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Raw Glycerol as Substrate for the Production of Yeast Biomass

Abstract: The possibility of using raw glycerol as a substrate for yeast biomass production as a source of proteins was investigated. Biomass concentration, protein content and total protein content of four yeasts (*Yarrowia lipolytica* NRRL YB-423, *Candida lipolytica* NRRL Y-1095, *Candida utilis* NRRL Y-900 and *Candida rugosa* NRRL Y-95) were determinate, comparing analytical grade glycerol and raw glycerol. *Y. lipolytica* NRRL YB-423 has been selected as promising for cultivation in a raw glycerol-based medium, mainly due to the higher biomass concentration in relation to the other strains. For this strain, four different culture media were tested. The best results were obtained with 50 g/L glycerol, 5.5 g/L ammonium phosphate, 5.5 potassium dihydrogen phosphate, 1 g/L ammonium sulphate, 0.25 g/L magnesium sulphate, 0.021 g/L calcium chloride dehydrate, 1 g/L yeast extract, 1 g/L peptone, pH adjusted to 5.5. In these conditions, it was possible to obtain 17.8 ± 0.6 g/L maximum biomass concentration, 18.2 ± 1.0% protein content and 3.1 ± 0.1 g/L total protein production. These results represent a 1.2-fold increase in biomass concentration, a 1.5-fold increase in protein content and a 1.9-fold increase in total protein production in relation to the results obtained with the previously medium composition.

Keywords: single cell protein, raw glycerol, biodiesel, yeast biomass

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1 Introduction

Biodiesel is produced by transesterification of vegetable oils and animal fats with a monovalent alcohol such as methanol and ethanol. Rapeseed oil, soybean oil, sunflower oil, African palm (*Elaeis guineensis*) oil, castor oil and *Jatropha curcas* oil are the raw materials most frequently used for the production of biodiesel, and glycerol is an inherent byproduct of this reaction [1].

Conversion of the crude product into pure glycerol requires several purification steps, all being energy-consuming and costly processes. Because of a constantly increasing biodiesel production, new applications for raw glycerol will become of great importance [2].

The utilization of raw glycerol as a feedstock for the establishment of bio-refineries can represent an alternative and "environmentally friendly" strategy, aiming to improve the economics of biodiesel industry, as well as to confront with the ongoing increment of glycerol waste streams. Raw glycerol constitutes a versatile carbon source with many possible applications in industrial microbial cultivations, due to its low cost and its wide availability [3]. Therefore, the development of technological processes to convert low priced glycerol into higher value products constitutes an excellent opportunity to add value to the production of biodiesel [4].

The potentiality of crude glycerol valorization without the need of additional treatment and purification steps is considered as a major challenge in order to confront the disposal of tremendous quantities of biodiesel-derived glycerol wastes globally [5]. However, although utilization of raw glycerol in the culture medium without prior purification offers a remarkable advantage against the traditional use of pure glycerol as substrate, only few reports have appeared in the literature on the use of this substrate as carbon source [6]. The raw glycerol obtained from biodiesel synthesis usually presents 55–90% of purity. The rest of the raw glycerol consists of unconverted triglycerides, unconverted methanol or ethanol, biodiesel, soaps and contamination [7].

On the other hand, microorganisms have the ability to upgrade low protein organic materials (as waste materials or relatively simple carbohydrates) to high protein food, and this has exploited on by industry [8]. Four types of microorganisms are used to produce biomass: bacteria, yeasts, fungi and algae. Yeasts were the first microorganisms known, the best studied and generally

best accepted by consumers [9]. Yeast biomass can be used for protein supplementation of a staple diet by replacing costly conventional sources like soymeal and fishmeal to alleviate the problem of protein scarcity. Moreover, the bioconversion of agricultural and industrial wastes to protein-rich food has an additional benefit of making the final product cheaper [10]. Yeast biomass has many applications in food and feed industries as it has high content of protein, high percentage of essential amino acids and other nutrients [11]. Yeasts are important as a raw material for the food, pharmaceutical and cosmetic industries, in addition to being an excellent source of nutrients, mainly proteins, vitamins of the B complex and essential minerals [12].

