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Use of Wastewaters from Ethanol Distilleries and Raw Glycerol for Microbial Oil Production

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Abstract: The production of biodiesel from single-cell oils (SCOs) utilizing industrial wastes as feedstock presents an economically viable approach. To date, studies have rarely reported the utilization of vinasse combined with industrial glycerol for the production of SCO. This study aimed to assess the performance of a *Rhodotorula toruloides* strain in vinasse from ethanol distilleries supplemented with pure/raw glycerol as an affordable carbon feedstock for SCO production. Several critical factors, including the C/N ratio, the impact of impurities in the crude glycerol, the proper nitrogen source, and the effects of the vinasse compositions, were evaluated. The results showed that the incorporation of urea and raw glycerol increased the lipid content to 51.8 ± 1.6% and the lipid productivity to 0.034 ± 0.001 g $L^{-1}h^{-1}$. Elevated biomass (42.5 g L^{-1}) and lipid (11.0 g L^{-1}) concentrations indicated that impurities in the raw glycerol positively affected the growth and lipid accumulation of this strain. Notably, supplementing raw glycerol to the vinasse led to a 16.1% increase in biomass concentration and a 25.7% rise in lipid content, significantly enhancing lipid productivity by 59.6%. The fatty acid profile predominantly featured unsaturated fatty acids (96.8%), including high percentages of stearic acid (41.8 ± 2.6%), palmitic acid (21.8 ± 1.5%), and oleic acid (18.3 ± 1.4%), aligning with the standards for vegetable-oil-based biodiesel manufacture. Fed-batch strategies using pulse-feeding turned out to be less effective than the constant-flow feeding strategy with vinasse supplemented with raw glycerol, which achieved a higher lipid productivity of 0.30 g $L^{-1}h^{-1}$.

Keywords: microbial oil; oleaginous yeast; biodiesel; vinasse; raw glycerol; fermentation

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1. Introduction

Concern over environmental issues and energy security has driven research interests in developing biofuels as potential supplements or replacements for conventional petroleum-based fuels [1–3]. Biodiesel is an alternative biofuel derived from the transesterification of vegetable or animal fats and has gained importance in recent decades as a biodegradable and renewable source of energy. However, its large-scale production from vegetable oils has some limitations. For the sustainable development of biodiesel, it is crucial to ensure that triacylglycerol (TAG) can be produced continuously from renewable feedstocks without competition with the food industry.

The potential of microbial oils as a viable alternative to vegetable oils in biodiesel manufacture has been recognized [4–7]. Some oleaginous microorganisms have exhibited

remarkable capabilities for lipid accumulation under special conditions, achieving lipid contents as high as 60–70% of their dry weight, with lipids sharing a similar fatty acid profile to those of vegetable oils. In contrast, the volumetric productivity of microbial lipids has not always been competitive, depending on the carbon source utilized [8]. Studies that have reported biomass concentrations exceeding 100 g L^{-1} and lipid contents greater than 60% have typically relied on glucose as the primary carbon source [9,10]. However, the reliance on glucose as a feedstock for microbial lipid production hampers its industrialization due to high production costs [11]. Substituting glucose with affordable carbon sources is vital for increasing the economic feasibility of the fermentation process. Accordingly, various fermentation substrates have been evaluated, including fermentable monosaccharides, glycerol, whey permeate, molasses, sewage sludge, lignocellulosic biomass, molasses, glycerol, organic acids, food waste, and a variety of agro-industrial wastes [12–21]. Additionally, some research teams from countries including Brazil, China, and Indonesia have reported work investigating the lipid production of oleaginous yeasts, utilizing stillage from the brewing industry and bioethanol distilleries [22,23].

Organic wastes generated by local industries can serve as a renewable raw material for SCO production. Both vinasse and industrial glycerol are by-products of biofuel production (bioethanol and biodiesel, respectively), the large volumes of which constitute a problem from an environmental point of view. With the dramatic increase in biodiesel production, a significant amount of raw glycerol is available in the market. Raw glycerol has been studied as an alternative feedstock for the production of SCO in recent years. Several studies have obtained promising results, considering raw glycerol an attractive alternative carbon source for transforming it into value-added products [24–28]. On the other hand, vinasse is an agro-industrial wastewater obtained in large amounts at the end of the distillation process from ethanol distilleries, and it has also been investigated for SCO production [29,30]. However, researchers have observed inhibited cell growth when cultivating oleaginous yeasts in vinasse [31–34], necessitating an initial assessment before their application at an industrial scale. Utilizing industrial glycerol and vinasse as substrates is a viable option for industrial-scale SCO production, and exploring the mixed use of these substrates constitutes an interesting and novel topic for the scientific community, particularly for those countries that produce ethanol from sugar cane with limited sources of fossil fuels [35]. In addition, this approach offers the benefit of reducing the organic load of vinasse applied to soils around agro-industrial facilities, which are often impacted by vinasse's acidic nature and high potassium content.

Rhodotorula species are considered promising candidates for lipid production due to their flexible substrate adaptability, superior lipid content, and potential of producing fatty-acid-derived chemicals and carotenoids [36,37]. In the present study, we evaluated the effect of impurities of raw glycerol and the synergistic effect of vinasse supplemented with raw glycerol on the kinetic behaviors of *Rhodotorula* MT372485. Additionally, we examined fed-batch strategies for scaled-up fermentation in SCO production. These results will provide valuable insights into the synergistic utilization of raw glycerol and vinasse as potential feedstocks for the industrial production of SCO, offering a sustainable pathway for biodiesel manufacturing.

2. Material and Methods

2.1. Microorganism and Raw Materials

Rhodotorula toruloides strain was sourced from the ICIDCA collection (Havana, Cuba), originally isolated from sugar cane molasses. The yeast strain, identified through sequencing of the D1/D2 region in ADNr 26S, was deposited in GenBank as *Rhodotorula* MT372485.

Yeast propagation involved initial cultivation on YPD agar for 96 h at 150 rpm, pH = 6.0 ± 0.2, and 30 °C on a rotary shaker (Sky-211b, Sukun) followed by liquid inoculation in YPD medium in 250 mL Erlenmeyer flasks at 30 °C for 48–72 h. Subsequently, propagation to 1000 mL Erlenmeyer flasks was carried out at initial pH of 5.0 and 30 °C utilizing an inoculum ratio of 1:10 (*v*:*v*) of *R. toruloides* in the exponential phase on YPD medium. During assays, samples were collected, and the supernatant was stored at −20 °C for analytical determinations.

Glycerol from BDH Prolabo was used in control media, while raw glycerol was obtained from Co. Rivers Bioengineering (Yiyang, Hunan, China). Vinasses were obtained from two ethanol distilleries located in different industrial complexes: vinasse A from Heriberto Duquesne distillery (Santi Spíritus, Cuba) and vinasse B from Rihui Lin distillery (Nanning, Guangxi, China). Fed-batch strategies were employed using an initial medium composed of diluted vinasse B supplemented with raw glycerol.

