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Biosurfactant Production by *Yarrowia lipolytica* in Corn Steep Liquor-Based Media for Hydrocarbon Bioremediation

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Abstract: Bioremediation using microorganisms offers a sustainable approach to addressing hydrocarbon contamination. This study explores biosurfactant production by *Yarrowia lipolytica* IMUFRJ 50682 during crude oil and asphaltene-free fraction biodegradation in corn steep liquor (CSL)-based media. By evaluating CSL concentrations (5–30 g/L) and combinations with glucose, molasses, and crude oil, this study demonstrates that CSL is an effective nutrient source for supporting microbial growth and biosurfactant production. The highest emulsification index (EI = 73.3%) was achieved with 20 g/L of CSL after 48 h, while media containing mixed carbon sources (glucose and crude oil) enhanced metabolic efficiency, yielding a maximum cell growth of 8 g/L after 150 h. Despite inhibiting cell growth, the asphaltene-free fraction promoted biosurfactant activity, with the EI reaching 35.8% after 264 h. The results emphasize the importance of pH control, with the optimal emulsification being observed at pH ~6. This work highlights the potential of CSL as a cost-effective and sustainable additive, advancing applications in bioremediation and biosurfactant production and contributing to the development of environmentally compatible hydrocarbon degradation strategies.

Keywords: bioremediation; biosurfactant; yeast; agro-industrial waste; hydrocarbon degradation

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1. Introduction

Hydrocarbon contamination poses severe environmental challenges due to its persistence, toxicity, and bioaccumulation potential. Oil spills and industrial discharges significantly impact aquatic ecosystems and soil quality, leading to long-term ecological and health risks [1]. According to the International Tanker Owners Pollution Federation (ITOPF), only one large spill (>700 tonnes) and nine medium spills (7–700 tonnes) were recorded in 2023. These data highlight a long-term decline in oil spill frequency, with incidents dropping by over 90% since the 1970s despite increasing global oil transport. The current decade averages about 6.8 spills over 7 tonnes annually, though most recent incidents involve smaller volumes. Even with this scenario, approximately 2000 tonnes of

oil spillage from tanker incidents was recorded globally in 2023, underscoring a continued need for advances in bioremediation technologies [2].

Bioremediation, an eco-friendly environmental restoration strategy, utilizes biological activity to eliminate pollutants from the environment effectively [3,4]. It offers significant advantages over conventional techniques, including maintaining ecological balance and reducing costs [5]. However, its effectiveness is often hindered by the structural complexity of hydrocarbons, such as asphaltenes, and the need for nutrient supplementation to sustain microbial activity [6,7]. Various microorganisms, particularly hydrocarbon-degrading yeasts, have demonstrated significant potential in bioremediation due to their ability to metabolize hydrocarbons. Among these, the yeast species *Yarrowia lipolytica* stands out for its remarkable efficiency in crude oil degradation [3,8–10].

The effectiveness of these microorganisms can vary widely across different environments due to factors like nutrient availability, pH, and temperature, as well as enzyme production capacity, which varies even within strains of the same species [11]. This variability highlights the importance of discovering and optimizing microbial strains for practical bioremediation applications.

Yarrowia lipolytica is a dimorphic yeast widely studied for its unique metabolic and genomic traits [12]. It exhibits a unique enzymatic toolkit, including monooxygenases and dioxygenases, enabling the degradation of long-chain alkanes and aromatic hydrocarbons [13,14]. This versatility makes it highly efficient in hydrocarbon biodegradation compared to other microbial species [4,12,15]. *Y. lipolytica*'s remarkable tolerance to extreme conditions, including high salinity, low temperatures, and toxic environments [4,12,16,17], allows it to thrive where other microorganisms fail, making it particularly suited for diverse remediation scenarios [4,12].

Microorganisms interact with hydrocarbons, which are only sparingly soluble in water, through mechanisms such as cell surface hydrophobicity or the production of surfactants and emulsifiers [3,12]. Sayed et al. [18] emphasized the potential of biosurfactants as eco-friendly agents in hydrocarbon bioremediation, particularly due to their ability to reduce surface and interfacial tensions, enhancing the bioavailability of hydrophobic pollutants. Despite their advantages, such as biodegradability, low toxicity, and environmental compatibility, the authors highlighted challenges related to optimizing microbial growth conditions and production costs. Ferreira et al. [10] demonstrated that *Yarrowia lipolytica* IMUFRJ 50682 can degrade complex hydrocarbons, including n-alkanes, isoprenoids, and aromatic compounds. Santos et al. [19] further observed that this strain produces high emulsification indexes in media containing residual frying oil and corn steep liquor (CSL). Miranda et al. [13] highlighted the importance of medium supplementation in enhancing hydrocarbon degradation by *Yarrowia lipolytica*. Their study demonstrated that low-cost components, such as corn steep liquor (CSL), effectively supported yeast growth, increased the production of microbial lipids, and stimulated the synthesis of enzymes like lipases and proteases, thereby improving the yeast's capacity to degrade hydrocarbons.

Most studies utilize CSL as a supplement rather than the sole carbon source, often in combination with ammonium sulfate to optimize microbial growth and metabolic activity [13,19]. Additionally, the majority of these studies focus on the degradation of a single pure hydrocarbon, such as hexadecane, as demonstrated by Miranda et al. [13], or a specific class of hydrocarbons, such as polycyclic aromatic hydrocarbons [20].

This study investigates the potential of *Yarrowia lipolytica* IMUFRJ 50682 to be used for biosurfactant production during hydrocarbon biodegradation in CSL-based media. It explores the underutilized potential of corn steep liquor as a cost-effective substrate for biosurfactant production, addressing the need for sustainable and economically viable bioremediation strategies.

2. Materials and Methods

A summary of the overall methodology is presented in Figure 1, which outlines the main steps of the analyses performed in this study.