An important aspect in the production of yeast biomass is the development of a culture medium based on low-cost substrates with high yield and productivity. In this context, glycerol may substitute traditional carbohydrates used in yeast cultivation, such as sucrose, glucose and starch [6].

In this study, four microorganisms were investigated in order to select the most appropriate strain for yeast biomass production as a source of protein. The biomass concentration and protein content obtained using analytical grade glycerol and raw glycerol as carbon sources were compared, and different medium compositions were tested for the most promising strain.

2 Material and methods

2.1 Yeast strains

Yarrowia lipolytica NRRL YB-423, *Candida utilis* NRRL Y-900, *Candida lipolytica* NRRL Y-1095 and *Candida rugosa* NRRL Y-95 were provided by the Northern Regional Research Laboratory (Peoria, USA) and certified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration). The strains were maintained on Yeast Malt (YM) agar tubes and stored at 4°C.

2.2 Glycerol

Analytical grade glycerol (98% w/w) was purchased from Synth (Diadema, Brazil). Raw glycerol was obtained from the synthesis of biodiesel by transesterification of soybean oil and anhydrous ethanol in alkaline catalysis. The raw glycerol contained 78.4% w/w of glycerol.

2.3 Inoculum

Two tubes of microbial culture, previously incubated at 25°C for 48 h, were used. They were scraped with 10 mL of 0.1% w/v peptone diluent for each tube and transferred to 500 mL Erlenmeyer flask containing 200 mL of a medium proposed by Papanikolaou and Aggelis [13], composed of (g/L): 30 analytical grade glycerol; 7 KH_2PO_4; 2.5 Na_2HPO_4; 1.5 $MgSO_4$ $7H_2O$; 0.15 $CaCl_2$; 0.15 $FeCl_3$ $6H_2O$; 0.02 $ZnSO_4$ $7H_2O$; 0.06 $MnSO_4$ H_2O; 0.5 $(NH_4)_2SO_4$; 0.5 yeast extract; pH adjusted to 6.0. The suspension was incubated at 30°C and 180 rpm, and growth was monitored by counting in a Neubauer chamber.

2.4 Shake-flask cultures

Cultivations of the four strains were performed with analytical grade and raw glycerol, using the medium proposed by Papanikolaou and Aggelis [13]. The amount of raw glycerol to be added had its composition considered in order to result in the same glycerol concentration when compared to the analytical grade glycerol-based medium.

Subsequently, once the most appropriate strain was found, cultivations with different culture media (Table 1)

Table 1 Glycerol-based media

Medium	Composition (g/L)	Reference
1	Glycerol 30; KH_2PO_4 7; Na_2HPO_4 2.5; $MgSO_4$ $7H_2O$ 1.5; $CaCl_2$ 0.15; $FeCl_3$ $6H_2O$ 0.15; $ZnSO_4$ $7H_2O$ 0.02; $MnSO_4$ H_2O 0.06; $(NH_4)_2SO_4$ 0.5; yeast extract 0.5; pH adjusted to 6.0.	Papanikolaou and Aggelis [13]
2	Glycerol 50; $(NH_4)_2HPO_4$ 5.5; KH_2PO_4 5.5; $(NH_4)_2SO_4$ 1.0; $MgSO_4$ 0.25; $CaCl_2$ $2H_2O$ 0.021; yeast extract 1.0; peptone 1.0; pH adjusted to 5.5.	Rivaldi et al. [14]
3	Glycerol 40; $(NH_4)_2HPO_4$ 12.4; citric acid 2; $CaCl_2$ $2H_2O$ 0.022; KCl 0.9; $MgSO_4$ $7H_2O$ 0.5; trace element solution 4.6 mL ($CuSO_4$ $5H_2O$ 6; NaI 0.08; $MnSO_4$ H_2O 3; Na_2MoO_4 $2H_2O$ 0.2; H_3BO_4 0.02; $CoCl_2$ 0.5; $ZnCl_2$ 20; $FeSO_4$ $7H_2O$ 65; biotin 0.2); H_2SO_4 5 mL; pH adjusted to 5.0.	Gasser et al. [15]
4	Glycerol 72; K_2HPO_4 3.53; NH_4Cl 28.8; $MgCl_2$ $6H_2O$ 18.29; KH_2PO_4 12.53; Na_2SO_4 12; citric acid 1.51; $ZnSO_4$ $7H_2O$ 0.05; biotin 2.9×10^{-5}; pH adjusted to 4.5.	Triboli et al. [16]

were carried out using raw glycerol. In this case, the amount of raw glycerol to be added had its composition considered in order to result in the required substrate (glycerol) concentration.