2.2. Culture Conditions

The basic media and culture conditions referred to previous studies employing *Rhodotorula* strains for SCO production [36,37]. The effect of impurities of raw glycerol was evaluated through a comparison of the biomass concentration, yield, and specific growth rate with a culture on glycerol at different substrate concentrations (20–120 g L^{-1}) over 48 h in media supplemented with 20 g L^{-1} peptone and 10 g L^{-1} yeast extract at pH 5.0.

Culture media with glycerol and raw glycerol supplemented with ammonium sulfate (0.7 g L^{-1}) or urea (0.31 g L^{-1}) were compared to evaluate the effect of inorganic and organic nitrogen sources, respectively. The total nitrogen concentration was adjusted to a carbon/nitrogen molar ratio (C/N) of 100.

The C/N ratio was investigated in culture media with glycerol (50 g L^{-1}) supplemented with K_2HPO_4 (0.84 g L^{-1}), $MgSO_4.7H_2O$ (1.0 g L^{-1}), and NH_4SO_4 concentrations equivalent to a C/N interval from 50 to 400.

The influence of the initial pH on growth and lipid accumulation was evaluated in medium with raw glycerol (100 g L^{-1}) supplemented with urea (0.3 g L^{-1}), $MgSO_4.7H_2O$ (1.0 g L^{-1}), and K_2HPO_4 (0.84 g L^{-1}) at pH intervals from 4.0 to 6.0.

The effect of the vinasse composition was assessed by comparing cell growth in vinasses A and B, both in their undiluted form (factor 1) and diluted form (factor 2 and factor 3). Factor 2 corresponded to a 50% dilution with water. Factor 3 corresponded to a mixture of 1/3 vinasse and 2/3 water, which equates to 33.33% vinasse.

The synergistic effect of raw glycerol impurities and the vinasse composition was evaluated using a medium with diluted vinasse B (factor 2) supplemented with raw glycerol (60 g L^{-1}).

Fermentation experiments were conducted using Chemap Bioreactors with an initial working volume of 2 L to evaluate different fermentation modes. Operational parameters were adjusted initially to 600 rpm, pH 5.0, 30 °C, PO_2 30%, and 1.0 vvm. Gradual increases in stirring speed and air flow up to 1000 rpm and up to 1.5 vvm, respectively, were performed when PO_2 decreased to 30%.

The initial culture medium used in the fed-batch strategies was composed of diluted vinasse B (factor 2) supplemented with raw glycerol (50.0 g L^{-1}). The experiments were performed with two replicates, and samples were analyzed per duplicate for the dry biomass, nitrogen concentration, chemical oxygen demand, and lipid content.

2.3. Analytical Methods

Cultures were centrifuged at 5000× *g* for 5 min. The yeast dry concentration (DCC) was determined from the value of biomass sample humidity determined in an infrared balance

(Goettingen, Sartorius GmbH, Gottingen, Germany) at 105 °C. The Conde method was utilized for chemical oxygen demand (COD) determination, involving refluxing centrifuged fermented medium and subsequent titration [38].

The quantitative analysis of organic compounds in vinasse and glycerol was performed with a Shimadzu 10AVP high-performance liquid chromatography (HPLC) system (Kyoto, Shimadzu Corp., Kyoto, Japan) equipped with an RID-10A refraction index detector and an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 65 °C with 5 mM H_2SO_4 as the eluent at a flow rate of 0.8 mL min^{-1}. The limit of detection was 100 ppm. Samples were diluted before measurement to ensure precise determination of analyte concentrations within the linear response range of the HPLC method.

The lipid content was determinate using the Bligh and Dyer protocol [39]. Samples were collected and centrifuged, and the lipids were extracted and weighed on an analytical balance. Fatty acid methyl esters (FAMEs) were obtained by converting the fatty acids in the oil samples and were analyzed by the gas chromatography–mass spectroscopy (GC-MS) method. Fatty acid compositions of the methyl esters were analyzed with a GC-14B gas chromatograph (Shimadzu, Japan) equipped with a CP-FFAP CB capillary column (25 m × 0.32 m × 0.30 μm) produced by Agilent. Heptadecanoic acid methyl ester was used as an internal standard. The column temperature was kept at 180 °C for 0.5 min and heated to 250 °C at 10 °C min^{-1}, and this was maintained for 6 min. The temperatures of the injector and detector were set at 245 °C and 260 °C, respectively. The determination was carried out using the internal standard method, with an internal standard concentration of 1 g L^{-1}. Samples were diluted to a range between 0.5 and 2 times the concentration of the standard sample for measurement.

2.4. Statistical Analysis

The graphics were elaborated with Microsoft Excel 2016. All values represent the mean values of three samples ± standard deviation (SD). The statistical analysis was performed using the ANOVA software (MINITAB 19) followed by Tukey's post hoc comparison ($p \leq 0.05$).

3. Results and Discussion

3.1. Effect of Raw Glycerol Concentration on Microbial Lipid Production

Some impurities present in crude glycerol, such as sodium oleate, methyl oleate, and glyceryl monooleate, have been found to exert a positive influence on cell growth and lipid production, resulting in enhanced yields. Some studies have demonstrated that an appropriate concentration of surfactants can be beneficial to the microbial fermentation process [40]. Additionally, research has indicated that an elevated concentration of sodium chloride in crude glycerol can be a factor leading to an increase in osmotic pressure, which may adversely affect the growth of yeasts. However, the addition of an optimal concentration of sodium chloride has been shown to promote cell growth and lipid accumulation [41,42].

Table 1 shows the impact of the glycerol and raw glycerol concentration on the growth parameters of the present study. No growth inhibition was observed within the examined range. Notably, the use of raw glycerol resulted in an increased biomass concentration, yield of biomass on substrate (Yx/s), and maximum specific growth rate (μ) compared to using pure glycerol under identical conditions.

This behavior aligns with findings by Xu et al. [36] using *R. toruloides* AS 21389, suggesting that raw glycerol can be directly converted to SCO by *R. toruloides*. However, it differs from the growth behavior observed in *R. toruloides* Y-1091, which exhibited a latency period during the initial hours. In the present study, the specific growth rates in glycerol

were similar to those observed of other high-lipid-producing strains cultured in glycerol, such as *Rhodotorula mucilaginosa* DISVA6094 and DISVAC7.1 [43].

Table 1. The effect of glycerol and raw glycerol concentration on dry cell concentration (DCC), yield of biomass on substrate (Yx/s), and maximum specific growth rate (μ) of *R. toruloides*.

