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Valorization of Sugarcane Vinasse and Crude Glycerol for Single-Cell Oils Production by *Rhodotorula glutinis* R4: A Preliminary Approach to the Integration of Biofuels Industries for Sustainable Biodiesel Feedstock

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Abstract: Single-cell oils (SCOs) offer a promising alternative to conventional biodiesel feedstocks. The main objective of this work was to obtain SCOs suitable for biodiesel production from the oleaginous yeast *Rhodotorula glutinis* R4 using sugarcane vinasse from a local sugar-derived alcohol industry as the substrate. Additionally, crude glycerol from the local biodiesel industry was evaluated as a low-cost carbon source to replace expensive glucose and as a strategy for integrating the bioethanol and biodiesel industries for the valorization of both agro-industrial wastes. R4 achieved a high lipid accumulation of 88% and 60% (*w*/*w*) in vinasse-based culture media, containing 10% and 25% vinasse with glucose (40 g L^{-1}), respectively. When glucose was replaced with crude glycerol, R4 showed remarkable lipid accumulation (40%) and growth (12.58 g L^{-1}). The fatty acids profile of SCOs showed a prevalence of oleic acid (C18:1), making them suitable for biodiesel synthesis. Biodiesel derived from R4 oils exhibits favorable characteristics, including a high cetane number (CN = 55) and high oxidative stability (OS = 13 h), meeting international biodiesel standards (ASTMD6751 and EN14214) and ensuring its compatibility with diesel engines. *R. glutinis* R4 produces SCOs from vinasse and crude glycerol, contributing to the circular economy for sustainable biodiesel production.

Keywords: agro-industrial waste; microbial lipid production; non-conventional yeasts; biorefinery; environmental sustainability; circular economy; cost-effective bioprocessing

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1. Introduction

The urgency to explore alternative sources to produce biofuels is evidenced by the growing demand for fossil fuels and social opposition to the use of vegetable or animal feedstocks, which compete with the availability of food and/or have a negative impact on the environment. In this sense, biodiesel is a biofuel that has several advantages over conventional diesel fuel, such as its biodegradability, non-toxicity, and superior engine

lubrication capacity. However, its production from vegetable oils, mainly rapeseed, soybean, and palm, among others, encounters economic limitations and several environmental challenges. Therefore, microbial oils, also called "single-cell oils" (SCOs) are emerging as alternative and renewable oil sources and as a viable and environmentally sustainable strategy. Moreover, when these biofuels are derived from inexpensive substrates, such as agro-industrial wastes, they become even more promising alternatives for liquid energy production [1–3].

The sugarcane industry in northwestern Argentina produces large volumes of vinasse, approximately 14 L for each liter of bioethanol produced [4]. Vinasse is a waste from the rectification and distillation of ethanol, characterized by high sulfur levels, low pH (3.5–5), dark brown color, and pungent odor [5]. It is considered a highly polluting waste, especially when discharged into bodies of water and soil [2]. Another waste generated by the biofuel industry is crude glycerol, the primary by-product of biodiesel production. For every ten tons of biodiesel produced, approximately one ton of crude glycerol is generated [6]. This waste mainly comprises glycerol (65–85% *w*/*w*), along with methanol, fatty acids, soaps, and salts [7]. Vinasse, like crude glycerol, encounters an imbalance between supply and demand, which poses economic and ecological challenges for its disposal [3]. Encouraging the exploration of alternative uses for these by-products is crucial to alleviate this burden.

Oleaginous yeasts such as *Rhodotorula* spp. represent a promising alternative source of SCOs because they possess the ability to efficiently accumulate 40–70% (*w*/*w*) of neutral storage lipids, primarily in the form of triglycerides (TAGs), under specific growth conditions [8,9]. Additionally, these yeasts exhibit superior physiology as microbial cell factories than other oleaginous yeasts, characterized by high cell density, rapid unicellular growth, the absence of endotoxins, high lipogenesis, and ease of large-scale fermentation, among other attributes [10].

In this context, the yeast strain R4—an isolate from the Argentine Antarctic region and later identified as *Rhodotorula glutinis*—could be considered an excellent candidate for SCOs production from industrial waste [11]. Viñarta et al. (2016) characterized it as an oleaginous yeast [8]. Subsequent research has highlighted its remarkable capacity for lipid production and accumulation compared to other oleaginous strains present in collections [9] and its ability to utilize different by-products and industrial wastes as substrates [12,13]. In contrast to other oleaginous yeasts, the fatty acids profile of the single-cell oils produced by *R. glutinis* R4 demonstrates a high degree of stability, exhibiting minimal variability even under different culture conditions. The importance of *R. glutinis* R4 lies in its consistent fatty acids composition, mainly rich in C18:1 and C16:0, which places it in a prominent position among oleaginous yeasts and even among other *Rhodotorula glutinis* strains [8,9,12–15]. This highlights its potential to produce SCOs with stable and desirable properties. Furthermore, the fatty acids profile proves to be suitable for biodiesel synthesis, complying with international standards [13]. This is an advantage when considering a biotechnological application based on the fatty acids composition. The use of sugarcane vinasse and crude glycerol in formulating a culture medium to produce SCOs by oleaginous yeasts could offer a promising approach for managing and repurposing industrial waste effectively. This approach not only addresses environmental concerns but also has the potential to generate economic benefits that contribute to the transition towards a circular economy.

The main objective of this work was to obtain SCOs, suitable for biodiesel production from the oleaginous yeast *Rhodotorula glutinis* R4 using sugarcane vinasse from the local sugar alcohol industry in Tucumán (the most important and largest sugarcane-producing region in Argentina) and crude glycerol from the biodiesel industry. Crude glycerol is proposed as a low-cost carbon source to replace expensive glucose and as an integration strategy between the bioethanol and biodiesel industries for the valorization of both wastes. The fatty acids profiles of SCOs produced by *Rhodotorula glutinis* R4 were determined by GC–MS, and the biodiesel quality properties were also estimated.