The flasks containing glycerol-based medium were inoculated with yeast suspension, previously prepared, in order to achieve 1×10^7 cells/mL. The flasks were maintained in a rotary shaker at 30°C and 180 rpm. Samples were taken at regular intervals and analytical determinations were performed in duplicate. Maximum biomass concentration (g/L), protein content (% w/w), total protein production (g/L) and maximum specific growth rate (h^{-1}) were obtained as responses. The total protein production was calculated according to Choi and Park [17]: multiplying the biomass concentration (g/L) by the protein content (% w/w) obtained at the end of cultivation and dividing by 100. The maximum specific growth rate (μ_{max}) was calculated from eq. (1) by using Microcal Origin® 6.0 software.

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

where μ is the specific growth rate (h^{-1}) and X is the biomass concentration (g/L).

2.5 Biomass concentration

The biomass was monitored by measuring the absorbance at 600 nm. Samples were centrifuged at $1{,}780 \times g$ for 15 min and cells were recovered after washing twice with distilled water. A calibration curve between OD_{600} and the cell dry-weight concentration (g/L) was first established for each microorganism [17].

2.6 Protein content

Total nitrogen was determined by the micro-Kjeldahl method at the end of cultivation. The conversion factor for protein content was 6.25 [18].

2.7 pH

The pH of supernatant was measured using a pH meter [18].

2.8 Statistical analysis

All assays were performed in triplicate. Results were evaluated statistically through variance analysis and Tukey's test at 95% confidence level ($p < 0.05$), using Statistica 6.0 software (StatSoft Inc., USA).

3 Results and discussion

3.1 Yeast selection

In order to investigate whether the impurities found in the raw glycerol could affect microbial growth, assays for *Y. lipolytica* NRRL YB-423, *C. lipolytica* NRRL Y-1095, *C. utilis* NRRL Y-900 and *C. rugosa* NRRL Y-95 were performed using raw glycerol as the main carbon source. They were then compared with the results obtained when analytical grade glycerol was used as a substrate.

Figure 1 shows microbial growth and pH variation for the yeasts *Y. lipolytica* NRRL YB-423, *C. lipolytica* NRRL Y-1095, *C. utilis* NRRL Y-900 and *C. rugosa* NRRL Y-95 in media containing analytical grade and raw glycerol. Figure 1A and B show that the four yeasts reached the stationary phase after 54 h of cultivation in both trials using analytical grade and raw glycerol. Biomass increase coincided with pH decrease (Figure 1C and D), probably caused by the organic acids production, such as citric, isocitric, α-ketoglutaric, acetic and pyruvic acid [6, 19–23]. It can also be seen that the initial pH of the culture of *C. lipolytica* NRRL Y-1095 was 4.5, probably due to the formation of organic acids in the inoculum. In addition, for *C. rugosa* NRRL Y-95, pH decline was less intense.

Table 2 shows a comparison between the yeasts growing on analytical grade glycerol and raw glycerol-based media. With regard to the maximum biomass concentration, *Y. lipolytica* NRRL YB-423 differed from the other strains, both in the medium containing analytical grade glycerol (13.8 ± 0.6 g/L) and the one with raw glycerol (14.7 ± 0.3 g/L). When maximum biomass concentrations using different substrates were compared for each strain, only *C. rugosa* NRRL Y-95 had significant difference at 95% confidence level, reaching 9.9 ± 0.4 g/L and 5.5 ± 0.3 g/L for analytical grade glycerol and raw glycerol-based medium, respectively.