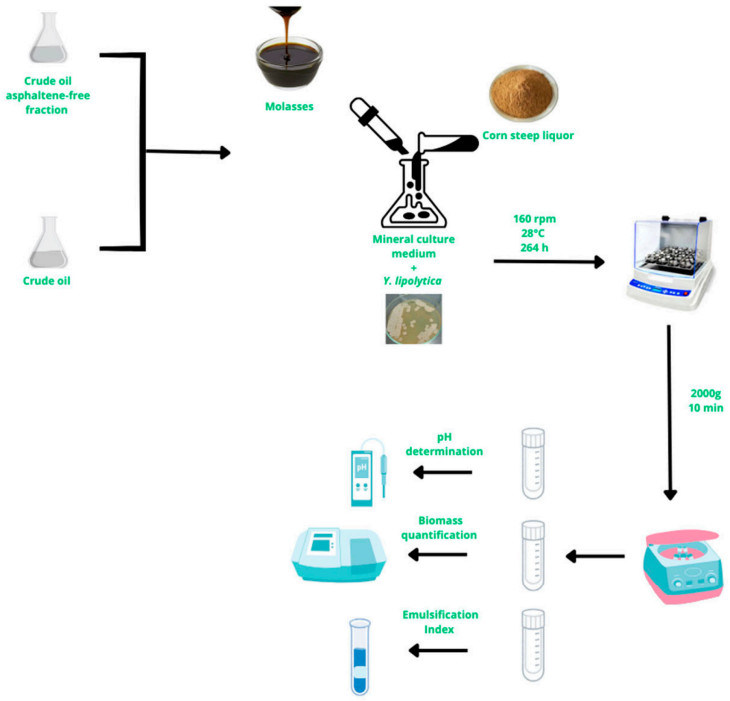

Figure 1. The workflow of the study, detailing biosurfactant production and the biodegradation experiments.

2.1. Materials

Corn steep liquor was provided by Ingredion (São Paulo, Brazil) and is the same batch characterized in detail by Santos et al. [21]. The Biochemical Engineering Department of the School of Chemistry (UFRJ) provided molasses, which was composed of 58% of sucrose/glucose/fructose. Peptone and yeast extract were sourced from Oxoid (Hampshire, UK), and glucose from Reagen (Colombo, Brazil). Additional reagents included $MnSO_4 \cdot H_2O$ from Reagen; Na_2HPO_4, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$ from Vetec Química Fina (Duque de Caxias, Brazil); KH_2PO_4 from Labsynth (Diadema, Brazil); and $MgSO_4 \cdot 7H_2O$ from ISOFAR Indústria e Comércio de Produtos Químicos (Duque de Caxias, Brazil).

Crude oil originating from Brazil's post-salt oil reserves and provided by DOPOLAB (Department of Organic Processes Laboratory, Rio de Janeiro, Brazil) is classified as heavy oil with 19.39 API gravity.

Crude oil asphaltene-free fraction was obtained by solvent extraction through the cold method, following the methodology proposed by Ferreira et al. [22] with adaptations. Two grams of petroleum was dissolved in 20 mL of limonene, and the mixture was left to rest for 24 h. Subsequently, the system was centrifuged at 2000× *g* for 15 min. The supernatant was then collected and used for bioremediation tests.

2.2. Strain and Preculture

The wild-type strain *Yarrowia lipolytica* IMUFRJ 50682, isolated from an estuary in Guanabara Bay, Rio de Janeiro, Brazil [23], was used in this study. The strain was maintained on a YPD agar medium (1% *w*/*v* yeast extract, 2% *w*/*v* peptone, 2% *w*/*v* glucose, 3% *w*/*v* agar, pH 6.8–7.0).

Following the preculture protocol, *Y. lipolytica* was inoculated into 200 mL of YPD medium (1% *w*/*v* yeast extract, 2% *w*/*v* peptone, and 2% *w*/*v* glucose, pH 6.8–7.0) in 500 mL Erlenmeyer flasks (Roni-Alzi, Rio de Janeiro, Brazil). The cultures were incubated at 28 °C on a rotary shaker (model TE-4200, Tecnal Equipamentos Científicos, Piracicaba, Brazil) at 160 rpm for 72 h. After 72 h of preculture, the cells were centrifuged at 2000× *g* and harvested in sufficient quantity to inoculate the biosurfactant production medium at a concentration of 1 mg of dry cell weight per mL.

2.3. Biosurfactant Production in Different Concentrations of Corn Steep Liquor (CSL)

Biosurfactant production was conducted in 1 L Erlenmeyer flasks containing 500 mL of production medium composed of varying concentrations of CSL (5.0 g/L, 10 g/L, 20 g/L, and 30 g/L), with and without ammonium sulfate (10 g/L), in triplicate. Initial pH was set to 4.0. After inoculation, the flasks were incubated at 28 °C and 250 rpm for 72 h. Samples were collected every 24 h for biomass quantification and pH measurement, which was not controlled. Samples were centrifuged at 2000× *g* and stored at −20 °C for subsequent emulsification index measurement.

2.4. Biosurfactant Production During Biodegradation in Corn Steep Liquor-Based Media

For biodegradation experiments with crude oil or crude oil asphaltene-free fraction, a mineral culture medium was used (g/L: KH_2PO_4, 7; Na_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.06 [24]) with corn steep liquor as the nitrogen source. The initial pH of the medium, without further adjustment, was measured to be 5.5–5.8.

For crude oil biodegradation assays, glucose, molasses, and/or crude oil were tested as carbon sources (Table 1). Limonene, the extraction solvent, was added to the mineral medium with CSL as a control for crude oil asphaltene-free fraction biodegradation assays. The crude oil asphaltene-free fraction was also tested with the mineral medium and CSL (Table 1).

Biosurfactant production was performed in 500 mL Erlenmeyer flasks containing 200 mL production medium. After inoculation, the flasks were incubated at 28 °C and agitated at 160 rpm for 264 h. Assays were conducted in duplicate, with samples collected after 0, 48, 96, 168, 216, and 264 h for biomass quantification and pH measurement. Post-measurement, samples were centrifuged at 2000× *g* and stored at −20 °C for glucose quantification and emulsification index determination.

Table 1. Carbon and nitrogen sources added to the mineral culture medium for crude oil biodegradation assays.

Medium	G (% *w*/*v*)	M (% *w*/*v*)	O (% *v*/*v*)	L (% *v*/*v*)	AF (% *v*/*v*)	CSL (% *w*/*v*)
G-CSL	1	-	-	-	-	1
O-CSL	-	-	1	-	-	1
G-O-CSL	1	-	1	-	-	1
M-CSL	-	1.7	-	-	-	1
M-O-CSL	-	1.7	1	-	-	1
L-CSL	-	-	-	1	-	1
AF-CSL	-	-	-	-	1	1

G: glucose; M: molasses; O: crude oil; CSL: corn steep liquor; L: limonene; AF: crude oil asphaltene-free fraction.

2.5. Analytical Procedures

2.5.1. Biomass Quantification and pH Determination

Cell concentration was monitored by measuring optical density (OD) at 570 nm, with values converted to grams of dry weight (d.w.) of cells per liter using a previously calculated factor based on a calibration curve. The calibration curve was linear within the range of 0.2–0.8 OD units, corresponding to 0.110–0.442 g/L of dry cell weight, with a coefficient of determination (R^2) of 0.994. The standard deviation of the calibration measurements was ±0.02 OD units.