2. Materials and Methods

2.1. Agro-Industrial Waste Used as Substrates

Two industrial wastes (sugarcane vinasse and crude glycerol) were utilized as substrates. The crude glycerol (ρ = 1.25 g cm^{-3}) used in this study was obtained directly from the biodiesel manufacturing plant (Santiago del Estero, Argentina) without undergoing any pretreatment or purification process in the laboratory before its use as the substrate. It was stored at 4 °C until use. The crude glycerol composition was as follows: 81.6% glycerol, 10.4% water, 6.2% ash, 1.8% non-glycerol organic matter, and 0.01% methanol [12]. In this work, the final concentration equivalent of 40 g L^{-1} glycerol was obtained by adjusting the crude glycerol (81.6% glycerol) to the required volume in each Erlenmeyer flask. Nitrogen content and pH of crude glycerol are described in Table 1.

Table 1. Physicochemical characterization of sugarcane vinasse used in this work.

Parameters	Vinasse	Crude Glycerol
pH	5.8	6.5
COD [mg O_2 L^{-1}]	106,252	NC
BOD_5 [mg O_2 L^{-1}]	64,360	NC
BOD_5/COD	0.6	NC
NO_2^-–N [mg L^{-1}]	4.8	0.1
NO_3^-–N [mg L^{-1}]	187	48.4
NH_4^+–N [mg L^{-1}]	1.4	ND
TN [mg L^{-1}]	193.2	48.5
PO_4^- [mg L^{-1}]	191.0	ND
K [g L^{-1}]	11.0	NC
Na [g L^{-1}]	0.6	NC
RS [g %]	0.9	NC

COD (chemical oxygen demand); **BOD_5** (biological oxygen demand); **NO_3^-–N** (nitrate-nitrogen); **NO_2^-–N** (nitrite-nitrogen); NH_4^+–N (ammonium-nitrogen); **TN** (total nitrogen); **PO_4^-** (phosphate); K (potassium); Ca (calcium); **BOD_5/COD** (biodegradability rate); **RS** (residual sugars). **NC** (not calculated); ND (not detected).

Sugarcane vinasse was obtained from a sugar factory located in the province of Tucumán, Argentina. It was collected and characterized as described by Ahmed (2018) [4]. COD was determined using the standard potassium dichromate method [16]. Sodium and potassium levels were analyzed by atomic absorption spectrophotometry.

For the determination of phosphate in vinasse (PO_4^-), a colorimetric method was employed following the HACH Company's method 10209 PlusTM 843 (0.15–4.5 mg L^{-1} PO_4^-) [17]. The concentration of nitrite-nitrogen (NO_2^-–N), nitrate-nitrogen (NO_3^-–N), and ammonium-nitrogen (NH_4^+–N) in vinasse was measured using HACH's methods. The nitrite (NO_2^-–N) test was based on HACH method (10237), with test vials of PlusTM 840 (0.6–6.0 mg L^{-1}) [17]. The nitrate (NO_3^-–N) concentration test was performed with test vials of PlusTM 835 (0.23–13.5 mg L^{-1} NO_3^-–N) according to HACH method (10206) [17]. Ammonium (NH_4^+–N) concentration was determinate using the HACH method 10205, with test vials of PlusTM 830 (0.015–2000 mg L^{-1} NH_3–N) [17]. The concentration of total nitrogen (TN) in the vinasse was determined by adding the values of ammonium-nitrogen (NH_4^+–N), nitrate-nitrogen (NO_3^-–N), and nitrite-nitrogen (NO_2^-–N) [17]. Table 1 presents the specific characteristics of the sugarcane vinasse and crude glycerol used in this study.

2.2. Yeast Strain, Medium, and Culture Conditions

Rhodotorula glutinis R4 was originally isolated from the Argentine Antarctic region, as reported by Rovati et al. in 2013 [11], and characterized as a psychrotolerant oleaginous yeast [8]. *R. glutinis* R4 strain was obtained from the Microbiological Resources Center Culture Collection (MIRCEN) of the PROIMI-CONICET research institute, located in San Miguel de Tucumán city, Argentina.

Lipid production experiments were conducted using vinasse-based culture media. The culture media were prepared by diluting vinasse in distilled water to achieve varying concentrations, including 90%, 50%, 40%, 30%, 25%, and 10% (*v*/*v*, vinasse/medium).

The dilution series of vinasse were made by mixing appropriate volumes of vinasse and distilled water, ensuring complete homogenization. In the initial assays, glucose (40 g L^{-1}) was added to the dilution series as the carbon source. In subsequent assays, vinasse-based media were prepared only with vinasse concentration of 10% (*v*/*v*). For this, a medium with vinasse 10% (*v*/*v*), glucose (40 g L^{-1}), and yeast extract (YE, 3 g L^{-1}) was prepared, and another medium containing vinasse 10% and crude glycerol (40 g L^{-1}) as carbon source was also considered. In all conditions, the pH of the medium was adjusted to 5.5. In addition, R4 was cultured in a control glucose-based medium (GMY) as a control of lipid production condition [9]. Except for glucose, all media components were autoclaved at 121 °C and 1 atm for 20 min. Glucose was subjected to sterilization-grade filtration.

To perform growth and lipid production experiments, *R. glutinis* R4 was aerobically cultured for 168 h in 100 mL of the different described culture media. The yeast inoculum was prepared using GMY medium, composed per liter: 8 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 3 g YE. Glucose was supplied as a carbon source (final concentration 40 g L^{-1}). The pH of the medium was adjusted to 5.5. A small amount from a colony of R4 grown on GMY agar was transferred to 50 mL of GMY medium and incubated at 25 °C with agitation at 250 rpm. After 24 h, this yeast culture was used to inoculate 100 mL of vinasse-based media in 500 mL Erlenmeyer flasks, resulting in an initial optical density (OD) at 600 nm of 0.1, equivalent to an initial cell count of 1×10^6 cells mL^{-1}. The Erlenmeyer flasks were fitted with cotton plugs, stirred at 250 rpm, and aerobically incubated at 25 °C for 168 h. Samples for analytical determinations, including biomass and lipid analysis, were collected after 72, 96, 120, 144, and 168 h of culture. All experiments were conducted in triplicate.

2.3. Analytical Determinations

The gravimetric method was used to determine the dry weight of the biomass (in g DW L^{-1}). Culture broth was centrifuged to separate the biomass, washed twice with an equal volume of distilled water, and dried at 105 °C until a constant weight. Total lipids were extracted from lyophilized and pulverized yeast biomass using chloroform:methanol (2:1, *v*:*v*) at room temperature, following the standard methodology described by Bligh and Dyer in 1959 [18]. This extraction process involved continuous agitation. Subsequently, the samples were centrifuged at 14,000× *g* for 10 min at 4 °C to separate the lipid-containing organic phase in the supernatant. The organic phase was then collected and evaporated to remove the solvents using vacuum evaporation (Savant Speedvac® Plus SC110A; UVS400A (Waltham, MA, USA) universal vacuum system plus). Gravimetric determination of lipids was conducted overnight at 105 °C until a constant weight was achieved [8]. Samples were collected at 72, 96, 120, 144, and 168 h of culture and all determinations were performed in triplicate.