Glycerol Concentration (g L^{-1})	Glycerol			Raw Glycerol		
	DCC (g L^{-1})	Yx/s (g g^{-1})	μ (h^{-1})	DCC (g L^{-1})	Yx/s (g g^{-1})	μ (h^{-1})
20	7.3 ± 0.3^{a}	0.28 ± 0.04^{d}	0.15 ± 0.00^{g}	10.2 ± 1.77^{j}	0.51 ± 0.09^{n}	0.19 ± 0.01^{q}
40	9.1 ± 0.2^{a}	0.19 ± 0.03^{e}	0.16 ± 0.00^{g}	13.5 ± 0.38^{k}	0.34 ± 0.01^{n}	0.20 ± 0.01^{q}
60	10.3 ± 1.6^{a}	0.14 ± 0.02^{f}	0.18 ± 0.00^{g}	15.9 ± 1.20^{k}	0.26 ± 0.02^{o}	0.24 ± 0.01^{q}
80	12.1 ± 1.9^{b}	0.13 ± 0.02^{f}	0.22 ± 0.00^{h}	17.4 ± 1.80^{kl}	0.21 ± 0.02^{o}	0.22 ± 0.01^{q}
100	15.7 ± 1.4^{c}	0.14 ± 0.01^{f}	0.20 ± 0.00^{h}	16.2 ± 1.45^{k}	0.16 ± 0.01^{p}	0.25 ± 0.01^{r}
120	17.7 ± 2.1^{c}	0.14 ± 0.04^{f}	0.26 ± 0.00^{i}	21.4 ± 1.62^{m}	0.18 ± 0.01^{p}	0.27 ± 0.03^{r}

Different letters in the same column in the table indicate that there were significant differences according to the Tukey test ($p \leq 0.05$).

The composition of the raw glycerol impurities depends on the biodiesel preparation methods and plays a crucial role in fermentation [44]. The observed increase in the biomass concentration, biomass yield, and specific growth rate with raw glycerol can be attributed to the presence of microelements as potassium, calcium, sulfur, and magnesium, which favored cellular growth and lipid accumulation.

Certain impurities, including inorganic salts, glycerides, and soaps, have positive impact on biomass and lipid production, while methyl esters exhibit no significant effects. Nevertheless methanol, a common impurity, has the largest inhibitor effect [45–47].

3.2. Effect of Nitrogen Source on Microbial Oil Production

As shown in Figure 1, there was no substantial variance in the peak cell density when either ammonium sulfate or urea was incorporated as the nitrogen source, regardless of using glycerol or raw glycerol as the carbon substrate. However, a noteworthy increase in the lipid content was observed when ammonium sulfate was replaced by urea in both substrates, with higher values attained in the raw glycerol media, reaching 7.9 ± 0.4 g L^{-1} for the biomass concentration, $51.8 \pm 1.6\%$ for lipids, and a lipid productivity of 0.034 ± 0.001 g $L^{-1}h^{-1}$.

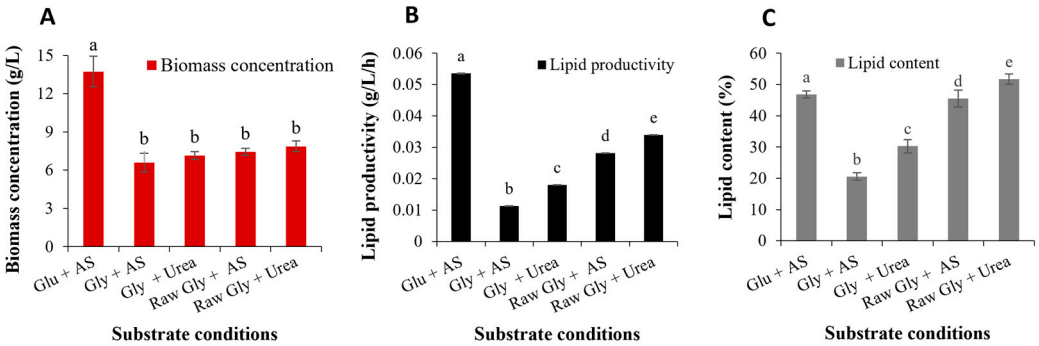


Figure 1. The effect of nitrogen source on (**A**) biomass concentration, (**B**) lipid productivity, and (**C**) lipid content grown in flask culture at initial pH of 5.0, 30 °C, and 150 rpm. Data are shown as mean ± SD from three experiments ($n = 3$). Glu: glucose, Gly: glycerol, AS: ammonium sulfate ($p < 0.05$, Fisher's test). Different letters in the same column in the table indicate that there were significant differences according to the Tukey test ($p \leq 0.05$).

The observed effect is consistent with those reported in the literature. Several researchers have found that the use of organic and inorganic nitrogen sources results in different effects on cell growth and lipid accumulation [45,46]. The advantageous effect of an organic nitrogen source on lipid accumulation has been demonstrated. *R. toruloides* CBS 14 reached 18% and 51% lipid contents, respectively, when cultured with different nitrogen sources, including ammonium sulphate and L-glutamate chloride [48,49]. Based on these results, urea was employed as the nitrogen source for the subsequent investigations.

3.3. Effect of C/N Ratio

Given that yeast accumulates lipids under conditions of excess carbon and limited nitrogen and that the optimal C/N ratio for yeast growth is approximately 35, a C/N ratio exceeding 50 was proposed to favor lipid production. In the present work, C/N ratios ranging from 50 to 400 were selected to evaluate the effect on lipid production, with the understanding that the elevation of the C/N ratio can be realized by supplementing the culture with glycerol when employing a feeding strategy. It was equally essential to take into account the nitrogen source contained in the vinasses. A glycerol concentration ranging from 20 to 120 g/L was selected to prevent the inhibition of yeast growth due to an excess substrate concentration. Furthermore, economic factors on an industrial scale were carefully considered.

As shown in Figure 2, the biomass concentration and biomass yield remained constant at an average value of 13.47 ± 0.8 g L^{-1} and 25.88 ± 1.6 g L^{-1}, respectively, with a C/N ratio ranging from 50 to 400. However, the lipid content and productivity reached maximum values of 39.3 ± 2.5% and 0.033 ± 0.004 g $L^{-1}h^{-1}$ in the C/N interval of 250–400, suggesting that the metabolism of *R. toruloides* was directed towards lipid biosynthesis rather than cell proliferation under the condition of higher C/N ratios.

