressed in the form of response surfaces to illustrate the individual and combinatorial effect of the factors evaluated on the responses. The point optimization method was used to optimize the level of each factor towards a maximum response (i.e. to predict the optimum concentration of each medium component for maximum surface tension reduction). Subsequently, the model was validated by performing the experimental runs suggested during the statistical optimization of the model (i.e. the combination of different optimized factors which yielded the maximum response).

After optimizing the selected culture medium components (yeast extract, ammonium sulfate and glucose), the effect of different olive oil concentrations (0, 10, 20, 30, 40 and 50 g/L) on the biosurfactant production was studied. The culture conditions used were the same as described above and the experiments were conducted in triplicate.

2.5. Biosurfactant production in bioreactor

A 5-L capacity bioreactor (BIOSTAT® A Fermentor, B. Braun Biotech International GmbH, Germany), equipped with agitation, temperature, pH and dissolved oxygen concentration online measurement and control was used. The experiments were performed at 28 °C, using 2 L of the previously optimized culture medium, with air injected in the bottom of the bioreactor at a constant flow rate of 1 L/min and without pH control. The fermentations were conducted as batch cultivations. The bioreactor was inoculated with a pre-culture of W. anomalus CCMA 0358 (grown overnight in YEPG medium at 28 °C and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. The effect of different agitation rates (250, 300, 350, 400 and 500 rpm) on biosurfactant production was studied. In order to evaluate cell growth and biosurfactant production, samples (10 mL) were taken at different time points during the fermentation. Cell concentration and surface tension were determined as described above. Whenever required, the culture broth supernatants were diluted 10 times with demineralized water, and the surface tension (ST^{-1}) was measured. All the experiments were performed in triplicate.

2.6. Effect of environmental parameters on biosurfactant activity

The effect of pH, temperature and salinity on the activity of the biosurfactant produced by W. anomalus CCMA 0358 was studied using the cell-free supernatants obtained at the end of the fermentation conducted with the optimized culture medium in bioreactor, as described by Varjani and Upasani [4]. The effect of pH on surface activity was studied by adjusting the cell-free supernatants to different pH values (2, 4, 6, 8, 10, 12) using 1 N NaOH or 1 N HCl solutions, and measuring the surface tension as described above. To assess the effect of salinity on biosurfactant activity, the cell-free

supernatants were supplemented with different NaCl concentrations (50, 100, 200 and 300 g/L); subsequently, the surface tension was measured as described above and compared with the values obtained without addition of NaCl. The stability of the biosurfactant at high temperature was also determined; for that purpose, the cell-free supernatants were incubated at 121 °C for 30 min and then allowed to cool to room temperature; the surface tension was measured and compared to the corresponding values before the heat treatment. All experiments were carried out in triplicate.

2.7. Removal of crude oil from contaminated sand

The potential applicability of the biosurfactant produced by W. anomalus CCMA 0358 in bioremediation was evaluated using artificially contaminated sand. Briefly, samples of 50 g of sand were mixed with 5 g of Arabian Light crude oil in 100 mL flasks and allowed to age at room temperature for 24 h. Afterwards, 50 mL of cell-free supernatant (obtained at the end of the fermentation conducted in bioreactor with the optimized culture medium) were added to each flask. The flasks were incubated at 80 rpm and 37 °C for 24 h. The oil removed was recovered from the surface and transferred to a graduated tube. In order to separate the water recovered together with the crude oil, the tubes were centrifuged ($2700 \times g$, 20 min), and subsequently the volume of crude oil was measured. The amount of crude oil recovered was calculated according to its density (0.837 g/mL). Control assays were performed using demineralized water under similar conditions. All the experiments were carried out in triplicate.

2.8. Statistical analysis

The Statistica 8.0 software was used for all the statistical analysis. The mathematical model that relates the factors optimized in the CCD was determined through a second-order adjustment of the experimental data. All the determinations of surface tension and cell concentration were performed at least three times.

3. Results and discussion

3.1. Optimization of biosurfactant production using response surface methodology (RSM)

The yeast strain W. anomalus CCMA 0358 produced a new type of glycolipid biosurfactant using a culture medium containing yeast extract, ammonium sulfate, glucose and olive oil [27]. In this study, a RSM based on a CCD was used to optimize the concentration of the different medium components in order to maximize biosurfactant production by this yeast strain. Yeast extract (X_1), ammonium sulfate (X_2) and glucose (X_3) were the independent factors selected to perform the optimization process. The corresponding levels for each factor are shown in Table 1. The design matrix (17 experiments) and the corresponding results of RSM experiments to determine the effect of the three abovementioned independent factors on the surface tension (response factor) are shown in Table 2 as observed and predicted values. Regression analysis of the optimization study indicated that yeast extract (X_1) was the only significant factor ($p < 0.1$) on surface tension (Table 3), whereas ammonium sulfate (X_2) and glucose (X_3) were found to be non-significant, as indicated by their high p values (Table 3). Moreover, the interactions between the different medium components were found to be non-significant.