The pH of cell-free samples was measured using a calibrated pH meter (DIGIMED, model DM-22, São Paulo, Brazil) with an accuracy of ±0.01 pH units and a measurement range of 0–14. Calibration was performed using standard buffer solutions at pH 4.1 and 6.8.

2.5.2. Glucose Concentration

Glucose concentration analysis was conducted using High-Performance Liquid Chromatography (HPLC) from Shimadzu (Kyoto, Japan), equipped with an Aminex® HPX-87H(300 mm × 7.8 mm column (Bio-Rad Laboratories Ltd., São Paulo, Brazil), and a refractive index (RI) detector (Shimadzu®, Kyoto, Japan). The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min with an injection volume of 20 µL. The column temperature was maintained at 55 °C, as previously described by Benevenuti et al. [25]. The method's detection limit was 0.1 g/L, with a standard deviation of ±0.02 g/L for repeated measurements.

2.5.3. Emulsification Index

The emulsification index (EI) was determined following the method of Cooper and Goldenberg [26], with slight modifications, which is widely used for evaluating the emulsifying capacity of biosurfactants. This method was selected for its reproducibility and relevance in studies involving hydrocarbon biodegradation, allowing for direct comparisons with previously published works.

Briefly, 1 mL of n-hexadecane was added to 1 mL of fermented cell-free culture medium in a test tube, followed by vortexing for 2 min. After 24 h, the emulsified layer's height and the sample's total height in the test tube were measured. The EI was calculated as the emulsified layer height (cm) percentage relative to the total liquid column height (cm), as shown in Equation (1). The reproducibility of the method was validated, with an error margin of ±2% for EI values.

$$E_{24} = (H_{el}/H_s) \times 100 \quad (1)$$

where E_{24} is the emulsification index after 24 h of fermentation, H_{el} is the emulsion height, and H_s is the total height.

2.6. Statistical Analysis

The data obtained for cell concentration and emulsification index (EI%) were analyzed using statistical methods to identify potential differences between experimental groups. Initially, a one-way Analysis of Variance (ANOVA) was performed to test the null hypothesis that all group means were equal. The ANOVA partitions the total variability into two components: between-group variability (due to differences among group means) and within-group variability (random variation within groups). The F-statistic and its associated *p*-value were used to assess statistical significance. A standard significance level of 0.05 was applied. When the ANOVA results suggested potential differences ($p \leq 0.05$), post hoc pairwise comparisons were conducted using Tukey's Honest Significant Difference (HSD) test. Tukey's test adjusts for multiple comparisons and provides the mean difference,

confidence intervals, and adjusted *p*-values for all possible pairwise group comparisons. For all analyses, the STATISTICA 7.1 software (StatSoft, Inc., Tulsa, OK, USA) was used.

3. Results

3.1. Influence of Corn Steep Liquor (CSL) Concentration on Biosurfactant Production

Cell growth, the emulsification index, and the pH profile during *Y. lipolytica* cultivation in CSL media, with and without ammonium sulfate, are presented in Figures 2 and 3 and Table 2, respectively. The results indicate that CSL alone did not significantly promote *Y. lipolytica* growth (Figure 2a), with only one condition reaching twice its initial concentration, and adding ammonium sulfate to increase nitrogen availability impacted cell growth only for the media with 10 and 30 g/L of CSL (Figure 2b). Even so, low values of cell concentration were obtained (around 2 g/L). Additionally, the pH values fluctuated consistently across all samples, ranging from approximately 4 to 7 within the first 24 h (Table 1), suggesting active metabolic activity within the culture medium.

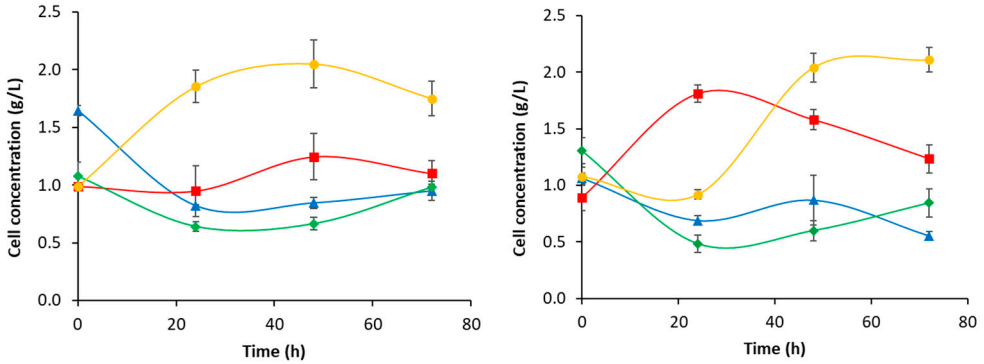

Figure 2. Cell growth of *Y. lipolytica* during cultivation in 1 L Erlenmeyer flasks containing 200 mL of medium, without (**a**) and with (**b**) ammonium sulfate, at 28 °C and 250 rpm. Four concentrations of corn steep liquor (CSL) were tested: 5 g/L (blue triangle), 10 g/L (red square), 20 g/L (green diamond), and 30 g/L (yellow circle).

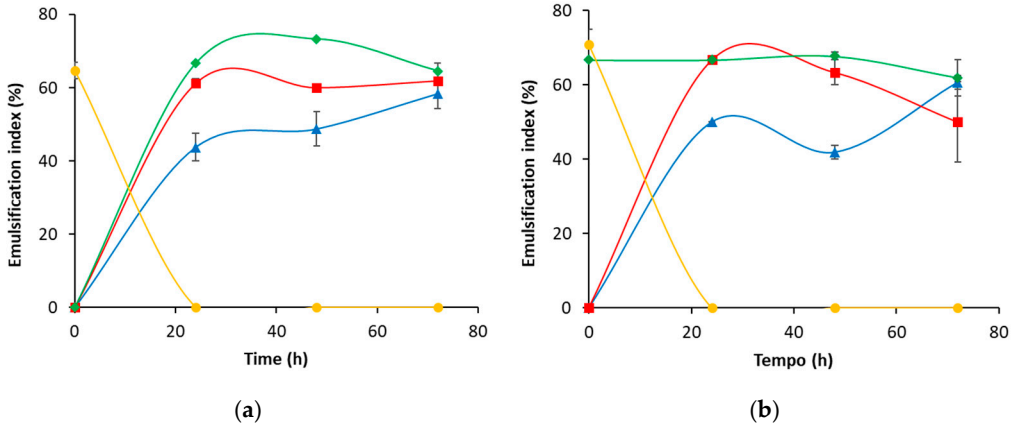

Figure 3. Emulsification index during *Y. lipolytica* cultivation in 1 L Erlenmeyer flasks containing 200 mL of medium, without (**a**) and with (**b**) ammonium sulfate, at 28 °C and 250 rpm. Four concentrations of corn steep liquor (CSL) were tested: 5 g/L (blue triangle), 10 g/L (red square), 20 g/L (green diamond), and 30 g/L (yellow circle). Media consisted of water, CSL, and, in some cases, ammonium sulfate. No mineral medium was added.