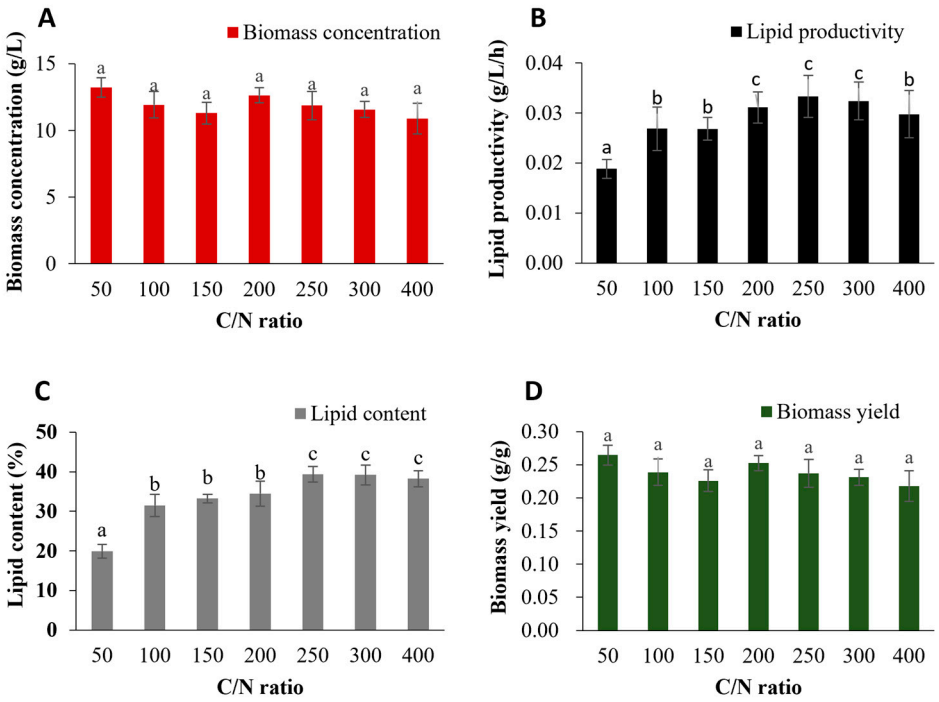


Figure 2. Effect of C/N ratio on (**A**) biomass concentration, (**B**) lipid productivity, (**C**) lipid content, and (**D**) biomass yield of *R. toruloides* grown in flask culture at initial pH of 5.0, 30 °C, and 150 rpm. Each point represents the mean value ± SD of three samples of a representative experiment of three runs ($p < 0.05$, Fisher's test). Different letters in the same column in the table indicate that there were significant differences according to the Tukey test ($p \leq 0.05$).

A nitrogen-limited culture condition with a C/N ratio of 250 was established using glycerol concentrations ranging from 20–120 g L^{-1} to evaluate the effect of the glycerol concentration on the cell biomass concentration, lipid content, and productivity. Figure 3 shows that the biomass concentration initially increased with the glycerol concentration and then remained constant (11.1 ± 0.4 g L^{-1}) when glycerol exceeded 40 g L^{-1}. However, the lipid concentration reached the maximal value of 5.7 g L^{-1} (48.1 ± 1.3%) at a glycerol concentration of 80 g L^{-1}. Lipid productivity showed a parabolic behavior, reaching maximal values (0.033 ± 0.002 g L^{-1} h^{-1}) within a glycerol concentration range of 60–80 g L^{-1}. Both the biomass yield and lipid yield decreased with increasing the glycerol concentration. This behavior is similar to that observed during the evaluation of *Torulaspora maleeae* Y30 feeding on glucose [50] and *Rhodotorula* strains feeding on raw glycerol [27,51].

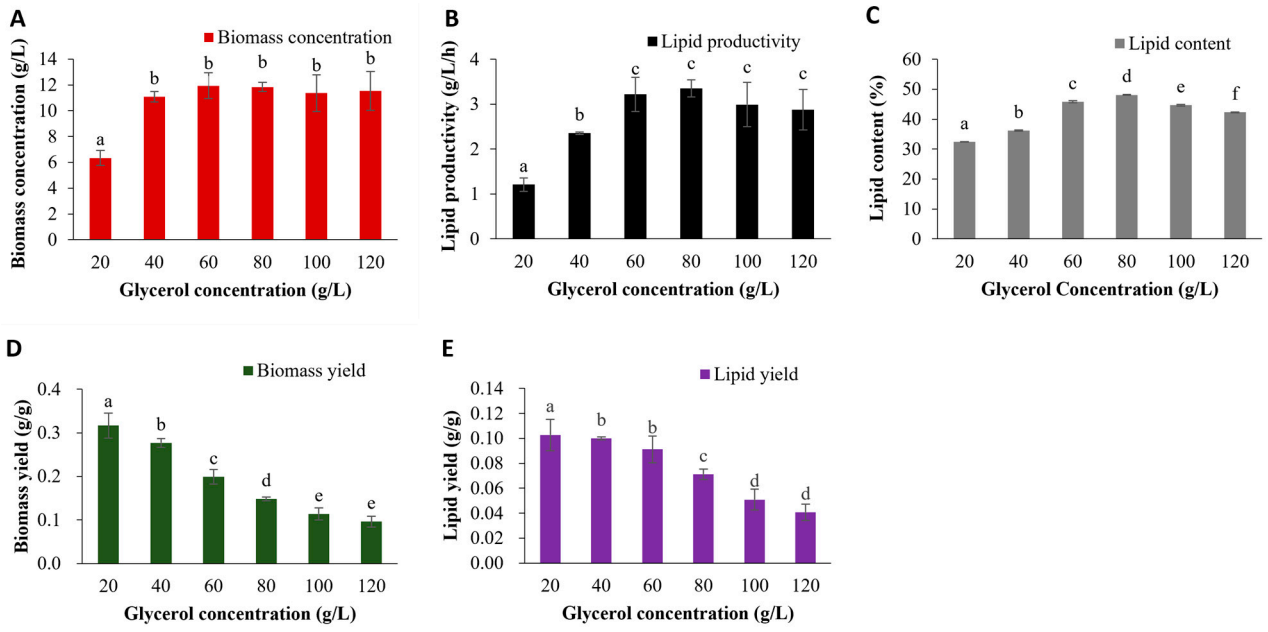


Figure 3. Effect of glycerol concentration on (**A**) biomass concentration, (**B**) lipid productivity, (**C**) lipid content, (**D**) biomass yield, and (**E**) lipid yield of *R. toruloides* using nitrogen-limited media (C/N 250). Each point represents the mean value ± SD of three samples from a representative experiment of three runs ($p < 0.05$, Fisher's test). Different letters in the same column in the table indicate that there were significant differences according to the Tukey test ($p \leq 0.05$).

3.4. Effects of Vinasse Composition on Microbial Lipid Production

The existing literature suggests that the primary organic components of sugarcane vinasse comprise sugars, alcohols, aldehydes, ketones, esters, and acids [20,29,52]. To effectively utilize vinasse, it is crucial to investigate its composition, given that the presence and concentration of toxic compounds can inhibit cell growth. Consequently, the use of vinasse may necessitate dilution or additional treatments.