Regarding the protein content, *C. utilis* NRRL Y-900 and *C. rugosa* NRRL Y-95 showed the best results in the medium containing analytical grade glycerol ($18.1 \pm 0.8\%$ and $19.6 \pm 1.0\%$, respectively), with no statistical differences between them. In the raw glycerol-based medium, *C. utilis* NRRL Y-900 showed the highest protein content ($18.8 \pm 0.1\%$), differing statistically from the other strains, at 95% confidence level. On the other hand, for *Y. lipolytica* NRRL YB-423 and *C. utilis* NRRL Y-900 there was no

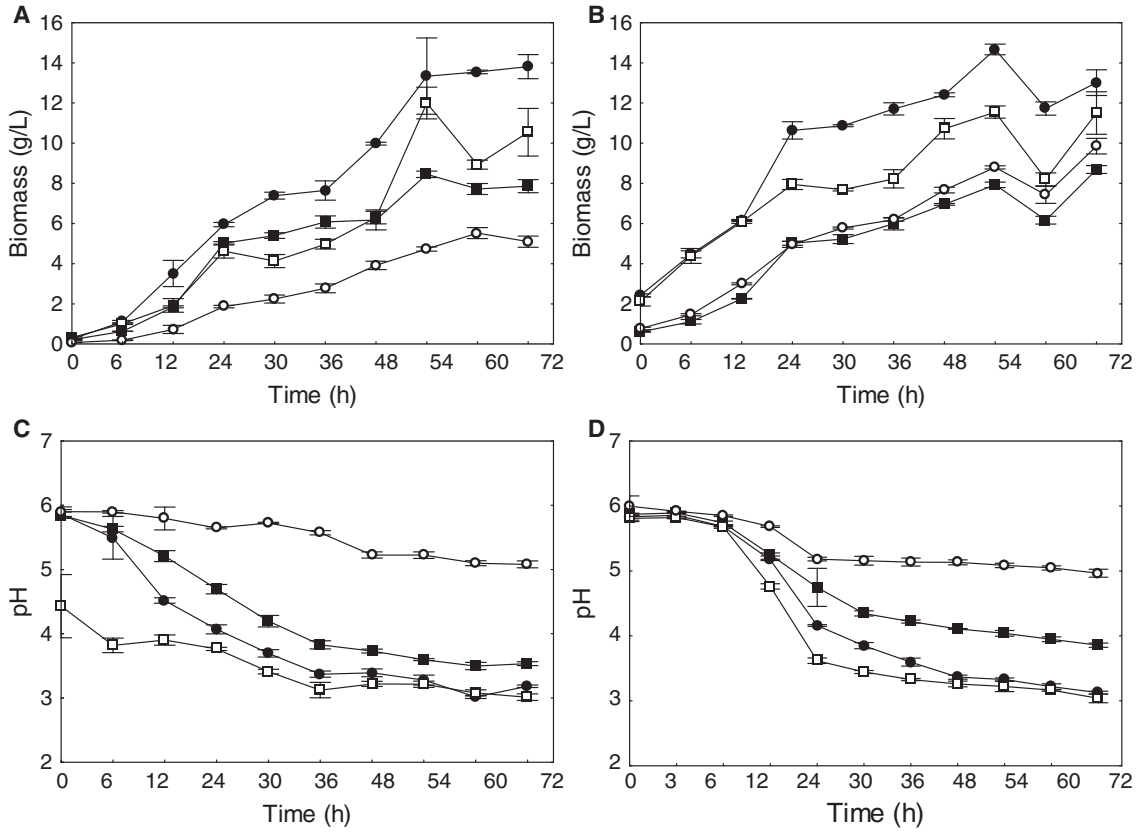

Figure 1 Growth curves and pH variation in a medium containing analytical grade glycerol (A and C, respectively) and raw glycerol (B and D, respectively). ● *Y. lipolytica* NRRL YB-423, □ *C. lipolytica* NRRL Y-1095, ■ *C. utilis* NRRL Y-900, ○ *C. rugosa* NRRL Y-95