The specific yield coefficients, lipid/biomass ($Y_{L/X}$), were determined using the weights of lipids and dry biomass. This coefficient is expressed as g g^{-1}. The productivity of biomass (Q_X) and lipids (Q_L) were determined by dividing their concentrations over time in the respective cultures, expressed as g L^{-1} h^{-1} [9].

2.4. Analysis of Fatty Acid Composition and Estimation of Biodiesel Properties

The relative abundance of fatty acids (FAs) in microbial oils derived from *R. glutinis* R4 was analyzed by GC–MS. SCOs were subjected to methanolysis, following the method described by Viñarta et al. (2016) [8], and the resulting fatty acids methyl esters (FAMEs) were analyzed using a Thermo Scientific™ TSQ 9000 Triple Quadrupole GC-MS/MS system (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with an automatic injector, and a capillary column DB5. The injection temperature was set at 270 °C. The initial temperature was 40 °C for 5 min, then increased to 190 °C at a rate of 23 °C min^{-1} and held for 4 min, followed by a further increase to 290 °C at a rate of 8 °C min^{-1} (held for 5 min). The detector temperature was maintained at 300 °C.

Fatty acids profiling was used to evaluate the quality of biodiesel produced by *R. glutinis* R4. Essential physical properties, such as cetane number (CN), iodine value (IV),

saponification value (SV), degree of unsaturation (DU), long-chain saturated factor (LCSF), cold filter plugging point (CFPP), oxidative stability (OS), high heating value (HHV), kinematic viscosity (ν), and density (ρ), were estimated using established empirical equations based on the fatty acid composition, according to Maza et al. (2020) [9].

2.5. Statistical Processing and Analysis of Data

Statistical data processing involved conducting all experiments in triplicate. Physiological data and correlation tests were analyzed using Sigma Plot 14.5 software, and the results are presented as the mean and standard deviation. Means were compared and analyzed using one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons tests, conducted at the $\alpha = 0.05$ level of significance.

3. Results and Discussion

3.1. Growth and Lipid Production Using Vinasse

This study investigated the potential of sugarcane vinasse as a substrate for formulating a culture medium to produce microbial lipids by *Rhodotorula glutinis* R4. Various vinasse-based culture media with different concentrations of vinasse (90%, 50%, 40%, 30%, 25%, and 10%, *v*/*v*) were evaluated. In the initial experiments, each culture medium was supplemented with 40 g L^{-1} of glucose as a carbon source. *R. glutinis* R4 was cultured under these conditions for 168 h. The early results revealed that *R. glutinis* R4 exhibited growth and metabolized sugarcane vinasse in media containing vinasse concentrations of 10 and 25% (*v*/*v*). This strain demonstrated tolerance to the inhibitors and toxic compounds present in the vinasse within this concentration range (Figure 1). In addition, R4 exhibited a notable capacity to accumulate neutral lipids under these conditions, achieving a higher lipid content of 86% (*w*/*w*) in 10% vinasse compared to 60% lipid content (*w*/*w*) in 25% vinasse (Figure 1).

The results indicated variations in cellular biomass production between 10% and 25% vinasse with glucose, ranging from 1.6 to 2.2 g L^{-1} and 2.3 to 3.5 g L^{-1}, respectively, with slightly higher values observed in the medium containing 25% vinasse. However, lipid production in both conditions was similar, around ~1.6 and 1.5 g L^{-1}, respectively (Figure 1). When biomass production and lipid accumulation by *R. glutinis* R4 were evaluated over culture time (72, 96, 120, 144, and 168 h) on media with 10% and 25% vinasse, no significant differences were evident (Figure 1A,B).

No growth of *R. glutinis* R4 was observed at concentrations higher than 25% vinasse, which could be attributed to its complex composition. This complexity is mainly attributed to the high organic matter content and the presence of compounds such as phenolics, phosphorus, and potassium, among other compounds. Nowadays, vinasse poses significant challenges due to its elevated chemical and biological oxygen demand, surpassing domestic wastewater by a hundredfold in terms of pollution. Moreover, its low biodegradability, often measured by the BOD_5/COD ratio, restricts microbial growth at high concentrations [19,20]. The sugarcane vinasse used in this study exhibited an acidic nature, with a pH of 5.8 and a substantial Chemical Oxygen Demand (COD) of 106,252 mg O_2 L^{-1}, indicating a considerable organic load. Additionally, its elemental composition revealed high potassium (K) levels at 11 g L^{-1}, sodium (Na) at 0.58 g L^{-1}, phosphate 191 mg L^{-1}, and total nitrogen (TN) at 193.2 mg L^{-1} (Table 1). The TN determined in this work is lower than the reference value for South American vinasse, which is estimated at 0.35 g L^{-1} [21]. However, a comparative analysis reveals that South American vinasse has the lowest nitrogen concentration compared to North and Central America (1.34 g L^{-1}), Europe (32.5 g L^{-1}), Asia (5.30 g L^{-1}), Australia (1.83 g L^{-1}), and Africa (0.83 g L^{-1}) [21].

Meanwhile, the observed growth of *R. glutinis* R4 at 10% and 25% (*v*/*v*) vinasse could be attributed to its capacity to tolerate these concentrations and metabolize the vinasse components. The nutrients present in vinasse moderately support cellular biomass growth and lipid accumulation. Recent investigations have highlighted that vinasse contains a readily available carbon source for microbial growth, as it contains compounds such as

residual glucose (0.21–2.61%) and ethanol (3.83 g L^{-1}), although in very low concentrations [22]. The sugarcane vinasse used in this study had a residual sugar content of 0.9%, which highlighted the need to add a carbon source to support R4 growth and establish a culture medium with a high C/N ratio [4]. These nutritional imbalances, often characterized by an excess of carbon sources and a deficiency of other essential nutrients such as nitrogen, are reported to initiate various physiological and metabolic adaptations that direct the carbon flux towards lipid synthesis [23]. At 10% and 25% vinasse concentrations, the nitrogen supply was 5.62% and 14.20% in relation to the supply in GMY (0.34 g L^{-1}), respectively. This explains the higher lipid accumulation and lower growth of R4 in culture media with vinasse. According to Sindhu et al. (2016), the balanced presence of macro and micronutrients in vinasse could function as a nutrient source for microbial growth or the production of industrially valuable compounds [24].