Table 2. pH values during *Y. lipolytica* cultivation at 28 °C and 250 rpm in 1 L Erlenmeyer flasks containing 200 mL of medium, with and without ammonium sulfate, with corn steep liquor (CSL) without mineral medium.

Time (h)	pH (Medium Without Ammonium Sulfate)			
	CSL Concentration			
	5 g/L	10 g/L	20 g/L	30 g/L
0	4.14 ± 0.06	4.11 ± 0.02	4.11 ± 0.00	4.07 ± 0.02
24	7.41 ± 0.11	7.33 ± 0.08	7.63 ± 0.04	7.02 ± 0.13
48	7.94 ± 0.02	7.70 ± 0.01	7.81 ± 0.12	7.70 ± 0.01
72	7.93 ± 0.09	7.79 ± 0.04	7.87 ± 0.04	7.98 ±0.05
Time (h)	**pH (Medium With Ammonium Sulfate)**			
	CSL Concentration			
	5 g/L	10 g/L	20 g/L	30 g/L
0	4.00 ± 0.00	3.99 ± 0.01	4.00 ± 0.02	4.11 ± 0.06
24	7.37 ± 0.00	7.64 ± 0.04	7.43 ± 0.02	7.13 ± 0.01
48	7.53 ± 0.04	7.91 ± 0.00	7.60 ± 0.04	7.72 ± 0.02
72	7.76 ± 0.07	8.06 ± 0.04	7.77 ± 0.04	7.93 ± 0.04

Regarding the emulsification index (EI), Figure 3a shows that the medium containing 20 g/L of corn steep liquor (CSL) without ammonium sulfate achieved the highest EI (73.30%) after 48 h of cultivation. However, some samples exhibited high EI values before cell addition, particularly those with higher CSL concentrations. At a CSL concentration of 30 g/L, the results were inconsistent and not comparable with those of other samples, likely due to the increased precipitation of CSL in the medium at this elevated concentration. The presence of ammonium sulfate (Figure 3b) increased the EI slightly when 5 and 10 g/L of CSL were used compared to when there was an absence of this nitrogen source. However, for 20 g/L of CSL, the presence of ammonium sulfate may have also induced CSL precipitation and interfered with the EI measurement since the samples before cell inoculation presented a high EI. Therefore, the addition of ammonium sulfate was discarded for subsequent experiments. Since there were no significant differences between the EI profiles of media with 10 and 20 g/L of CSL, and considering that the presence of other media components may induce CSL precipitation, 10 g/L of CSL was adopted for the biodegradation studies.

3.2. Biosurfactant Production During Crude Oil Biodegradation in Corn Steep Liquor-Based Media

To investigate biosurfactant production during crude oil biodegradation in a CSL-based medium, the addition of carbon sources concomitant to the crude oil was tested. Glucose (G) is a simple carbon source that can induce cell growth and improve yeast metabolism for crude oil (O) biodegradation. Therefore, it was added to the CSL-based medium as a control (G-CSL) and to crude oil (G-O-CSL). As a cheaper carbon source, molasses (M) was also tested in this media with a control (M-CSL) and in the presence of crude oil (M-O-CSL). The crude oil alone in the CSL-based medium was also assessed (O-CSL); the results are shown in Figure 4.

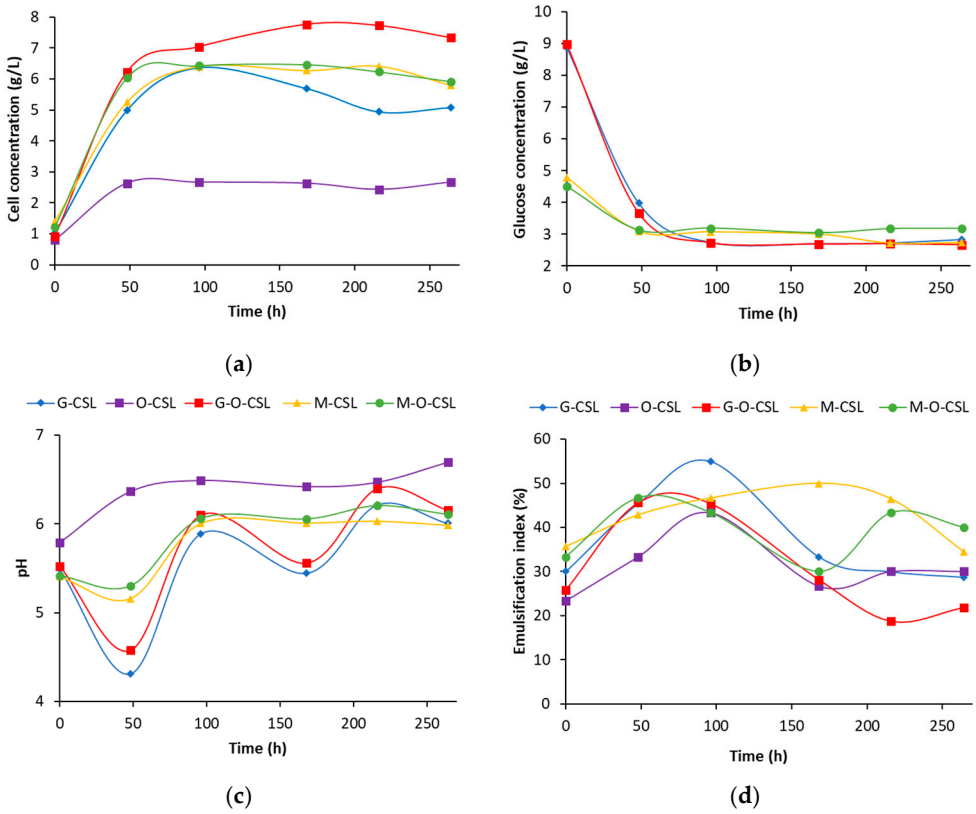

Figure 4. Cell growth (**a**), glucose consumption (**b**), pH profile (**c**), and emulsification index (**d**) during *Y. lipolytica* cultivation in 1 L Erlenmeyer flasks containing 200 mL of mineral medium at 28 °C and 250 rpm. G: glucose; M: molasses; O: crude oil; CSL: corn steep liquor.