Table 2 Mean values ± standard deviations for maximum biomass concentration (X_{max}), protein content, total protein production and maximum specific growth rate (μ_{max}) for analytical grade glycerol and raw glycerol-based media and statistical analysis of the data for different strains

Yeast	X_{max} (g/L)		Protein content (%)		Total protein production (g/L)		μ_{max} (h^{-1})	
Strain	Analytical grade glycerol	Raw glycerol	Analytical grade glycerol	Raw glycerol	Analytical grade glycerol	Raw glycerol	Analytical grade glycerol	Raw glycerol
Y. lipolytica YB-423	$13.8 \pm 0.6^{a.A}$	$14.7 \pm 0.3^{a.A}$	$13.6 \pm 0.4^{c.A}$	$11.9 \pm 0.3^{c.A}$	$1.9 \pm 0.1^{a.A}$	$1.6 \pm 0.1^{a.B}$	$0.25 \pm 0.05^{a.A}$	$0.14 \pm 0.01^{a.B}$
C. lipolytica Y-1095	$12.0 \pm 0.8^{b.A}$	$11.5 \pm 0.3^{b.A}$	$16.7 \pm 1.5^{b.A}$	$12.1 \pm 0.5^{c.B}$	$1.8 \pm 0.1^{a.A}$	$1.4 \pm 0.2^{a.B}$	$0.13 \pm 0.03^{b.A}$	$0.15 \pm 0.04^{a.A}$
C. utilis Y-900	$8.4 \pm 0.2^{c.A}$	$8.7 \pm 0.2^{d.A}$	$18.1 \pm 0.8^{ab.A}$	$18.8 \pm 0.1^{a.A}$	$1.4 \pm 0.1^{b.A}$	$1.6 \pm 0.0^{a.A}$	$0.23 \pm 0.01^{ab.A}$	$0.17 \pm 0.01^{a.A}$
C. rugosa Y-95	$5.5 \pm 0.3^{d.A}$	$9.9 \pm 0.4^{c.B}$	$19.6 \pm 1.0^{a.A}$	$14.5 \pm 0.3^{b.B}$	$1.0 \pm 0.1^{c.A}$	$1.4 \pm 0.1^{a.B}$	$0.26 \pm 0.06^{a.A}$	$0.17 \pm 0.01^{a.A}$

Note: Different lowercase letters within the same column for the same parameter represent significant differences ($p < 0.05$). Different uppercase letters within the same row for the same parameter represent significant differences ($p < 0.05$).

significant difference, at 95% confidence level, between analytical grade glycerol and raw glycerol-based media. However, the other strains showed a reduction in protein content in the medium containing raw glycerol when compared to analytical grade glycerol. Moreover, the use of raw glycerol has the advantage of reducing production costs, since it does not require a purification process.

When the total protein production was evaluated, at 95% confidence level, *Y. lipolytica* NRRL YB-423 and

C. lipolytica NRRL Y-1095 showed the highest values (1.9 ± 0.1 g/L and 1.8 ± 0.1 g/L, respectively) for analytical grade glycerol, differing statistically from the other yeast strains. In the medium containing glycerol derived from biodiesel production, there was no statistically significant difference between the yeasts. Comparing analytical grade glycerol and raw glycerol, *C. utilis* NRRL Y-900 showed no statistically significant difference between the media, while *Y. lipolytica* NRRL YB-423 and *C. lipolytica* NRRL Y-1095 showed a decrease in total protein production in the medium containing raw glycerol, compared to the analytical grade glycerol-based media. Moreover, *C. rugosa* NRRL Y-95 showed higher total protein production in raw glycerol-based media.

In relation to the specific growth rate, all yeast strains showed no statistically significant differences at 95% confidence using raw glycerol; however, *C. lipolytica* NRRL Y-1095 showed a lower specific growth rate, differing statistically from other strains, at 95% confidence, using pure glycerol.

In general, the use of raw glycerol and, consequently, the presence of impurities such as ethanol, soaps and salts did not have any impact on maximum biomass concentration reached for each yeast strain, but protein content was more influenced. According to Yang et al. [24], impurities in crude glycerol can greatly influence the conversion of glycerol into other products, resulting in lower production rates and product yields (compared with pure or commercial glycerol under the same culture conditions).