The results herein gathered were then fitted to a second-order polynomial equation that describes the surface tension (response
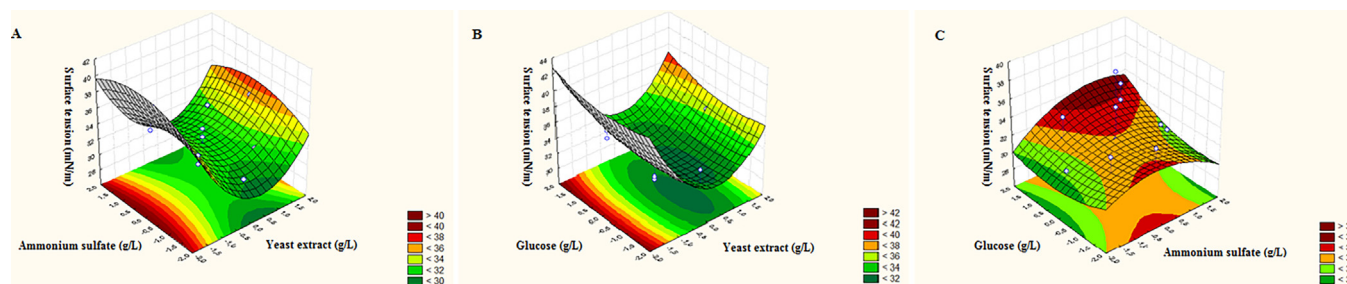
Fig. 1. Response surfaces representing the effect of medium components (yeast extract, ammonium sulfate and glucose) on the surface tension, which is the indicative of the production of biosurfactant. (A) Yeast extract *vs.* ammonium sulfate; (B) Yeast extract *vs.* glucose; (C) Ammonium sulfate *vs.* glucose.

Table 3
Coefficient regression values for the model that describes the effects of the medium components in the surface tension.

Factor	Terms	Regression coefficients	Standard error	p-value
Yeast Extract (X_1)	Linear	−1.1238	0.31865	0.009641
Ammonium sulfate (X_2)	Linear	0.2777	0.31865	0.412291
Glucose (X_3)	Linear	0.3446	0.31865	0.315358
X_1X_2	Interactive	0.0312	0.41616	0.942244
X_1X_3	Interactive	0.0812	0.41616	0.850755
X_2X_3	Interactive	0.2937	0.41616	0.503088
X_1^2	Squared	1.8169	0.35106	0.001287
X_2^2	Squared	−0.4860	0.35106	0.208688
X_3^2	Squared	0.3288	0.35106	0.380125

factor) based on the concentration of the significant medium components:

$$Y = 31.8106 - 1.1238\,X_1 + 1.8169\,X_1^2 \qquad (2)$$

where Y is the surface tension, and X_1 the concentration of yeast extract (the only component that was found to be significant). As ammonium sulfate and glucose were found to be non-significant, they were not included in the model equation. The regression equation obtained from ANOVA showed that R^2 (coefficient of determination) was 0.8792, indicating that 87.9% of the variability in the responses could be explained by the second-order polynomial equation given above (Eq. (2)).

To establish the optimum level of each independent factor for maximum biosurfactant production, three-dimensional response surface plots were constructed by plotting the response (surface tension) as a function of two independent factors at a time, while maintaining the value of the other factor at its central level (Fig. 1). Finally, the optimum levels of yeast extract (X_1 = 4.64 g/L), ammonium sulfate (X_2 = 4.22 g/L) and glucose (X_3 = 1.39 g/L) that allow the highest biosurfactant production (i.e. the lowest surface tension value, estimated to be 31.5 mN/m) can be obtained from the minimization of the previous equation.

In order to validate the optimum culture medium predicted by the model, *W. anomalus* CCMA 0358 was grown in flasks at the same conditions previously described, using a culture medium with the following composition: 4.64 g/L yeast extract; 4.22 g/L ammonium sulfate; 1.39 g/L glucose; and 20 g/L olive oil. The initial surface tension of this culture medium (49.0 ± 0.1 mN/m) was reduced to 31.1 ± 0.1 mN/m after 24 h of growth, which is very similar to the surface tension value predicted by the model (31.5 mN/m). Hence, it can be concluded that the model adequately adjusts the experimental data and clearly describes the effect of the culture medium composition on the biosurfactant production, as the experimental values obtained are in accordance with the expected values determined by the model.