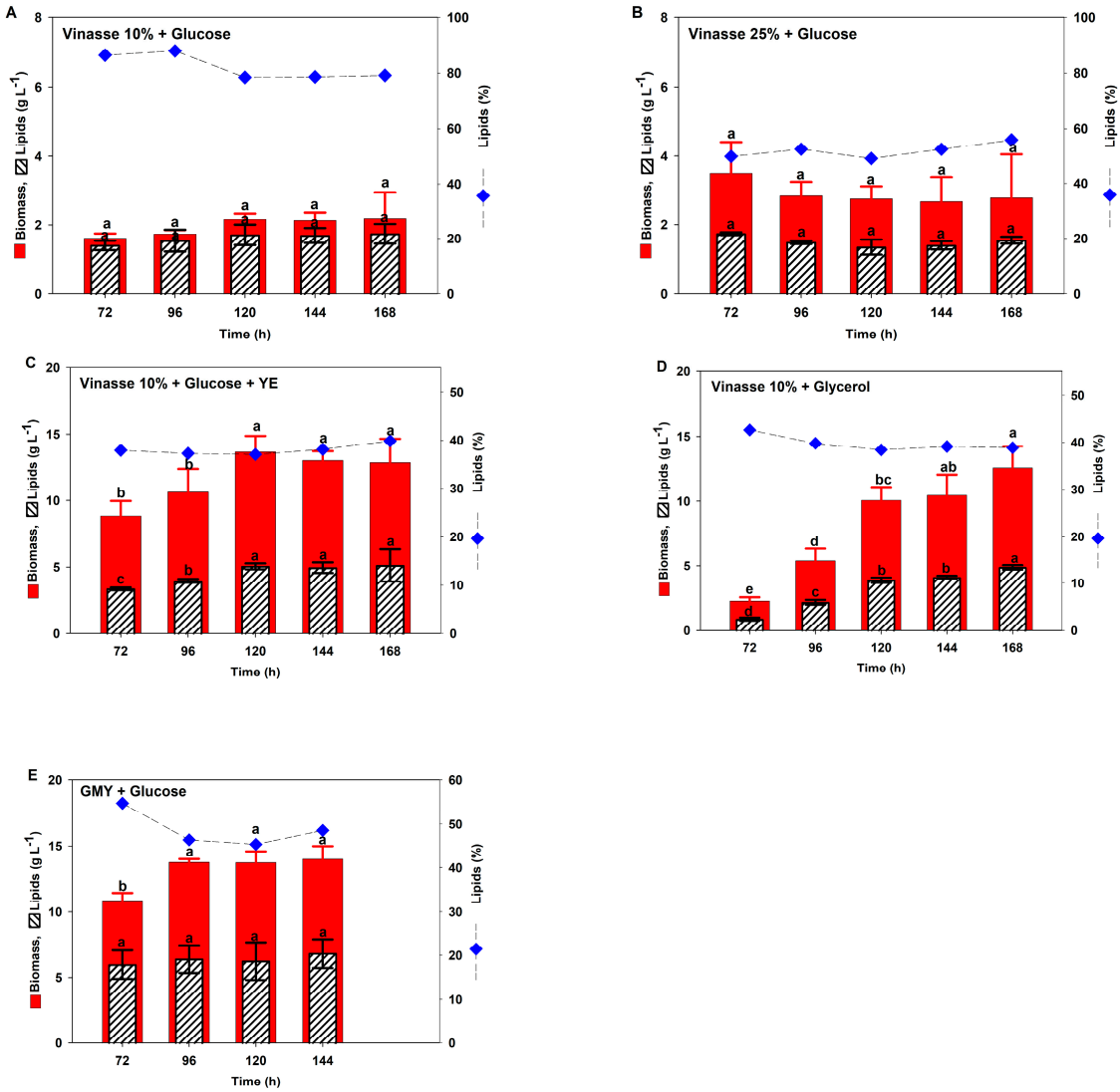

Figure 1. (**A**–**E**). Biomass (g L^{-1}), lipid production (in g L^{-1}), and lipid accumulation (%, *w*/*w*) by *Rhodotorula glutinis* R4 under different culture conditions. (**A**,**B**) show the growth of *R. glutinis* R4 in media containing 10% and 25% sugarcane vinasse (*v*/*v*) supplemented with glucose (40 g L^{-1}) as the sole carbon source, respectively. (**C**) shows the growth of R4 in vinasse (10%, *v*/*v*) supplemented with glucose (40 g L^{-1}) and YE (3 g L^{-1}) to promote cellular growth. (**D**) shows R4 culture in vinasse (10%) supplemented with crude glycerol (40 g L^{-1}) as the sole carbon source, with the aim of replacing glucose. (**E**) shows growth of R4 in GMY, culture used as control, with glucose as carbon source. The data represent the mean ± standard deviation derived from three independent experiments. Values with the same letters indicate no significant differences based on Tukey's test with $\alpha = 0.05$.

On the other hand, the lower cellular biomass of *R. glutinis* R4 observed in the presence of vinasse compared to the control medium (GMY) could be attributed to limitations in nitrogen and other essential nutrients for heterotrophic microorganisms [25]. Various studies have highlighted the ability of *Neurospora intermedia*, *Rhizopus oligosporus*, and *Mucor circinelloides* to grow using vinasse as a culture substrate. However, the addition of ammonium sulfate and potassium dihydrogen phosphate to the vinasse required supplying essential nitrogen and phosphorus sources [5,22,26]. In this work, neither ammonium nor sulfate salts were added to the vinasse used for the culture media to promote lipid accumulation. Despite this nitrogen deficiency, consistent growth of R4 was observed under the conditions evaluated. These results suggest that sugarcane vinasse could provide an optimal environment for the biological culture of *R. glutinis* R4.