Considering that the yeasts were not significantly different from each other regarding total protein production in raw glycerol-based media, *Y. lipolytica* NRRL YB-423 was selected for biomass concentration and protein content improvement. *Y. lipolytica* is an unconventional yeast which in general shows a lower but useful protein content, with a content of essential amino acids complied with the FAO standards [25] and is known for its ability to excrete metabolites such as organic acids and enzymes that can also be explored.

3.2 Medium composition

Figure 2 shows *Y. lipolytica* NRRL YB-423 growth and pH profile using raw glycerol-based media with different compositions (Table 1). As shown in Figure 2A, stationary phase was reached between 54 h and 72 h cultivation. Lag phase was pronounced in the cultivation with the medium 3, and with the medium 4 the growth was limited in comparison with the media 1 and 2. In addition, biomass increase coincided with the pH decrease for both medium 1 and 2 (Figure 2B), clearly observed, probably due to organic acids excretion [6, 22, 26]. On the other hand, the decrease in pH with the medium 4 was low. Therefore, media 3 and 4 showed the worst performances, which can be associated with the absence of organic nitrogen sources, such as yeast extract and peptone, present in the media 1 and 2 (Table 1).

As shown in Table 3, medium 2 [14] had the best results for maximum biomass concentration (17.8 ± 0.6 g/L), protein content ($18.2 \pm 1.0\%$) and total protein production (3.1 ± 0.1 g/L), differing statistically from the other culture media at 95% confidence level. Such results represent a 1.2-fold increase in biomass concentration, a 1.5-fold increase in protein content and a 1.9-fold increase in total protein production in relation to the results obtained with the previously medium composition. Comparing these media, it can be observed a high nitrogen addition with medium 2, and this increase of nitrogen content in the medium can lead to the augment of cellular protein content [27].

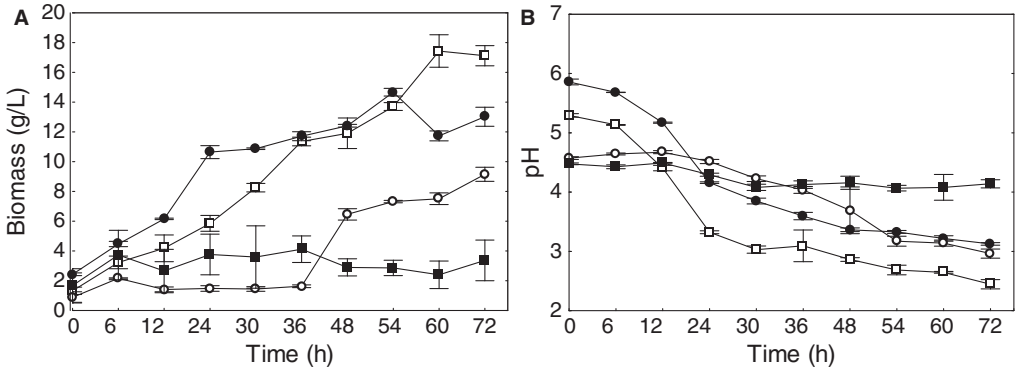

Figure 2 Growth curves (A) and pH profile (B) for *Y. lipolytica* NRRL YB-423 growing on different culture media using raw glycerol as carbon source. ● Medium 1; □ Medium 2; ○ Medium 3; ■ Medium 4

Table 3 Mean values ± standard deviations for maximum biomass concentration (X_{max}), protein content, total protein production and maximum specific growth rate (μ_{max}) and statistical analysis of the data

Medium	X_{max} (g/L)	Protein content (%)	Total protein production (g/L)	μ_{max} (h^{-1})
1	14.7 ± 0.3^{b}	11.9 ± 0.3^{c}	1.6 ± 0.1^{b}	0.14 ± 0.02^{b}
2	17.8 ± 0.6^{a}	18.2 ± 1.0^{a}	3.1 ± 0.1^{a}	0.19 ± 0.02^{b}
3	9.1 ± 0.5^{c}	16.1 ± 0.2^{b}	1.5 ± 0.1^{b}	0.18 ± 0.04^{b}
4	4.1 ± 0.9^{d}	17.0 ± 0.9^{ab}	0.6 ± 0.2^{c}	0.30 ± 0.06^{a}

Note: The same letters indicate that there is no significant difference between the studied media at 95% confidence level ($p < 0.05$).