Several authors reported the effect of yeast extract as nitrogen source (at concentrations between 1 and 10 g/L) for the production of biosurfactants by yeast strains belonging to the genera *Candida*, *Pseudozyma* and *Pichia* [18,19,28–31]. For instance, Konishi

et al. [18] reported that growth and MEL production by *Pseudozyma hubeiensis* SY62 increased as the concentration of yeast extract increased from 1 to 10 g/L. However, yeast extract does not always have a positive effect on biosurfactant production. Daverey and Pakshirajan [17] reported that yeast extract at concentrations higher than 2 g/L reduced the sophorolipid production by *Starmerella bombicola* NRRL Y-17069 (in this case, the highest sophorolipid production occurred under nitrogen-limiting conditions). It has also been reported that supplementing the culture medium with yeast extract at a concentration of 10 g/L did not have any effect on the sophorolipid production by *S. bombicola* ATCC, 22214 [32].

In our previous assays, the different culture media were supplemented with olive oil at a concentration of 20 g/L. To further optimize the biosurfactant production by *W. anomalus* CCMA 0358, additional experiments were performed using the optimized culture medium supplemented with olive oil at different concentrations (0–50 g/L). The assays were performed using the conditions previously described. The culture media supplemented with olive oil at concentrations between 10 and 50 g/L led to similar surface tension values (around 31 mN/m) after 24 h of growth (*data not shown*). For this reason, an olive oil concentration of 10 g/L was selected to perform the following experiments to reduce the production costs of this biosurfactant.

Moreover, these results demonstrated that the presence of a hydrophobic carbon source (in this case olive oil) in the culture medium is essential for biosurfactant production by this yeast strain. The assays performed without olive oil led to surface tension values considerably higher (40.6 ± 0.1 mN/m) when compared with those observed with the other culture media containing olive oil at different concentrations. The results herein obtained are in accordance with the general notion in the literature, as it has been widely reported that biosurfactant production by yeasts usually requires the simultaneous presence of a hydrophilic and a hydrophobic carbon source in the culture medium [17–19,21,28,29,32]. As an exception, Monteiro and co-workers [33] reported that the maximum glycolipid biosurfactant production by *Trichosporon montevideense* CLOA72 was achieved using sunflower oil (20 g/L) as the sole carbon source.

Table 4

Surface tension values (ST and ST^{-1}, mN/m) and cell concentrations (cells/mL) obtained with *Wickerhamomyces anomalus* CCMA 0358 grown in bioreactor using the optimized culture medium at different agitation speeds (250–500 rpm). ST: surface tension of the cell-free supernatant; ST^{-1}: surface tension of the cell-free supernatant diluted 10 times with demineralized water. The results presented correspond to the values obtained at the optimum time for each condition (24 h). Results represent the average of three independent experiments ± standard deviation.

Agitation speed (rpm)	ST (mN/m)	ST^{-1} (mN/m)	Growth (cells × 10^8/mL)
250	34.5 ± 1.0	42.2 ± 0.7	4.7 ± 0.1
300	32.8 ± 0.5	39.9 ± 0.3	4.5 ± 0.2
350	31.2 ± 0.4	38.0 ± 0.9	2.7 ± 0.2
400	31.0 ± 0.2	38.4 ± 0.3	2.0 ± 0.1
500	29.3 ± 0.4	33.9 ± 0.1	2.0 ± 0.2

Table 5

Effect of pH and NaCl concentration on surface activity. Surface tension values (ST, mN/m) obtained with the cell-free supernatants from *Wickerhamomyces anomalus* CCMA 0358 (grown in bioreactor using the optimized culture medium) supplemented with different NaCl concentrations and at different pH values. The surface tension values were determined at room temperature (25 °C). Results represent the average of three independent experiments ± standard deviation.

		ST (mN/m)
[NaCl] (g/L)	0	29.3 ± 0.4
	50	28.2 ± 0.5
	100	28.9 ± 0.1
	200	29.2 ± 0.3
	300	29.1 ± 0.2
pH	2	34.7 ± 0.9
	4	33.9 ± 0.7
	6	31.5 ± 0.8
	8	30.7 ± 0.3
	10	31.5 ± 0.6
	12	30.5 ± 0.6

Furthermore, it has to be pointed out that the concentration of the hydrophobic carbon source used in this study was considerably lower when compared with other works, where oleic acid, cotton seed oil, olive oil, coconut oil or soybean oil at concentrations between 75 and 150 g/L were added to the culture medium [17–19,28,32].