In the present study, vinasse samples A and B were characterized and are summarized in Table 2. The findings revealed that vinasse samples A and B could be distinguished as different, with six compounds exhibiting significant variations, among which glucose, glycerol, and nitrogen were the most significant.

In vinasse A, no inhibition of cell growth was observed. However, cell growth in undiluted vinasse B experienced a significant inhibitory effect, characterized by an initial lag phase of approximately 24 h, followed by a slow growth rate with a maximum specific growth rate of 0.034 h^{-1}. In contrast, the dilution of vinasse B allowed for a significant reduction in the adaptation phase time and a significant increase in the specific growth rate, reaching up to 0.143 h^{-1}(Figure 4). The inhibitive effect of vinasse has also been

documented in previous research. Lima et al. proposed that the initial COD of vinasse is the most influential variable affecting cell growth and lipid accumulation, outpacing the influence of other variables such as inoculum size, temperature, and culture time [52]. Given that vinasse typically contains some growth-inhibiting compounds like furfural and 5-hydroxymethyl furfural (HMF), it should be diluted to decrease the organic load to below 25,000 mg L^{-1} in order to alleviate the toxic impacts [53].

Table 2. Composition of vinasses A and B.

Composition	Concentration (g L^{-1})	
	Vinasse A	**Vinasse B**
Glucose *	4.60 ± 0.35	12.8 ± 1.10
Fructose	4.34 ± 0.23	5.01 ± 0.60
Ethanol *	0.36 ± 0.06	0.71 ± 0.15
Arabinose *	0.43 ± 0.03	3.77 ± 0.09
Glycerol *	4.90 ± 0.70	9.94 ± 1.20
Acetic acid	1.66 ± 0.31	1.23 ± 0.65
Lactic acid	0	0
Furfural	0	0
5-HMF	0.01 ± 0.01	0
Vanillin *	0	0.02 ± 0.01
Nitrogen *	0.70 ± 0.20	3.50 ± 0.12

Data show the mean values of 3 samples ± standard deviation (SD). * Significant difference in the compound was observed between vinasses A and B.

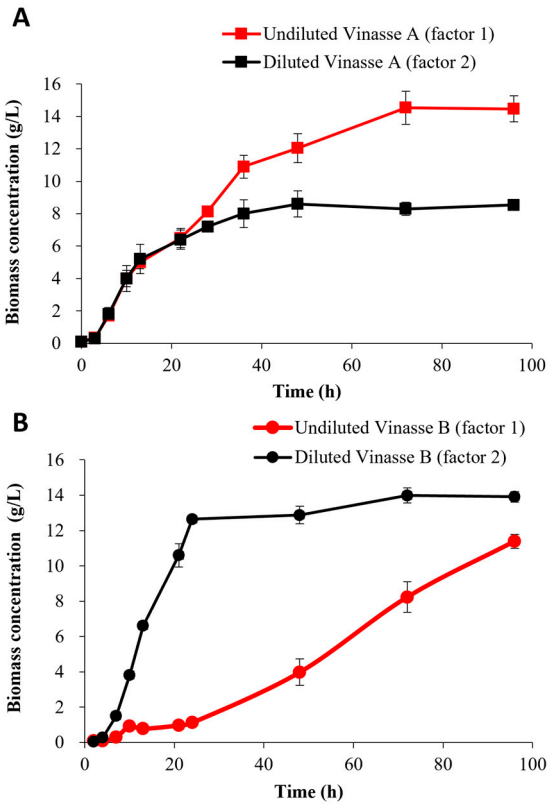


Figure 4. Growth kinetics of *R. toruloides* strain in two vinasse samples. (**A**) Cell growth in undiluted vinasse A (factor 1) and diluted vinasse A (factor 2). (**B**) Cell growth in undiluted vinasse B (factor 1) and diluted vinasse B (factor 2). Each point represents the mean ± SD of three samples from a representative experiment.

Here, we summarize the results of the biomass concentration, maximum specific growth rate, and removal of organic load at the end of the culture period (Table 3). The

results obtained from using undiluted vinasse A and diluted vinasse B, both with an organic load exceeding 35,000 mg L^{-1}, were inconsistent with previous reports, as no inhibition of cell growth was observed under these conditions. This indicates that the COD value alone is not necessarily the determining variable for the inhibition of cell growth in vinasse, but rather the presence of toxic compounds in the vinasse appears to be a more critical determinant.

Table 3. Biomass concentration, maximal specific rate, initial COD, and COD removal of *R. toruloides* cultured in undiluted vinasse A, undiluted vinasse B (factor 1), diluted vinasse A, and diluted vinasse B (factor 2).

Factor	DCC (g L^{-1})	μ_{max} (h^{-1})	Time (h)	Initial COD (g L^{-1})	COD Removal (%)
			Vinasse A		
1	14.5 ± 0.04^{a}	0.116 ± 0.015^{c}	74^{d}	40.5 ± 4.90^{f}	70.5 ± 1.33^{h}
2	9.40 ± 0.16^{b}	0.116 ± 0.021^{c}	51^{e}	21.2 ± 1.31^{g}	55.3 ± 0.45^{i}
			Vinasse B		
1	11.5 ± 0.51^{a}	0.034 ± 0.006^{c}	96^{e}	69.8 ± 2.30^{g}	68.6 ± 0.52^{i}
2	13.9 ± 0.41^{b}	0.143 ± 0.022^{d}	24^{f}	34.5 ± 2.45^{h}	71.3 ± 0.76^{j}

Data show the mean values of 3 samples ± standard deviation (SD). Different letters in the same column of each vinasse indicate that there were significant differences according to the Tukey test ($p \leq 0.05$).

Typically, inhibitory effects largely depend on the features of microorganisms and are always a result from the simultaneous presence of two or more toxic compounds, producing a synergistic inhibition of growth and intracellular metabolism. Several studies have highlighted the effect of organic acids on yeast metabolism and have identified specific concentration thresholds of these compounds as warning signs for potential fermentation issues in industrial processes. These issues can lead to diminished yields and alterations in the fatty acid profile of microbial oils, where the extent of inhibition varies depending on the particular yeast strain [53,54]. Notably, furfural, HMF, vanillin, and certain organic acids such as acetic, formic, and levulinic acids are among the most extensively studied inhibitors [55,56]. In studies with *R. toruloides* Y4, acetic acid was tolerated up to 40 g L^{-1} as a sole substrate, yet completely inhibited growth could occur when other inhibitory compounds were present [57]. The coexistence of 5-HMF (16 mmol) and acetic acid (100 mmol) in the medium showed a very slight effect. However, cell growth practically ceased in the presence of furfural and vanillin at concentrations ranging from 8 to 12 mmol. Noticeably, the synergistic effect of acetic acid and vanillin had a more pronounced effect on cell growth than other synergistic effects. According to the composition of vinasse B used in this study, it is likely that the growth inhibition was due to the synergistic effect of 1.23 g L^{-1} acetic acid, 0.02 vanillin, and other undetermined toxic compounds.