Cell growth profiles (Figure 4a) were similar when glucose was present, whether with pure glucose or molasses. However, a slightly higher growth rate can be visually detected in the media with crude oil as an additional carbon source. An ANOVA (Analysis of Variance) was used to check whether there were significant differences between the different media (G-CSL, O-CSL, G-O-CSL, M-CSL, and M-O-CSL) in relation to cell concentrations over time. The ANOVA resulted in an F-value of 3.40 and a *p*-value of 0.0236, indicating that there are significant differences between the cell concentrations in the different media. A post hoc test, Tukey's HSD test, was performed to identify the differences between two media. The G-O-CSL medium achieved the highest cell concentration, reaching approximately 8 g/L at around 150 h of fermentation, differing significantly from the other media. No significant differences were found for the G-CSL, M-O-CSL, and M-CSL media, and they also supported relatively high cell concentrations, demonstrating the adaptability of *Y. lipolytica* to utilize molasses-based substrates, either alone or in combination with crude oil. The inclusion of molasses likely supplies a diverse range of sugars and nutrients, which, when combined with hydrocarbons in M-O-CSL, enhances the metabolic efficiency of *Y. lipolytica*. Even with evidence that *Y. lipolytica* does not produce invertase-like enzymes [27] and would therefore not be able to consume sucrose, cell growth in the M-CSL media shows that the glucose present in this material is sufficient to sustain cell growth in comparable amounts to the glucose medium (G-CSL). These findings underscore the potential of complex media containing hydrophilic and hydrophobic carbon sources to maximize cell growth. When crude oil was used alone (O-CSL), cell growth was significantly lower but still higher than in the absence of oil (Figure 2a, red rectangle), reinforcing that crude oil is being used as a carbon source.

A significant decrease in the glucose concentration (Figure 4b) is observed within the first 50 h in the glucose-containing media (G-CSL and G-O-CSL). This rapid consumption

indicates that *Y. lipolytica* preferentially utilizes simple sugars over more complex substrates, as supported by previous studies [28,29]. The swift depletion of glucose likely provides a metabolic advantage, serving as an immediate energy source and precursor for biosynthetic pathways, enabling rapid initial growth and early adaptation to the fermentation environment. An inferior glucose concentration was detected in the molasses media (Figure 4b), which was expected since molasses mainly comprises sucrose. Even so, glucose depletion occurs within 48 h, similar to the glucose media. This result shows that when crude oil is added to molasses, the yeast preferentially consumes simple sugars, as in glucose media, indicating a possible substitution for this cheaper carbon source.

Similar pH oscillatory profiles are observed for glucose-containing media (G-CSL, G-O-CSL, M-CSL, and M-O-CSL), mainly in the first 100 h. The oscillatory amplitude of the pH profiles is inferior for the molasses media, which can be attributed to the lower glucose levels. For the crude oil medium (O-CSL), a completely different profile is detected, with a continuous increase in pH values, indicating that the oscillatory profile of media with crude oil and glucose is attributed to the presence of the sugar molecule.

When evaluating the emulsification index (Figure 4d), the G-CSL medium exhibits the highest value, peaking at 55% after 96 h of cultivation. However, the ANOVA for the emulsification index resulted in an F-statistic of 2.20 and a *p*-value of 0.098, indicating that there is no statistical evidence of significant differences between media. Also, it can be noted that the initial EI values are high, probably because of the presence of a mineral medium and the additional carbon source that could have induced CSL precipitation. So, the EI values must be analyzed by subtracting the initial value (ΔEI). The ΔEI values of 96 h of process were compared, as shown in Figure 5. The ANOVA performed for these values resulted in an F-statistic of 58.61 and a *p*-value of 0.00005, indicating statistical evidence of significant differences between media.

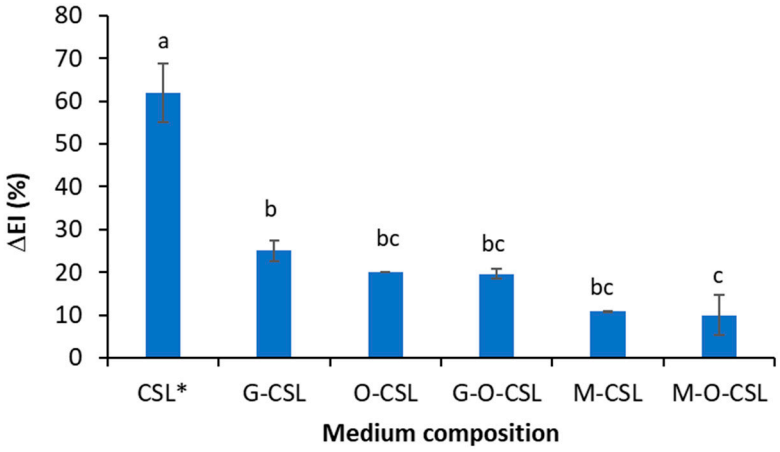

Figure 5. Emulsification index (EI) after 96 h subtracted by initial EI value (ΔEI) of *Y. lipolytica* cultivation in 1 L Erlenmeyer flasks containing 200 mL of medium at 28 °C and 250 rpm. G: glucose; M: molasses; O: crude oil; CSL: corn steep liquor. * Data from CSL medium were obtained after 72 h of cultivation. Different letters (a–c) indicate that values differ significantly from each other, according to Tukey's test, at significance level of 5% ($p < 0.05$).

In this context, none of the media tested presented similar or higher values than the CSL-based medium. However, it is essential to understand that when oil is present, two processes are occurring: the biodegradation of crude oil and biosurfactant production. With that in mind, since the EI values of the media with crude oil and the additional carbon source (G-O-CSL and M-O-CSL) are similar or lower than the media with only

crude oil as a carbon source (O-CSL), there is no need for an additional carbon source. The media with crude oil without these sugars (O-CSL) resulted in a variation in EI values (ΔEI, from 0 to 96 h) of 20%, which was slightly inferior to the medium with only glucose (G-CSL, ΔEI of 25%).