Based on the preliminary results of vinasse-based media, subsequent efforts focused on enhancing *R. glutinis* R4 cell growth and promoting neutral lipid accumulation. The impact on cellular biomass and lipid production was assessed under two additional conditions: one with 10% vinasse, glucose (40 g L^{-1}), and YE (3 g L^{-1}) and another with 10% vinasse supplemented solely with crude glycerol (40 g L^{-1}) as the carbon source. Both conditions exhibited favorable effects on cell growth and total lipid accumulation in *R. glutinis* R4 (Figure 1).

However, notable differences were observed between the two conditions. After 72 h of culture, the medium with 10% vinasse, glucose, and YE demonstrated a significant 5.5-fold increase in *R. glutinis* R4 growth, resulting in 8.87 g L^{-1} of biomass, and 2.4-fold higher lipid production compared to the medium with 10% vinasse and glucose, which recorded totals of 3.36 g L^{-1} and 1.74 g L^{-1} of lipid, respectively. (Figure 1). Moreover, it is important to highlight that the culture medium containing 10% vinasse, glucose, and YE achieved biomass values similar to the GMY control medium. After 120 h of culture, a cell biomass of 13 g L^{-1} was reached, whereas in the control culture, it required 96 h to reach a comparable level. Under these conditions, lipid production was only 1.2 g L^{-1} lower compared to the control culture medium (Figure 1). These results could be attributed to the similar nitrogen supply of the GMY medium and the culture medium containing vinasse, glucose, and YE, the content of the latter being 5.6% more nitrogen, represented by the 10% diluted vinasse. In this sense, Martinez-Silveira et al. (2022) also observed that the addition of YE to vinasse-based culture media increased the cell biomass, but lipid production decreased under these conditions [27].

On the other hand, substituting glucose with crude glycerol positively influenced the cellular growth of *R. glutinis* R4 when cultivated in 10% vinasse. Under these conditions, using a culture medium containing 10% vinasse (*v*/*v*) with crude glycerol (40 g L^{-1}) resulted in a substantial increase in biomass production, rising from 2.30 g L^{-1} to 12.58 g L^{-1} after 168 h of culture, representing a ~6-fold rise compared to the medium containing 10% vinasse and glucose. Similarly, lipid production surged approximately 2.80-fold, escalating from 1.74 g L^{-1} to 4.88 g L^{-1} after 168 h of culture when compared with the culture grown in 10% vinasse and glucose. The positive effect on growth could be attributed to the presence of trace impurities in glycerol, which act as growth factors mitigating the stress induced by vinasse [8,13]. In this regard, the nitrogen contribution from crude glycerol is insignificant (0.0021 g L^{-1}) compared to that of 10% vinasse (0.019 g L^{-1}).

In the culture medium containing 10% vinasse and crude glycerol, the biomass and lipid production reached a maximum value of 12.58 g L^{-1} and 4.88 g L^{-1} (39% *w*/*w*), respectively, by the end of the culture, which was similar to that obtained using 10% vinasse, glucose, and YE (12.87 g L^{-1}, and ~5 g L^{-1}), as shown in Figure 1.

The growth delay observed in the presence of 10% vinasse and crude glycerol medium, compared to the control medium GMY or 10% vinasse, glucose and YE, could be attributed to impurities present in the crude glycerol (Figure 1). These impurities, such as soap, methanol, ash, fatty acid methyl esters (FAMEs), and salts, have been identified in previous studies [7,28].

The lower lipid levels in the medium with vinasse and glycerol compared to the GMY control, as well as with the 10% vinasse with glucose and YE, could also be attributed to the composition of glycerol. The crude glycerol used was not subjected to any pretreatment or purification process before being employed as a substrate. Gao et al. (2016) reported that the presence of sodium oleate, methyl oleate, and sodium chloride increased lipid concentration in *Rhodotorula toruloides* by 68%, 47%, and 64%, respectively, compared to pure glycerol. Conversely, the presence of methanol decreased lipid production by 17.7%. Therefore, the removal of methanol to acceptable levels [29] generates a great deal of interest due to the abundant supply of glycerol, a by-product derived from the biodiesel industry, which is considered waste [7,12,30]. Crude glycerol production currently exceeds 7.66 million tons per year, a figure that far exceeds market demand. In addition, several impurities present in crude glycerol hinder its direct commercial application [7].

In summary, *R. glutinis* R4 demonstrated its capability to produce and accumulate lipid under all the conditions evaluated, with the highest lipid content (88%, *w*/*w*) and lipid/biomass yield ($Y_{L/X}$ = 0.88) at 96 h in a 10% vinasse-based culture medium and glucose (Table 2). The addition of YE in a medium containing 10% vinasse and glucose increased the cellular biomass 10- to 13-fold compared to medium with only 10% vinasse and glucose, showing values similar to the control culture GMY (~13 g L^{-1}). Although the highest productivity values were obtained at 72 h in the culture medium with vinasse, YE, and glucose (Table 2), the total lipid production was 2.6 times lower than the control culture GMY and slightly higher than the 10% vinasse culture with glucose. Therefore, the results of this study, particularly using a vinasse-based medium with crude glycerol as a carbon source, are relevant and highlight the ability of *R. glutinis* R4 to develop, synthesize, and accumulate significant amounts of lipids. In this medium, the volumetric productivities of the lipids (Q_L) and biomass (Q_x) reached 0.032 and 0.084 g L^{-1} h^{-1}, respectively—slightly lower than the control culture (Q_L = 0.052 g L^{-1} h^{-1} and Q_x = 0.114 g L^{-1} h^{-1}) after 120 h of cultivation (Table 2).

The production of biodiesel through oleaginous yeasts requires the optimization of various parameters, including strain selection, culture composition, and physicochemical properties [31]. Consequently, the scalability of this process is currently considered economically unprofitable. A promising avenue for improvement involves the development of more cost-effective culture media, potentially taking advantage of industrial waste.

While the primary objective of this work was to investigate lipid production in oleaginous yeast using vinasse and crude glycerol, we recognized the value of analysing the cost components of the culture media to enhance the interpretation of our results. From the results obtained, a cost analysis was conducted to underscore the potential of the R4 strain when utilizing two agro-industrial residues, vinasse and crude glycerol, as substrates. The estimated cost per liter of medium:

- GMY medium composed of yeast extract, glucose, and salts ($MgSO_4 \cdot 7H_2O$ and KH_2PO_4) is estimated at ~USD 51.16. Glucose represents 76% of the total cost.
- Medium 10% Vinasse + Glucose + Yeast Extract = USD 41.35, representing a 19% reduction in medium cost compared to GMY medium.
- Medium 10% Vinasse + Glycerol = USD 0 (the cost of materials for this medium was considered "0" in this study, as vinasse and crude glycerol substrates are local industry residues with no current application).