3.5. Effect of Using Raw Glycerol and Vinasse as a Carbon Source on Lipid Production

The synergistic effect of the raw glycerol impurities and vinasse composition was evaluated using a medium enriched with diluted vinasse B supplemented with glycerol or raw glycerol (60 g L^{-1}). Although no significant differences were observed for the growth-specific rate and the lipid content between vinasse B supplemented with either purified or raw glycerol, there were significant differences in DCC and lipid productivity, with the raw glycerol supplementation yielding superior results. Table 4 indicates that after 148 h, the biomass concentration increased by 16.1% and the lipid content by 25.7% in the presence of raw glycerol, leading to a significant enhancement in lipid productivity of 59.6%.

Table 4. Fermentation parameters of *R. toruloides* cultured in diluted vinasse B supplemented with glycerol and raw glycerol.

Parameters	Vinasse Supplemented with Glycerol	Vinasse Supplemented with Raw Glycerol
DCC (g L^{-1}) *	36.60 ± 2.40	42.5 ± 0.40
Maximum specific growth rate (h^{-1})	0.16 ± 0.01	0.18 ± 0.02
Lipid content (%)	20.60 ± 2.40	25.9 ± 3.10
Lipid productivity (g $L^{-1}h^{-1}$) *	0.047 ± 0.008	0.075 ± 0.012

Data show the mean values of 3 experimental samples ± standard deviation (SD). * Significant difference in the parameter was observed between vinasse B supplemented with glycerol and raw glycerol.

Figure 5 shows the profile of the methyl ester composition of the yeast oils when glycerol and raw glycerol were incorporated into the media, respectively. The fatty acid profiles were similar, with C16–C18 fatty acids exceeding 85%. The predominant fatty acids were oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0), and linoleic acid (C18:2) (Figure 5).

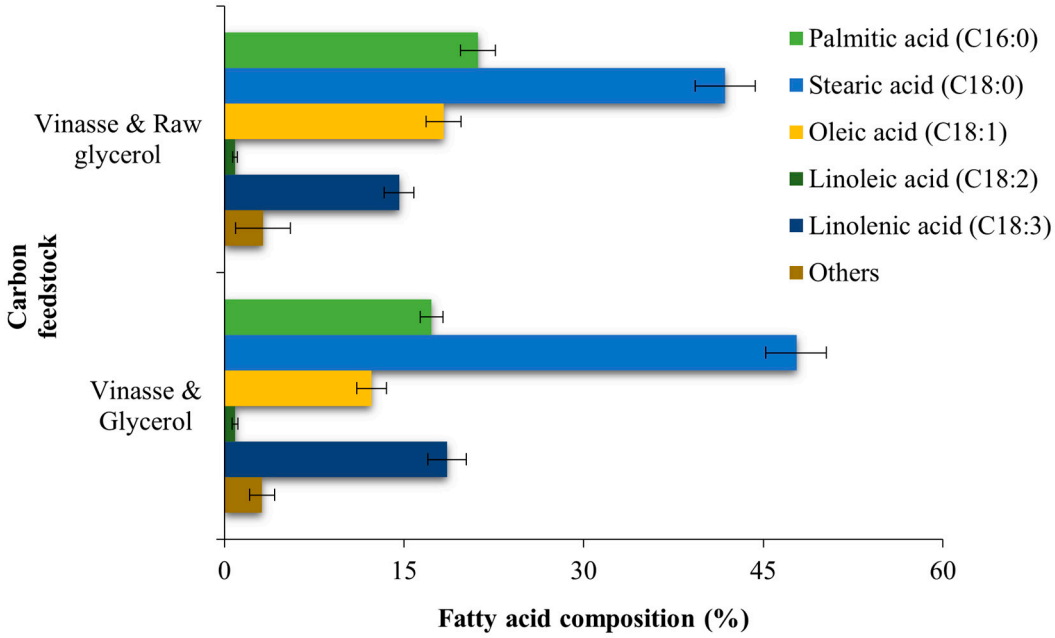


Figure 5. Average percentage of major fatty acid compositions of oils extracted from *R. toruloides* biomass grown on diluted vinasse B supplemented with pure and raw glycerol. Each bar represents the average of each sample.

Although the lipid content and yield could vary with the different proportions of vinasse and with the use of either purified or industrial glycerol, the fatty acid profiles observed under the tested conditions generally corresponded to those reported by other researchers for various oleaginous yeast species [58–61]. Consequently, it can be inferred that the combined use of vinasse and industrial glycerol as a carbon source did not substantially affect the quality of the microbial oils, which are intended for use as raw materials in biodiesel production.

3.6. Evaluation of Batch and Fed-Batch Strategies

3.6.1. Batch System

The maximal biomass concentration of the batch cultures in 4 L fermenters reached 57.3 ± 4.1 g L^{-1} after 196 h, with a maximal specific growth rate of 0.092 h^{-1} and a glycerol consumption rate of 0.72 g L^{-1} h^{-1}. At the end of the batch culture, the lipid content reached

34.7 ± 1.8%. The specific lipid production rate was 0.10 g L^{-1}, and the lipid productivity reached 0.12 ± 0.015 g $L^{-1}h^{-1}$. Comparable lipid contents in *R. toruloides* strains have been documented by other research groups when cultured on raw glycerol as the sole substrate, as well as on vinasse from ethanol distilleries using sweet potato hydrolysate and in rice wine factories, under nitrogen-limited conditions [31,35,44]. However, these studies observed significantly lower biomass concentrations compared to the yields obtained in the current study.

3.6.2. Fed-Batch Strategies

In order to enhance the lipid productivity, three fed-batch strategies were further explored. These strategies were initiated following the consumption of over 85% of the initial glycerol supply and the cessation of the yeast culture's exponential growth phase.

Strategy I

As is shown in Figure 6A, glycerol was added in pulses to maintain the concentration within a range of 50–60 g L^{-1}, with each addition occurring as the glycerol level dropped to 5.0 g L^{-1}. The culture reached a maximum specific growth rate of 0.15 h^{-1} during glycerol feeding, comparable to the rate observed with the *R. toruloides* Y4 strain in a glucose medium at 60 g L^{-1} [62]. The rate of glycerol consumption during the first 160 h of culture was 0.75 ± 0.02 g $L^{-1}h^{-1}$; however, it dropped to 0.55 g $L^{-1}h^{-1}$ after the third glycerol feeding. Concurrently, the lipid accumulation rate increased from 0.14 g $L^{-1}h^{-1}$ to 0.39 g $L^{-1}h^{-1}$. At the end of the culture, the biomass, lipid concentration, lipid content, and lipid productivity reached 129.0 ± 5.6 g L^{-1}, 66.2 ± 4.5 g L^{-1}, 51.4%, and 0.23 ± 0.01 g $L^{-1}h^{-1}$, respectively.