3.2. Biosurfactant production in bioreactor

Growth and biosurfactant production by *W. anomalus* CCMA 0358 were evaluated in a 5-L bioreactor aiming at an increase of the production scale. The culture medium used in these assays was that optimized previously in flasks, and the effect of the agitation speed (250–500 rpm) on biosurfactant production (assessed as the reduction of the surface tension of the culture medium) was evaluated. In all cases, air was injected into the culture medium at a constant flow rate of 1 L/min (0.5 vvm (volume of air per culture volume and minute)). The results obtained (Table 4) indicated that a higher agitation speed resulted in a lower surface tension value at the end of the fermentation. The initial surface tension of the culture medium was 49.1 ± 0.1 mN/m, and the lowest surface tension value (29.3 ± 0.4 mN/m) was achieved in fermentations performed at 500 rpm, after 24 h of growth. However, as it can be seen from Table 4, an increase in the agitation speed led to a decrease in the number of cells at the end of the fermentation.

Other authors earlier reported results similar to those obtained in this study whereby a high agitation speed is more favorable for biosurfactant production by different yeast strains; agitation rates between 350 and 550 rpm have been commonly used for biosurfactant production by different yeasts in bioreactor, with air-flow rates between 1 and 2 vvm [17,19,21]. Contrarily, the optimum conditions for biosurfactant production by *Candida lipolytica* UCP0988 in bioreactor were a low agitation rate (200 rpm) without air injection [23].

One parameter that contributes to the production costs of biosurfactants is the fermentation time. In the case of *W. anomalus* CCMA 0358, the lowest surface tension value (i.e. the highest biosurfactant production) was achieved after 24 h of growth. However, in most of the yeast strains reported in the literature, longer incubation times (usually 144–240 h) are required to achieve the maximum biosurfactant production [17,19,21,23]. This represents an important advantage of *W. anomalus* CCMA 0358 over other commonly used yeasts.

Fig. 2 shows the evolution of growth and surface tension for the fermentation performed in bioreactor at 500 rpm. As it can be seen, up to 14 h of fermentation there is an inverse relationship between both parameters, indicating a growth-associated biosurfactant production. After that point, the number of cells decreased, whereas the surface tension remained constant up to 24 h. Afterwards, a slight increase in the surface tension was observed, probably due to a partial degradation of the biosurfactant. On the contrary, the

production of sophorolipids by several yeast strains occurs mainly during the stationary growth phase [21,32]. However, Monteiro and co-workers [33] reported a partially growth-associated biosurfactant production by *T. montevideense* CLOA72 (70% of the biosurfactant was produced during the exponential growth phase).

The great advantage of the present study when compared with others previously reported is the faster (24 h) biosurfactant production achieved with *W. anomalus* CCMA 0358.

3.3. Effect of temperature, pH and salinity on biosurfactant activity

The applicability of biosurfactants can be conditioned by the effect of pH, temperature and salinity on their stability and performance. For instance, the stability of biosurfactants at high temperature is of great importance for their use in the food and oil industries, as well as for their incorporation in laundry detergents [25]. The biosurfactant produced by *W. anomalus* CCMA 0358 was found to be stable at high temperature. The surface tension of the cell-free supernatant remained constant (around 29 mN/m) after incubation at 121 °C for 20 min, indicating no loss of activity. In a similar way, other biosurfactants produced by yeasts were not affected by incubations at high temperatures (80–120 °C) [17,22,28,29,33].

Regarding the effect of salinity, NaCl concentrations as high as 300 g/L did not have a negative effect on biosurfactant activity, as it can be seen from the surface tension values obtained (Table 5). The lowest surface tension value (28.2 ± 0.5 mN/m) was obtained for a NaCl concentration of 50 g/L. This property makes this biosurfactant useful for applications involving high salinities, such as bioremediation of marine environments. Other biosurfactants produced by yeasts have been reported to remain stable at NaCl concentrations up to 100 g/L [28], 120 g/L [22], 150 g/L [29], 200 g/L [17] and 300 g/L [33].