This analysis revealed varying costs per liter across different media, with the GMY Medium being the most expensive and 10% Vinasse + Glycerol Medium emerging as the most cost-effective, as both components were provided at no cost. This study highlights the potential of using a more cost-effective culture medium, leading to a reduction in the overall cost of the process. By using a medium based on crude glycerol and 10% vinasse, the cost of the medium is eliminated, while water use is reduced by 10%. These results emphasize the economic implications of choosing the appropriate culture medium for lipid production in oleaginous yeasts. Furthermore, the foundation is laid for future optimizations using vinasse and crude glycerol as substrates. Regarding vinasse, it is a local waste that requires

urgent treatment and disposal at the local level in our region. As for crude glycerol, we could consider the cost incurred by the company to extract and bottle the by-product in the future. Based on market prices for crude glycerol, it can be acquired at a price of USD 0.2–2.65/kg or USD 200/ton. Thus, if crude glycerol were to be purchased for culture media based on 10% vinasse, the 40 g/L of crude glycerol required for the experiments would represent a cost of USD 0.008–0.106/L of medium compared to USD 51/L for GMY or USD 41/L for vinasse + glucose + yeast extract medium. In other words, the cost of crude glycerol in the culture medium prepared with this component and 10% vinasse would represent the total medium cost (excluding water), nearly eliminating the cost of the culture medium when replacing glucose.

Table 2. Lipid and biomass production by *R. glutinis* R4 in vinasse-based medium at 25 °C between 72 and 168 h of culture.

Conditions	Time (h)	$Y_{L/X}$ (g g^{-1})	Q_L (g $L^{-1}h^{-1}$)	Q_x (g $L^{-1}h^{-1}$)
Vinasse 10% + glucose	72	0.864 ± 0.012^{a}	0.019 ± 0.001^{a}	0.023 ± 0.002^{a}
	96	0.880 ± 0.009^{a}	0.016 ± 0.002^{b}	0.018 ± 0.001^{ab}
	120	0.784 ± 0.007^{b}	0.014 ± 0.001^{c}	0.018 ± 0.001^{bc}
	144	0.786 ± 0.005^{b}	0.012 ± 0.002^{d}	0.015 ± 0.003^{d}
	168	0.791 ± 0.005^{b}	0.010 ± 0.001^{e}	0.013 ± 0.004^{e}
Vinasse 25% + glucose	72	0.498 ± 0.014^{c}	0.024 ± 0.001^{a}	0.049 ± 0.002^{a}
	96	0.559 ± 0.011^{bc}	0.016 ± 0.001^{b}	0.028 ± 0.001^{b}
	120	0.600 ± 0.009^{ab}	0.011 ± 0.003^{c}	0.019 ± 0.003^{c}
	144	0.506 ± 0.016^{b}	0.010 ± 0.001^{d}	0.019 ± 0.003^{d}
	168	0.559 ± 0.008^{ab}	0.009 ± 0.001^{e}	0.017 ± 0.002^{e}
Vinasse 10% + glucose + YE	72	0.379 ± 0.001^{b}	0.047 ± 0.002^{a}	0.123 ± 0.001^{a}
	96	0.372 ± 0.014^{c}	0.041 ± 0.001^{b}	0.111 ± 0.002^{b}
	120	0.370 ± 0.019^{d}	0.042 ± 0.005^{b}	0.114 ± 0.001^{bc}
	144	0.381 ± 0.024^{ab}	0.034 ± 0.001^{c}	0.090 ± 0.002^{d}
	168	0.399 ± 0.012^{a}	0.031 ± 0.003^{d}	0.077 ± 0.003^{e}
Vinasse 10% + glycerol	72	0.426 ± 0.010^{a}	0.014 ± 0.005^{b}	0.032 ± 0.001^{d}
	96	0.398 ± 0.002^{b}	0.022 ± 0.002^{bc}	0.057 ± 0.001^{c}
	120	0.384 ± 0.005^{c}	0.032 ± 0.003^{a}	0.084 ± 0.002^{ab}
	144	0.391 ± 0.019^{b}	0.028 ± 0.004^{b}	0.073 ± 0.001^{b}
	168	0.388 ± 0.018^{bc}	0.029 ± 0.001^{b}	0.075 ± 0.003^{b}
GMY control + glucose	72	0.548 ± 0.012^{a}	0.083 ± 0.001^{a}	0.151 ± 0.002^{a}
	96	0.464 ± 0.008^{bc}	0.066 ± 0.005^{bc}	0.143 ± 0.001^{ab}
	120	0.454 ± 0.014^{bc}	0.052 ± 0.001^{c}	0.114 ± 0.003^{bc}
	144	0.486 ± 0.009^{bc}	0.047 ± 0.003^{d}	0.097 ± 0.003^{c}

R. glutinis R4 was cultured in GMY (control) and vinasse-based media (for details, see the Materials and Methods, Section 2.2) at 25 °C during 168 h. The data represent the mean ± standard deviation (SD) derived from three independent experiments. Lipid/biomass yield ($Y_{L/X}$), and the volumetric productivities of lipids (Q_L) and biomass (Q_X). Volumetric lipid efficiency refers to the lipids produced per 1 L of medium. Values in the same column with the same letters indicate no significant differences based on Tukey's test with $\alpha = 0.05$. YE: yeast extract.

It is essential to note that other factors, such as energy consumption, sterilization procedures, culture times, equipment expenses, transportation, storage, and downstream processing costs, remained consistent across all media or components. We believe that this supplementary analysis will offer valuable insights into the practical applications and commercial viability of our findings.

The valorization of vinasse and crude glycerol into value-added products could positively contribute to the biodiesel industry. Furthermore, this has the potential to embody a robust integration of different industries for a sustainable source of biodiesel feedstock.