3.3. Biosurfactant Production During the Biodegradation of Crude Oil Asphaltene-Free Fraction in Corn Steep Liquor-Based Media

The chemical composition of petroleum includes a complex combination of aromatic hydrocarbons, aliphatic hydrocarbons, heterocyclic hydrocarbons, asphaltenes, and non-hydrocarbon compounds. Both the resin and asphaltene fractions contain polar non-hydrocarbon chemicals in contrast to the aliphatic and aromatic fractions. Asphaltenes are high-molecular-weight chemicals that are insoluble in solvents like n-heptane. Due to their highly complex structure, asphaltenes have very condensed carbon chains that are difficult for microorganisms to access, making their bioconversion less viable [30]. In this sense, these compounds were extracted by a recently developed green-extraction process with limonene [22] to investigate whether this extraction could improve cell growth and biosurfactant production. The same CSL-based medium was used with the addition of crude oil asphaltene-free fraction (AF-CSL) in comparison to the control, which was the CSL-based medium with the solvent used for the extraction of asphaltenes, limonene (L-CSL). These results were compared to the crude oil medium (O-CSL), as shown in Figure 6.

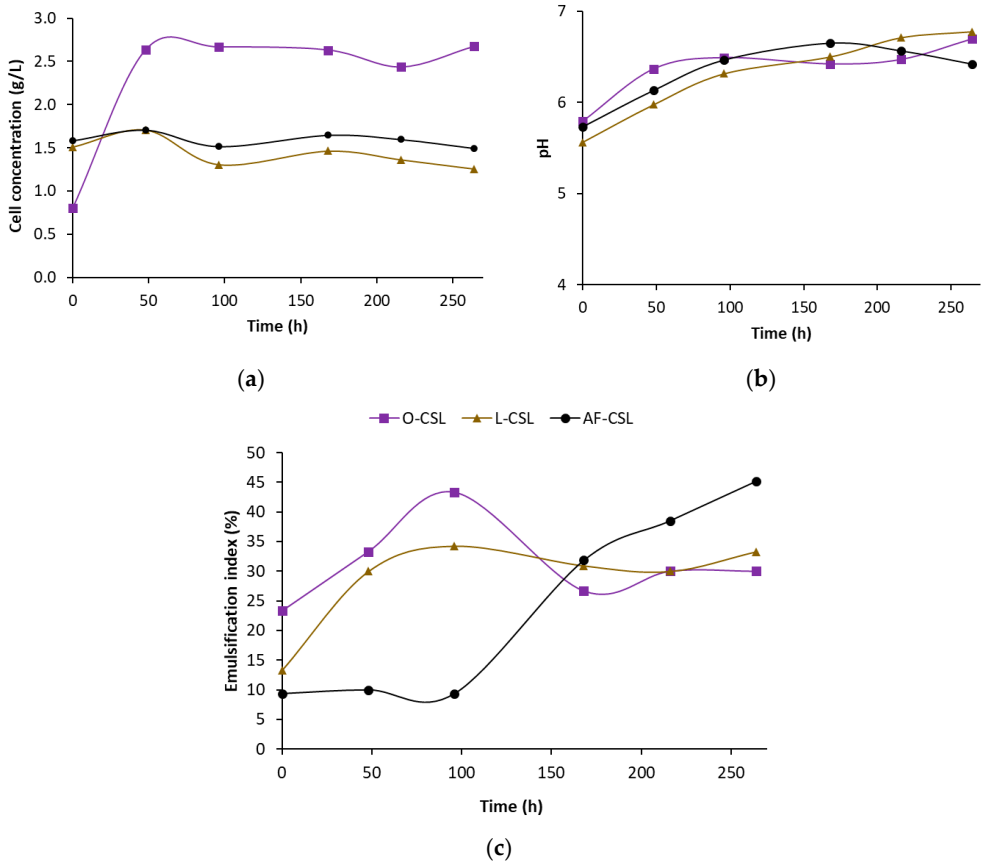

Figure 6. Cell growth (**a**), pH profile (**b**), and emulsification index (**c**) during *Y. lipolytica* cultivation in 1 L Erlenmeyer flasks containing 200 mL of mineral medium at 28 °C and 250 rpm. O: crude oil; CSL: corn steep liquor; L: limonene; AF: crude oil asphaltene-free fraction.

Y. lipolytica's cell growth was inhibited by the presence of the solvent used for the asphaltene extraction (limonene), since no significant increase in cell concentration was

detected in this medium (L-CSL) (Figure 6a). For the asphaltene-free fraction (AF-CSL), cell growth was also not detected, but it could be attributed to the presence of the solvent. The ANOVA revealed an F-statistic of 6.68 and a *p*-value of 0.0084, suggesting statistical differences between the media tested. Tukey's test confirmed significant differences between the AF-CSL medium and the L-CSL medium in relation to the O-CSL medium, but not between them. Despite the different cell growth profiles of these media compared to the crude oil medium (O-CSL), the pH profiles of all three media were similar, with an increasing tendency (Figure 6b).

Regarding the emulsification index, the ANOVA only revealed no significant differences between media (F-value: 0.61; *p*-value: 0.555) for the results after 96 h (*p*-value: 0.0225). For the ΔEI, significant differences between the media were detected for 96 h (*p*-value: 0.0478), 216 h (*p*-value: 0.0473), and 264 h (*p*-value: 0.0309). Although the AF-CSL presented no increase in EI values during the first 100 h, in this medium, a higher ΔEI, considering the end of the process (264 h, ΔEI = 35.8%), was detected. Therefore, this crude oil fraction shows potential to be explored in future works of biosurfactant production concomitant to the biodegradation process.

This study did not include abiotic controls to evaluate changes in the medium or biosurfactant activity in the absence of microbial activity. While biological controls were used to establish baselines, future studies should include abiotic controls to better distinguish between biological and chemical contributions to the observed effects.

4. Discussion

We have shown that CSL is an excellent raw material for biosurfactant production by *Y. lipolytica* and can be used solely in the culture medium to obtain a high EI. It was also a suitable base medium for biosurfactant production during crude oil biodegradation, including an asphaltene-free fraction. The scalability of using CSL as a nutrient source for biosurfactant production lies in its availability and low cost as an agro-industrial byproduct. Incorporating CSL into industrial-scale processes can significantly reduce production costs as it eliminates the need for expensive supplements such as yeast extract or peptone. Additionally, the valorization of CSL aligns with the principles of the circular economy, as it involves transforming a waste product into a valuable input for biotechnological applications. This reduces the environmental impacts associated with CSL disposal and minimizes the carbon footprint of the production process.