Regarding the effect of pH, the biosurfactant produced by *W. anomalus* CCMA 0358 was more stable under neutral and alkaline conditions (pH 6–12), where the surface tension remained almost constant (Table 5). On the contrary, at acidic pH values, a slight increase in the surface tension values was observed, meaning that the biosurfactant was less active in those conditions. The stability of this biosurfactant at pH values between 6 and 12 is useful for its application in laundry detergent formulations, which pH is usually in the range of 9–12 [25]. Other biosurfactants produced by yeasts remained stable at pH values between 2 and 12 [28,29]. In other cases, a partial loss of activity was observed at high pH values (pH 12) [17,33], whereas other biosurfactants remained stable only at pH values between 6 and 10 [22].
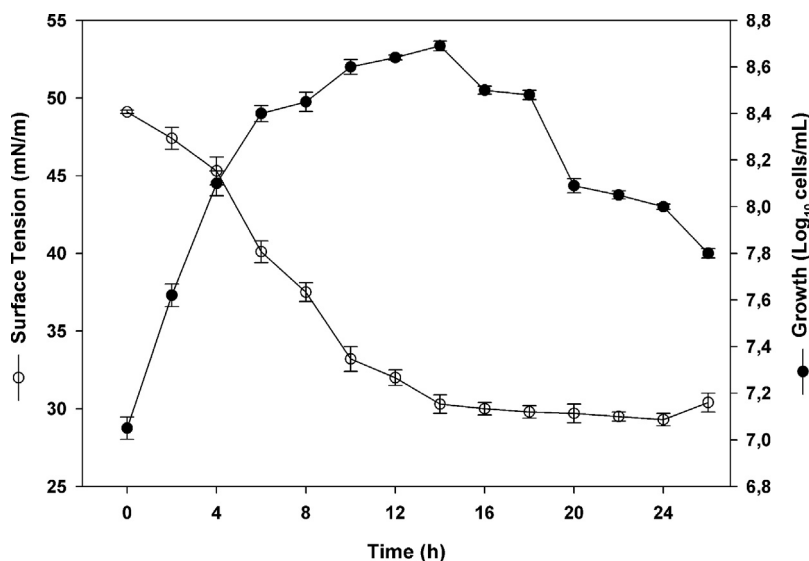
Fig. 2. Evolution of growth (Log_{10} cells/mL) and surface tension (ST, mN/m) in fermentations performed with *Wickerhamomyces anomalus* CCMA 0358 grown in bioreactor using the optimized culture medium at 28 °C and 500 rpm. Results represent the average of three independent experiments ± standard deviation.

3.4. Bioremediation assays

The biosurfactant produced by *W. anomalus* CCMA 0358 was evaluated for its ability to remove crude oil from artificially contaminated sand. The cell-free supernatant obtained at the end of the fermentation performed using the optimized culture medium allowed the recovery of about 20 ± 4% of the oil impregnated in the sand when compared with the control assays (performed with demineralized water at the same conditions). One advantage of biosurfactants for application in bioremediation is their possible use without purification (i.e. as cell-free supernatants), which contributes to increase their competitiveness with chemical surfactants, as the purification steps can account for up to 60–70% of their total production costs [3,9].

The potential use of biosurfactants produced by several yeasts in bioremediation has been reported by other authors. The biosurfactants produced by *C. lipolytica* UCP0988 and *Candida glabrata* UCP1002 were found to remove between 19% and 43% (depending on the biosurfactant concentration and the sand particle size) of motor oil from contaminated sand when compared with a control performed with demineralized water [22,28]. Moreover, sophorolipid biosurfactants produced by *S. bombicola* ATCC 22214 recovered up to 27% of crude oil in core-flooding experiments [29]. The results herein obtained were similar to those reported for the lipopeptide biosurfactant surfactin produced by *Bacillus subtilis* #573, which led to recoveries between 19 and 26% (depending on the culture medium used to grow the microorganism) of crude oil from contaminated sand in similar assays [13,20].

These results, together with the stability of this biosurfactant at high temperature, as well as over a wide range of salinities and pH values, make it a promising candidate for application in bioremediation processes.

4. Conclusions

The biosurfactant produced by *W. anomalus* CCMA 0358 exhibited excellent surface active properties, as demonstrated by the low surface tension values achieved (from 49 mN/m up to 29 mN/m). Furthermore, it remained stable at high temperature (121 °C), high salinities (300 g/L NaCl) and pH values between 6 and 12. The crude biosurfactant allowed the recovery of 20% of crude oil from contaminated sand. The excellent surface tension reducing ability

exhibited by this biosurfactant, together with its stability under extreme environmental conditions, as well as its ability to recover crude oil from contaminated sand make it a promising candidate for application in bioremediation or in the petroleum industry, as an alternative to the traditional chemical surfactants.

Conflicts of interest

None.

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