3.2. Fatty Acids Profile and Biodiesel Properties

The fatty acids composition significantly influences the quality of the fuels obtained by transesterification from triacylglycerols (TAG). The characteristics that affect the properties of biodiesel are the chain length, degree of saturation, and branching of the fatty acids [32]. The SCOs of R4, analyzed by GC–MS after methanolysis, exhibited a predominant presence of long-chain fatty acids (C16–C18) similar to those commonly found in vegetable oils (Table 3), which are currently the main source of lipids for biodiesel production [9,12–15,33,34]. The order of relative abundance of fatty acids (FA) present in SCOs from R4 was C18:1 > C16:0 > C18:2 in sugarcane vinasse (at 10 or 25%, *v*/*v*) with glucose or crude glycerol as the sole carbon sources, similar to the control condition (GMY) (Table 3). These results align with the typical composition reported for microbial lipids produced by other strains of *R. glutinis*, and other oleaginous yeasts, indicating these oils are highly suitable for biodiesel synthesis [8,9]. For instance, in a culture medium based on 10% (*v*/*v*) vinasse and glucose, the FA profile of R4 oils was similar to that of the GMY control medium. After 120 h of culture, C16:0 constituted ~19%, and C18:1 more than 50%, of the FA content. These proportions increased to 26.6% and 57.8%, respectively, after 168 h of culture under the same conditions (Table 3).

On the other hand, the use of 10% (*v*/*v*) vinasse and crude glycerol resulted in a C18:1 content exceeding 63%. In the rest of the conditions evaluated, C18:1 and C16:0 predominated, constituting between 72% and 86% in the oils from *R. glutinis* R4, highlighting its potential as an excellent raw material for biodiesel [9,15,32].

Monounsaturated fatty acids (MUFAs) were the most abundant (60–73%), mainly represented by C18:1, similar to those found in vegetable oils (Table 3). In lower abundance, saturated fatty acids (SFAs) ranged from 19 to 27%, depending on the culture medium composition. Finally, polyunsaturated fatty acids (PUFAs) were represented only by C18:2, while C18:3 was not detected under the GC–MS-described conditions (Table 3).

The literature emphasizes the significance of an FA profile on fuel properties of FAMEs. The high C18:1 content is crucial for biodiesel quality, resembling the profiles found in various vegetable oils (Table 3) that are currently used to produce biodiesel. Utilizing sugarcane vinasse as a substrate for *R. glutinis* R4 cultivation showcases its potential as a cost-effective medium for biodiesel feedstock production. This is advantageous, as a high C18:1 content balances low-temperature fluidity and oxidative stability, critical parameters for biodiesel quality [32]. Moreover, combining sugarcane vinasse (10%, *v*/*v*) with crude glycerol as the sole carbon source results in notably high oleic acid content (63.15%), offering a promising approach for low-cost cultivation media for *R. glutinis* R4 as a source of SCOs suitable for biodiesel production (Table 3). Previous studies reporting similar oleic acid contents further support the feasibility of achieving optimal biodiesel characteristics [13,35].

Monounsaturated fatty acids such as oleate (C18:1) play a key role in enhancing cold-temperature flow properties. Biodiesel with high methyl oleate levels exhibits excellent characteristics, including superior ignition quality, reduced NOx emissions, and enhanced fuel stability [36]. Reducing the content of polyunsaturated FAs, as observed in the high C18:1 content of microbial oils from *R. glutinis* R4, enhances fuel stability, which is crucial for balancing low-temperature performance and oxidative stability in biodiesel [36]. The ability of *R. glutinis* R4 to produce SCOs rich in oleic acid, especially under specific culture conditions, positions it as a promising candidate for biodiesel production with desirable fuel properties. The biodiesel quality parameters obtained from *R. glutinis* R4 complied with international standards, including Cetane Number (CN), Iodine Value (IV), Oxidative Stability (OS), Density (ρ), and Kinematic viscosity (ν), among others, which are estimated and presented in Table 4. These parameters are crucial for fuel suitability. The analysis revealed a CN ranging between 54 and 56.8, exceeding the threshold established by biodiesel standards (47 to 51), but its value is considered adequate (Table 4) and is comparable to that of petrodiesel (57.8). According to EN 14214 standards, oxidative stability values equal to or greater than 6 h indicate significant resistance to oxidation [37]. The results from the R4 oils ranged between 10 and 17 h, surpassing those found in vegetable oils

and meeting the requirements established by the EN 14214 standard (Table 4). Overall, all physicochemical properties were in line with biodiesel from vegetable oils and petrodiesel. Notably, the absence of linolenic acid (C18:3) in the SCOs from R4, remaining below 12% in all tested conditions, met the European biofuel requirements (Tables 3 and 4). The findings highlighted that cultivating *R. glutinis* R4 in a medium with vinasse and crude glycerol produced FAMEs (fatty acid methyl esters) from SCOs within the physicochemical ranges established by international biodiesel standards, ensuring high-quality fuel for diesel engines. Specifically, the R4 oil biodiesel generated from vinasse and crude glycerol exhibited a CN of 55, comparable to petrodiesel (CN = 57.8), an IV of 80 (g I_2 100 g^{-1}), and an OS of 13 h. These parameters indicated efficient combustion and stability against oxidation.

Biodiesel with low iodine values, such as that exhibited by R4, typically signifies a more combustible and efficient fuel compared to those with higher values [37]. Additionally, the density (ρ = 0.88 g cm^3) and kinematic viscosity (ν = 3.94 mm^2 s^{-1}) values aligned well with international standards and mirrored those of petroleum-derived diesel (ρ = 0.84 g cm^3; ν = 3.43 mm^2 s^{-1}). Moreover, previous studies conducted by our research group have shown that R4 oils could be transformed with an efficiency exceeding 90% into FAMEs through transesterification [12,13].

The results of this study highlight the significance of the high content of oleic (C18:1) and palmitic (C16:0) acids in microbial oils derived from R4 for biodiesel synthesis. The observed fatty acids profile aligns with the requirements for achieving a balance between low-temperature flow characteristics and oxidative stability, as recommended by European biodiesel standards [27,38]. The use of sugarcane vinasse as a substrate further enhances the potential for cost-effective biodiesel feedstock production. These findings contribute valuable insights to the ongoing efforts in developing sustainable and high-quality biodiesel sources. These outcomes collectively suggest promising prospects for considering *R. glutinis* R4 as a source of microbial oils, providing a sustainable alternative for biodiesel production with high performance and quality assurance.

Table 3. Relative abundance of fatty acids produced by *R. glutinis* R4 under different vinasse-based culture media and GMY medium, after 120 and 168 h of culture, incubated at 25 °C and 250 rpm. In addition, the relative composition of vegetable oil fatty acids is detailed.