Maximum biomass concentration was quite significant when compared with some studies found in the literature. Kim et al. [28] reported that recombinant *Y. lipolytica* CX161-1B strain produced about 14 g/L of biomass during cultivation on pure glycerol-based media (25 g/L), after 30 h cultivation. For *Y. lipolytica* LGAM S (7)1, Papanikolaou and Aggelis [13] reached about 7.5 g/L biomass using glycerol as carbon source, while Papanikolaou et al. [6] obtained 7.9 g/L biomass for *Y. lipolytica* ACA-DC 50109 cultivated in a medium containing 45.9 g/L residual glycerol from biodiesel synthesis (65% w/w). Both works used a nitrogen-limited medium, in order to induce lipid accumulation and citric acid production. Makri et al. [29] reported biomass of 4.68 g/L when growing *Y. lipolytica* in repeated batch culture using pure glycerol (27.8 g/L). Chatzifragkou et al. [30] obtained 7.1 g/L biomass production during growth of *Y. lipolytica* LFMB 20 using a nitrogen-limited medium containing 30 g/L raw glycerol.

In the work of Galvagno et al. [31], a chemically induced mutant of *Y. lipolytica* Y-1095 was able to produce 17.84 g/L in an optimized medium containing 13.0 g/L biodiesel-derived glycerol and 10.0 g/L peptone after 48 h incubation. Taccari et al. [32] obtained for *Y. lipolytica* DiSVA C 12.1 biomass of 25.7 g/L in an optimized medium containing 60 g/L of crude glycerol, 20 g/L of peptone and 10 g/L of yeast extract. Therefore, it is important to note that we achieved a similar value using a lower concentration of organic nitrogen, contributing to a lower cost of production.

On the other hand, the protein content, in general, was lower compared with other yeasts used for single cell biomass production. In the work of Zheng et al. [27], *C. utilis* OZ993 showed a protein content of 26% in a medium containing salad oil processing residue. Choi et al. [33] found higher protein content (36.5%) using *Pichia stipitis* CBS 5776 in a medium containing waste Chinese cabbage as a carbon source and ammonium sulphate as a nitrogen source. Finogenova et al. [34], with the mutant *Yarrowia lipolytica* N1, obtained similar results (protein content of 18.0%), using ethanol (18.0 g/L) as carbon source to produce citric acid.

The values of maximum specific growth rate were similar that those mentioned by Papanikolaou et al. [26], Papanikolaou et al. [6] and Taccari et al. [32] for *Yarrowia lipolytica* strains, respectively, 0.21 h^{-1}, 0.21 h^{-1} and 0.15 h^{-1}.

However, in relation to the total protein production, the results obtained in this study were greater than those obtained by Choi and Park [17] for *P. stipitis* CBS 5776 growing on YM broth (2.8 g/L). Thus, *Y. lipolytica* NRRL YB-423 may have highly potential application for industrial production of yeast biomass using raw glycerol.

4 Conclusion

Among the studied yeasts, *Y. lipolytica* NRRL YB-423 was selected as promising for cultivation using a raw glycerol-based medium, mainly due to the high biomass concentration. For this yeast, the replacement of pure glycerol by raw glycerol had no impact on biomass production and protein content. Thus, it was possible to use raw glycerol without prior purification, representing an economical advantage. In order to improve some cultivation parameters, the modification of medium composition was considered. This strategy led to a 1.2-fold increase in biomass concentration, a 1.5-fold increase in protein content and a 1.9-fold increase in total protein production, reaching 17.8 ± 0.6 g/L, $18.2 \pm 1.0\%$ and 3.1 ± 0.1 g/L, respectively. Therefore, the composition of the culture medium was found to be crucial for the improvement of *Y. lipolytica* NRRL YB-423 performance in batch cultivation.

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