Strategy II

This strategy aligned with the results achieved by Wieber et al. [9], where the highest lipid accumulation and the highest substrate consumption occurred when the culture medium was enriched with a carbon source alongside a limited supply of nitrogen. Therefore, once the batch stage was completed, the culture was supplemented with 400 mL increments of diluted vinasse and raw glycerol, adjusting the glycerol concentration to 60 g L^{-1}. The incorporation of diluted vinasse proved to be a viable strategy, as it supplied the necessary nitrogen concentration to enhance lipid accumulation. Additionally, the added vinasse volume helped to reduce the medium viscosity due to the high cell density and to reduce the energy consumption associated with agitation and air flow. Similar increments were added whenever the glycerol concentration fell to 5 g L^{-1}.

As is shown in Figure 6B, the maximum biomass concentration of 145.8 ± 5.8 g L^{-1} occurred after 170 h of culture. The maximum specific growth rate in the exponential stage was 0.15 ± 0.002 h^{-1}. At the end of this culture at 306 h, the lipid content was 41.3%, with a lipid productivity of 0.2 ± 0.02 g $L^{-1}h^{-1}$. The accumulated biomass reached up to 429.6 g in a total volume of 3 ± 0.2 L. The rate of lipid accumulation increased from 0.12 to 0.29 g $L^{-1}h^{-1}$ after 170 h.

Strategy III

In contrast to strategies I and II, strategy III carried out continuous supplementation of a medium composed of diluted vinasse B (factor 3) supplemented with raw glycerol at a rate of 500 g $L^{-1}h^{-1}$ (C/N = 220). Figure 6C demonstrates that the cell concentration continued to rise until the specific growth rate started to decline due to insufficient substrate supply, which occurred as the cell culture density became elevated in the fermenter.

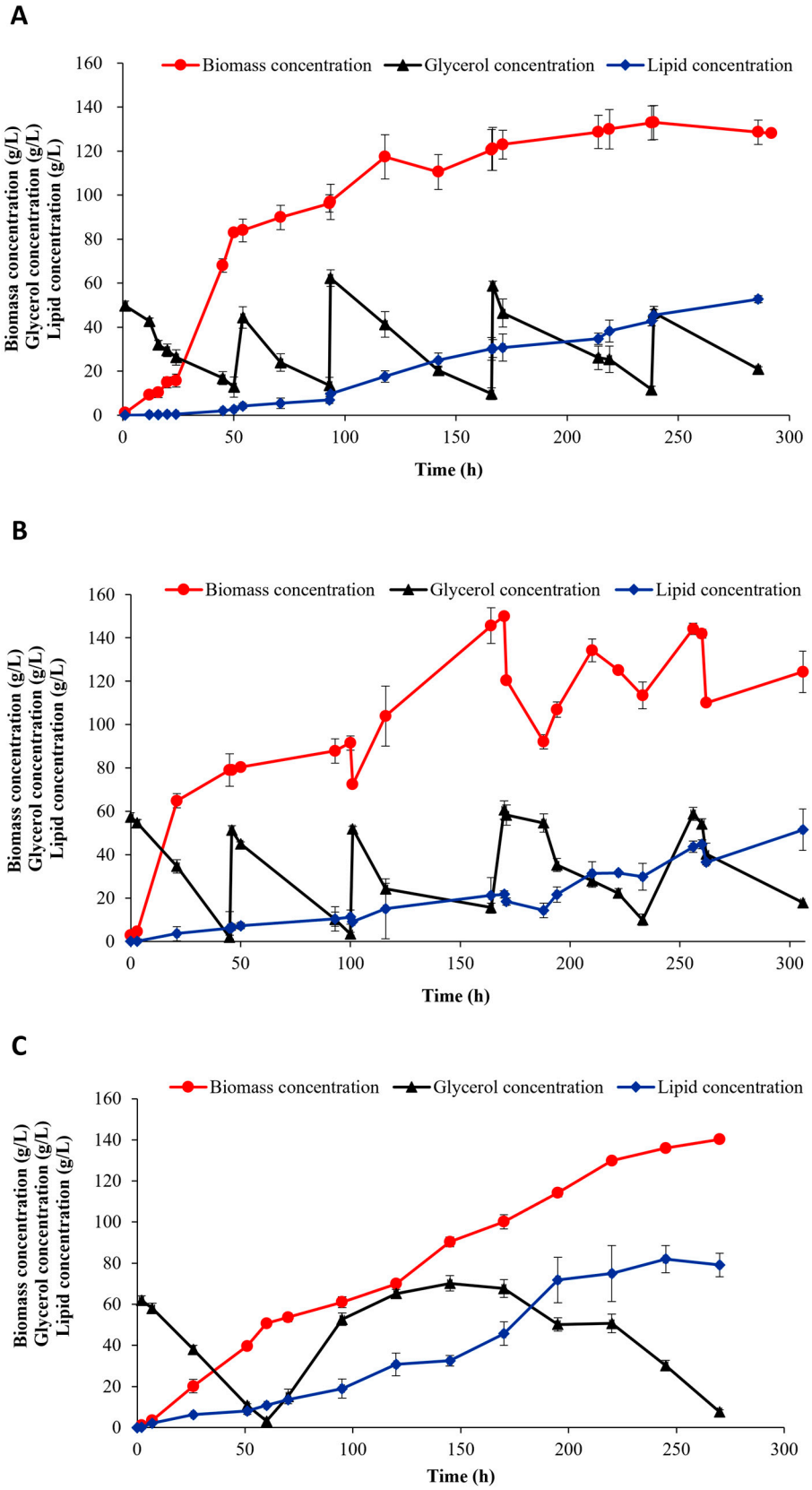


Figure 6. Kinetic behavior of biomass concentration (o; g L^{-1}), glycerol concentration (Δ; g L^{-1}), and lipid concentration (◊; g L^{-1}) of cultures employing fed-batch strategies with raw glycerol and vinasse. (**A**) Strategy I, (**B**) strategy II, and (**C**) strategy III.

In terms of the behavior of glycerol, as shown in Figure 6C, the glycerol concentration rose to a peak of 72.3 ± 3.8 g L^{-1}, with the lipid accumulation rate reaching 0.98 g $L^{-1}h^{-1}$.

Subsequently, glycerol accumulation ceased, with its concentration starting to decline as the glycerol consumption rate increased. As a result of this strategy, a biomass concentration of 136.0 ± 1.9 g L^{-1} and a lipid content of 52.5 ± 5.5% were reached, resulting in a lipid productivity of 0.30 ± 0.04 g $L^{-1}h^{-1}$ and a total dry biomass of 523.6 ± 2.4 g.