CSL is a nutrient-rich byproduct of the corn wet-milling process, and it is commonly used as a supplement in microbial fermentations and as animal feed due to its composition. It is mainly composed of approximately 50% water, with the remaining portion being rich in proteins, free amino acids, minerals, and vitamins, along with reducing sugars (like dextrose) and organic acids such as lactic acid [31,32]. These components contribute to CSL's use as a cost-effective nitrogen and mineral source in fermentation industries.

A comparative analysis of the current study with previous research on biosurfactant production is presented in Table 3. In a previous study, Santos et al. [19] evaluated biosurfactant production using *Y. lipolytica* IMUFRJ 50682 with CSL (1–5 g/L) as the sole nutrient source, with or without ammonium sulfate supplementation. The results showed improved biosurfactant production when CSL was combined with ammonium sulfate. In the present study, higher CSL concentrations were tested (5–30 g/L) with and without ammonium sulfate, and no significant difference was observed between the two conditions. This suggests that CSL alone may provide sufficient nitrogen for biosurfactant production at higher concentrations. However, the precipitation of CSL was observed at 30 g/L, which may result from the oversaturation of proteins, minerals, or other components in the medium. This phenomenon can impact biosurfactant production by

limiting the bioavailability of nutrients and introducing variability in emulsification index measurements. Future studies can explore pre-treatment methods, such as filtration or centrifugation, to remove insoluble components and improve medium homogeneity.

Table 3. A comparative analysis of biosurfactant production conditions and findings reported in the current study and in previous works.

Study	Condition	Findings	Implications
Santos et al. [19]	CSL (1–5 g/L)	Improved biosurfactant production when CSL was combined with ammonium sulfate; Highest EI: 58%	Demonstrates potential of CSL and ammonium sulfate as effective nutrient combinations
Present study	CSL (≥10 g/L)	No significant difference in biosurfactant production with or without ammonium sulfate; Highest EI: 73%	Suggests CSL alone provides sufficient nitrogen for higher concentrations, reducing reliance on additional supplements
Santos et al. [19]	Butter whey + CSL	Comparable EI achieved with butter whey and CSL within 24 h, faster than butter whey + CSL + ammonium sulfate; Highest EI: 67%	Highlights potential negative impact of ammonium sulfate in media with certain carbon sources like butter whey
Santos et al. [33]	Animal fat + CSL	Surface tension reduction (50 to 28 mN/m) and highest EI with petroleum (100%) and motor oil (94%)	Versatility of biosurfactant produced with CSL, including enhanced oil recovery and bioremediation
Present study	Crude oil/asphaltene-free fraction + CSL	Highest EI achieved (35.8% after 264 h) in asphaltene-free fraction medium; limonene inhibited cell growth	Suggests asphaltene-free fractions are promising substrates, with further optimization needed for solvent compatibility

CSL: corn steep liquor; EI: emulsification index.

Santos et al. [19] also observed a high EI during *Y. lipolytica* IMUFRJ 50682 cultivation in media containing residual frying oil or dairy byproducts (butter whey, cheese whey, or ricotta whey) as carbon sources, with CSL and ammonium sulfate as vitamin and nitrogen sources. However, only the medium containing butter whey, CSL, and ammonium sulfate achieved an emulsification index comparable to those obtained in the present study, requiring 96 h of fermentation. Notably, when the authors grew the strain in a medium containing only butter whey and CSL, the same emulsification index was achieved within 24 h, suggesting a potential negative impact of ammonium sulfate in the presence of butter whey as the primary carbon source. Furthermore, media containing only butter whey and

ammonium sulfate resulted in an emulsification index ten times lower than that of media containing butter whey and CSL.

Nevertheless, CSL contains various components that can act as natural emulsifiers, such as proteins, peptides, phospholipids, and polysaccharides. At higher CSL concentrations, some media exhibited a high EI at a timepoint of zero. Biosurfactant extraction was performed on samples with significant emulsification indices at a timepoint of zero. Still, no surfactant was obtained, confirming the influence of the natural emulsifiers present in CSL. Therefore, 10% CSL was selected as the optimal supplementation condition for bioremediation assays of petroleum fractions, as it promotes biosurfactant production and does not contain suspended solids, which can interfere with the quantification of cell growth and the emulsification index.

Santos et al. [33] evaluated various media formulations containing animal fat and corn steep liquor (CSL) combined with glucose, yeast extract, urea, and other inorganic nitrogen sources to produce a biosurfactant using *Candida lipolytica* (current *Yarrowia lipolytica*). The medium containing 5% animal fat as the primary carbon source and 2.5% CSL as the nitrogen and vitamin source was identified as optimal, achieving the highest reduction in surface tension (from 50 to 28 mN/m) after a 6-day fermentation period. The biosurfactant exhibited high emulsification indices with hydrophobic substrates like petroleum (100%) and motor oil (94%). Additionally, the biosurfactant demonstrated remarkable stability across a wide range of pH values, temperatures (4 °C to 120 °C), and salinity levels (up to 12% NaCl), underscoring its suitability for applications in harsh industrial environments and bioremediation.

Several studies have shown that *Y. lipolytica* can efficiently metabolize diverse carbon sources, including sugars and hydrocarbons, supporting its versatility and potential for biotechnological applications such as biosurfactant production [10,34]. The metabolic pathways involved in biosurfactant production and hydrocarbon degradation by *Yarrowia lipolytica* rely on activating enzymes that facilitate the assimilation and breakdown of complex substrates. Hydrocarbon degradation is initiated by enzymes such as monooxygenases and dioxygenases, which introduce oxygen into hydrophobic compounds, rendering them more accessible for further metabolism. Additionally, lipases may play a role in breaking down lipid components of hydrocarbons into smaller, metabolizable units.

The production of biosurfactants is closely linked to these metabolic processes as it enhances the solubility of hydrophobic substrates and facilitates their transport into cells. Genes involved in the synthesis of surfactant molecules, such as those encoding hydrophobins or lipid biosynthetic enzymes, may be upregulated in response to hydrocarbon exposure. Previous studies have identified specific genes, such as LIP2 (lipase) and POX genes (peroxisomal β-oxidation), as being upregulated during hydrocarbon metabolism in *Y. lipolytica* [10,29].

The adaptability of *Y. lipolytica* to grow on molasses-based substrates (Figure 4a,b), with or without the addition of crude oil, was demonstrated by the high cell concentrations observed for M-O-CSL and M-CSL media, likely due to the diversity of sugars and nutrients in molasses and the metabolic boost provided by hydrocarbons [35–37].

The increase in pH in oil-containing media (crude oil or crude oil asphaltene-free fraction) (Figures 4c and 6b) can be attributed to the metabolic activity of *Y. lipolytica*, which likely produces alkaline byproducts, such as long-chain fatty acids and ammonia, as part of its metabolism of hydrocarbons. These alkaline byproducts contribute to the gradual rise in medium alkalinity, a phenomenon observed in other studies on hydrocarbon fermentation with *Y. lipolytica*. In contrast, the pH oscillations throughout the fermentation period in media containing either glucose or molasses suggest a more balanced production of acidic and neutral metabolites when more straightforward carbon sources are the primary substrates.

This stability indicates that the metabolism of glucose and molasses does not induce the same level of alkalinization as oil-based substrates, likely because these more straightforward carbohydrates are processed through different metabolic pathways that produce fewer alkaline byproducts [38]. Maintaining an optimal pH is crucial in fermentation processes because many enzymes involved in biosurfactant synthesis operate most efficiently within specific pH ranges.

The highest EI values (Figures 4d and 6c) were achieved when the medium pH was around 6, supporting the findings of Bouchedja et al. [39], who demonstrated that pH significantly impacts the dispersion quality of oil microdroplets, in turn influencing their assimilation by the *Y. lipolytica* population. Significant deviations from this pH range can lead to reduced biosurfactant production. Future studies can further optimize pH control in oil-based fermentation processes with *Y. lipolytica*, aiming to maximize biosurfactant yield and functional properties.

No emulsification activity is typically observed when *Y. lipolytica* is grown using glucose as the carbon source and/or without water-immiscible compounds [29]. However, aligning with our findings (Figure 4d), biosurfactant production has been reported with *Y. lipolytica* grown solely on glucose as a carbon source [29,40,41] without hydrocarbons. This suggests that the biosynthesis of these compounds may not be exclusively triggered by the yeast's catabolism of hydrocarbons [29]. Moreover, the rapid biosurfactant production in the G-CSL medium (Figure 4d) is beneficial for processes requiring a fast-acting emulsifier, such as in food and pharmaceutical industries and environmental applications [8,29]. Considering that all assays presented an EI before cell addition (Figure 4d), the CSL-AF medium was the one that presented the highest EI (≈35% in 264 h), excluding its initial value. This delayed peak might be related to the inhibitory effect of limonene, the solvent used for asphaltene extraction, on cell growth. Li et al. [42] demonstrated that 0.5 g/L of limonene was sufficient to increase the lag phase and reduce the cell growth of a strain of *Y. lipolytica*. In the present study, we used 1% (*v*/*v*) of limonene in the control medium (L-CSL), which is approximately the same amount in the asphaltene-free fraction when added to the medium (AF-CSL). Considering limonene's specific density (0.8411 g/cm^3), 1% (*v*/*v*) is equivalent to 8.4 g/L of limonene in the culture medium, which is far from the amount tolerated by this species. However, this extended time for biosurfactant production may be advantageous in applications requiring sustained biosurfactant production over extended periods, such as in continuous bioremediation processes where prolonged emulsification is necessary [34–37].

The insolubility of petroleum hydrocarbons is a limiting factor for their microbial degradation [43]. Surfactants can reduce the surface tension of petroleum hydrocarbons and thus promote emulsification, thereby improving their solubility and consequently increasing the access of *Y. lipolytica* to carbon chains, culminating in their degradation [43–45]. Biosurfactants produced by *Y. lipolytica* have higher environmental compatibility and lower toxicity [43,46,47], which means they can better promote oil degradation [48]. According to the results, *Y. lipolytica* presents a moderate production of surfactant and concomitant cell growth, indicating a potential practical application in oil degradation [47,49]. Therefore, biosurfactant-producing and petroleum-degrading microorganisms tend to promote accelerated petroleum hydrocarbon biodegradation [49–53]. The biodegradation process can be conducted ex situ, i.e., away from the site of the oil spill, in a controlled environment such as bioreactors, treatment facilities, or engineered biopiles. In such systems, contaminated material, such as oil-soaked soil or water, is collected and transported to a designated treatment area where conditions are optimized for microbial activity [54]. This controlled approach allows for the precise regulation of parameters such as nutrient concentration, pH, temperature, aeration, and microbial inoculum. In the case of this study, the nutrient medium based on corn steep liquor (CSL) could be efficiently applied to promote the growth of *Yarrowia lipolytica* and enhance biosurfactant production, thereby improving the emulsification and breakdown of

hydrocarbons. The production of biosurfactants by *Y. lipolytica* not only accelerates oil degradation, making the process more economical, but also plays a crucial role in the microbial conversion of toxic hydrocarbons into less toxic or non-toxic metabolic intermediates. These intermediates can then be further metabolized through the tricarboxylic acid cycle [55–57].

5. Conclusions

This study demonstrates the significant potential of *Yarrowia lipolytica* IMUFRJ 50682 in biosurfactant production and hydrocarbon biodegradation using corn steep liquor (CSL)-based media. The results highlight the adaptability of this yeast strain to diverse carbon sources, including crude oil and molasses, emphasizing its metabolic flexibility and efficiency in emulsification processes. Higher biosurfactant production was achieved with 20% CSL supplementation, but 10% CSL is better for biosurfactant production when other components are present in the media. These results show CSL's viability as a cost-effective and sustainable nutrient source. The findings underline the role of CSL not only as a substrate for microbial growth but also as a critical enhancer of biosurfactant activity. However, some limitations must be acknowledged, including the inhibitory effects of limonene as a solvent for asphaltene extraction, the laboratory-scale nature of the study, and the precipitation issues observed at higher CSL concentrations, which can affect medium consistency. Despite the inhibitory effects of limonene, the use of asphaltene-free crude oil fractions revealed promising emulsification indices, paving the way for further studies on substrate optimization and solvent alternatives. Moreover, the results suggest that pH control is critical for maximizing biosurfactant synthesis, with pH ~6 being identified as an optimal range. Future research should focus on optimizing pH control, testing alternative solvents to limonene, improving medium formulations to ensure consistency in industrial applications, and conducting field trials under real-world environmental conditions to validate the practical applicability of the results. This work advances the application of *Y. lipolytica* in sustainable biotechnological processes, positioning CSL as a cost-effective and efficient nutrient source for biosurfactant production and hydrocarbon bioremediation, with further optimizations and practical validations paving the way for economically viable and environmentally compatible solutions.

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