Despite the fact that many studies on lipid production from oleaginous yeasts have investigated feeding strategies, the majority have employed glucose as the carbon source, with only a few of them investigating the fermentation performance of alternative carbon sources [63,64]. Meester et al. (1996) [65] obtained a lipid productivity of 0.59 g $L^{-1}h^{-1}$ in a fed-batch fermentation experiment with glycerol as the carbon and energy source. Additionally, a study by Zhou et al. [31] demonstrated that by supplementing a culture of *R. toruloides* Y2 with distillery vinasse and feeding glucose at a flow rate of 1.2 g $L^{-1}h^{-1}$, the lipid content was enhanced from 39.5% to 53.8%.

In the present study, a fermentation time of 240 h was set to compare the productivity of the three feeding strategies, and the results are summarized in Table 5. The results indicated that the lipid content obtained in strategy III was 1.5 and 1.8 times greater than that in strategies I and II, respectively, reaching the highest lipid productivity.

Table 5. Biomass concentration, lipid content, lipid productivity, and total biomass after 240 h of fermentation using different feeding strategies.

Strategy No.	Supplemented Medium	Feeding Mode	DCC (g L^{-1})	Lipids (%)	Lipid Productivity (g $L^{-1}h^{-1}$)	Total Biomass (g)
I	Glycerol 50 g L^{-1}	Pulses	132.0 ± 7.7	34.3 ± 2.5	0.19 ± 0.02	330.8 ± 7.7
II	Glycerol 50 g L^{-1} and diluted vinasse B (factor 2)	Pulses	143.6 ± 6.2	28.8 ± 3.5	0.17 ± 0.08	402.1 ± 2.9
III	Glycerol 500 g L^{-1} and diluted vinasse B (factor 3)	Constant flow	136.0 ± 1.9	52.5 ± 7.5	0.30 ± 0.04	523.6 ± 2.4

It is important to note that comparable lipid contents have been reported in both batch and continuous systems by various researchers, including those working with genetically modified strains [22,57]. When comparing the results of the present study with the findings of previous works involving *Rhodosporidium* sp. strains and those with genetically engineered oleaginous yeasts, particularly in terms of lipid accumulation using glucose, glycerol, raw glycerol, or distillery vinasse, it can be concluded that employing mixtures of ethanol distillery vinasse and raw industrial glycerol yields similar or even superior results compared to their individual use as carbon substrates [9,20,25,31,66–71].

In this study, we comprehensively examined various factors influencing cell growth and lipid production from vinasse and glycerol, including the nitrogen source, the presence of inhibitory compounds in vinasse, the composition of crude glycerol, and the synergistic effect of the inhibitors and the impurities. Table 6 presents an overview of the relevant research, indicating that industrial glycerol exerts a beneficial effect on lipid accumulation, with levels surpassing or equivalent to those achieved using glucose and pure glycerol. Furthermore, the table reveals that supplementing vinasse with crude glycerol can enhance the biomass concentration and lipid accumulation, depending on the composition of the vinasse employed.

Table 6. Comparison of lipid production by *Rhodotorula* strains utilizing vinasse or glycerol as substrates.

Strain	Carbon Source	Nitrogen Source	Biomass (g L^{-1})	Lipid Content (%)	References
R. toruloides DSM 4444	Glucose	Peptone and yeast extract	37.2	64.5	[69]
	Raw glycerol	$(NH_4)_2SO_4$ and yeast extract	41.0	60.0	[24]
R. toruloides AS2.1389	Glycerol	$(NH_4)_2SO_4$	26.5	38.1	[44]
	Raw glycerol	$(NH_4)_2SO_4$	26.7	69.0	[36]
	Vinasse + domestic wastewater	Peptone and yeast extract	8.12	43.6	[33]
R. toruloides DSMZ 4444	Glucose + glycerol	$(NH_4)_2SO_4$ and yeast extract	17.4	63.0	[13]
R. glutinis R4	Vinasse + glucose	Yeast extract	2.3	88.0	[34]
	Vinasse + raw glycerol	Yeast extract	12.58	40.0	
Rhodotorula MT372485	Raw glycerol	$(NH_4)_2SO_4$	7.4	45.5	This study
	Raw glycerol	Urea	7.86	51.7	
	Vinasse + raw glycerol	$(NH_4)_2SO_4$	36.6	20.6	
	Vinasse + raw glycerol	Urea	42.5	25.9	

4. Conclusions

The results obtained in this study demonstrated that *Rhodotorula* MT372485 is a promising yeast candidate for SCO production using vinasse and crude glycerol as substrates. The fatty acid composition of the lipids extracted from the yeast closely resembled that of vegetable oils intended for utilization in biodiesel production. The yeast is able to grow without inhibition in a raw glycerol concentration up to 120 g L^{-1}. The impurities present in the raw glycerol had a positive influence on cell growth and lipid accumulation. The optimal lipid production was obtained with a raw glycerol concentration of 60–80 g L^{-1}, supplemented with urea at a C/N ratio of 250, at pH 6.0, and at 25 °C. The investigation of the kinetic behaviors of yeast in vinasse from various distilleries indicated that the presence of toxic compounds, particularly furfural and vanillin, can inhibit its growth. Consequently, the vinasse composition is a crucial factor in determining if vinasse dilution is required for preparing the culture media. Considering that these feedstocks are derived from the waste streams of local industries, the inherent variability in the compositions presents a significant challenge for SCO production. To facilitate the potential scaling-up of production, the tolerance of the target strain should be further investigated and strengthened, which can be achieved through process optimization and strain engineering.

The development of fed-batch strategies can effectively promote cell growth and lipid accumulation. The fed-batch strategy employing a constant flow of vinasse supplemented with 500 g L^{-1} raw glycerol allowed for achieving a higher lipid productivity than pulse-feeding strategies, reaching 0.30 g $L^{-1}h^{-1}$ at 240 h. This productivity is comparable with other studies using glucose. These findings suggest that vinasse supplemented with raw glycerol serves as a viable alternative to conventional carbon substrates, which can reduce production costs, mitigate the environmental problems associated with the disposal of vinasse and crude glycerol, and enhance the economic feasibility for the industrial production of biodiesel. The results of this study can be further used to carry out a technical–economic assessment of biodiesel production utilizing agro-industrial residues associated with the production of ethanol from sugar cane distilleries.

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J.X. and M.D.; resources, D.L. and M.G.A.-U.; writing—original draft preparation, E.F.; writing—review and editing, J.X.; visualization, W.D.; supervision, D.L.; project administration, E.F.; funding acquisition, E.F. and J.X. All authors have read and agreed to the published version of the manuscript.

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