	Relative Abundance of FAs (*w*/*w* %)														
SCOs from *R. glutinis* R4	**C14:0**	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C22:1**	**Others**	**Total C16**	**Total C18**	**Total SFA**	**Total MUFA**	**Total PUFA**	**Reference**
Control GMY 120 h	ND	19.8	8.3	2.9	52.2	13.0	ND	ND	ND	28.2	68.1	22.7	60.5	13.0	This work
Vinasse 10% + glucose 120 h	ND	19.0	6.6	3.6	55.7	15.1	ND	ND	ND	25.6	74.4	22.5	62.3	15.1	This work
Vinasse 10% + glucose 168 h	ND	26.6	3.6	3.4	57.8	8.7	ND	ND	ND	30.2	69.8	30.0	61.3	8.7	This work
Vinasse 25% + glucose 120 h	ND	15.9	2.7	3.1	70.5	7.9	ND	ND	ND	18.6	81.4	19.0	73.1	7.9	This work
Vinasse 25% + glucose 168 h	ND	25.9	2.7	2.8	57.6	11.0	ND	ND	ND	28.6	71.4	28.7	60.4	11.0	This work
Vinasse 10% + glucose + YE 120 h	ND	23.9	1.7	3.5	60.7	9.3	ND	ND	ND	25.6	73.5	27.4	62.5	9.3	This work
Vinasse 10% + glucose + YE 168 h	ND	23.0	3.4	4.1	55.4	14.1	ND	ND	ND	26.4	73.6	27.1	58.8	14.1	This work
Vinasse 10% + glycerol 120 h	ND	19.3	2.6	3.4	63.1	11.4	ND	ND	ND	21.9	78.1	22.8	65.7	11.5	This work
Vinasse 10% + glycerol 168 h	ND	24.0	2.3	3.4	60.5	9.7	ND	ND	ND	26.4	73.6	27.4	62.9	9.7	This work
Oils from vegetables															
Rapeseed	0.07	4.9	0.2	1.6	66.6	17.1	7.8	0.3	1.4	5.2	93.1	6.6	67.1	24.8	[38]
Sunflower	0.05	4.8	0.1	4.8	67.7	21.3	0.1	0.9	0.3	5.0	93.8	9.7	68.7	21.4	[38]
High oleic sunflower	0.03	3.9	0.2	2.6	85.3	6.7	0.1	0.9	0.3	4.1	94.7	6.5	86.4	6.8	[38]
Jatropha curcas	0.07	14.0	1.1	7.9	42.7	33.8	0.1	0.2	0.2	15.0	84.5	22.0	44.0	33.8	[38]
Palm	0.04	38.8	ND	3.2	37.6	8.8	0.2	ND	ND	38.8	49.7	42.0	37.6	8.9	[39]

Values indicate wt. %; ND: Not determined.

Table 4. Estimated biodiesel properties based on the fatty acids profile of *R. glutinis* R4 under different tested growth conditions, according to ASTM D6751 and EN 14214 International Standards for Biofuels.

	Biodiesel Properties											
Oils	**SV (mg KOH)**	**IV (gI_2 100 g^{-1})**	**CN**	**OS (h)**	**DU (%wt.)**	**LCSF (%wt.)**	**CFPP (°C)**	**HHV (MJ/Kg)**	**v at 40 °C (mm^2 s^{-1})**	**ρ (g cm^{-3})**	**C18:3 (%)**	**Reference**
ASTM D6751	≤0.5	NS	≥47	>3	NS	NS	NS	NS	1.9–6	0.86-0.89	NS	[27,38]
EN 14214	≥0.5	≤120	≥51	≥6	NS	NS	+5 to −20	~35	3.5–5	0.86-0.90	≤12	[27,38]
Diesel	218.1	63.8	57.8	23.7	ND	ND	−20	48.5	3.43	0.84	ND	[40,41]
Vinasse 10% + glucose	203.7	84.1	54.2	10.4	92.6	3.7	−4.9	39.5	3.9	0.88	ND	This work
Vinasse 25% + glucose	202.1	80.4	55.2	17.5	88.9	3.1	−6.7	39.5	4.0	0.87	ND	This work
Vinasse 10% + glucose + YE	201.9	73.2	56.9	15.2	81.1	4.1	−3.5	39.2	3.9	0.87	ND	This work
Vinasse 10% + crude glycerol	202.8	80.1	55.2	12.9	88.6	3.7	−5.0	39.5	3.9	0.88	ND	This work

Table 4. *Cont.*

Oils	Biodiesel Properties											
	SV (mg KOH)	**IV (gI_2 100 g^{-1})**	**CN**	**OS (h)**	**DU (%wt.)**	**LCSF (%wt.)**	**CFPP (°C)**	**HHV (MJ/Kg)**	**v at 40 °C (mm^2 s^{-1})**	**ρ (g cm^{-3})**	**C18:3 (%)**	**Reference**
Control GMY	196.8	78.8	56.3	11.7	86.5	3.4	−5.7	38.0	3.7	0.84	ND	This work
Soybean	190.7	120.6	42.6	ND	143.4	ND	−4.0	39.8	4.0	0.89	ND	[38]
Cynara cardunculus	189.2	114.8	43.2	ND	136.5	ND	−3.0	40.0	4.7	0.89	ND	[38]
High oleic sunflower	187.5	84.6	53.2	ND	99.9	ND	2.0	40.5	4.7	0.88	ND	[38]
Brassica carinata	188.1	106.1	46.4	ND	126.4	ND	5.0	40.1	5.3	0.90	ND	[38]
Sunflower	186.9	96.7	49.2	ND	112.8	ND	1.0	40.3	4.5	0.88	ND	[38]
Rapeseed	185.0	101.5	48.3	ND	116.8	ND	−7.0	40.3	4.4	0.88	ND	[38]
Jatropha	186.0	91.2	54	3.95	ND	ND	1.0	40.4	4.6	0.86	ND	[38]
Palm	ND	57	61	4	64.2	7.7	10.0	ND	4.5	ND	0.2	[42]

NS: Not specified by international biodiesel standard, ND: Not determined. SV: Saponification Value, IV: Iodine Value, CN: Cetane number, OS: Oxidation Stability, DU: Degree of Unsaturation, LCSF: Long-Chain Saturated Factor, CFPP: Cold Filter Plugging Point (°C), HHV: Higher Heating Value, v: Kinematic Viscosity, ρ: Density, C18:3, Linolenic acid content, YE: Yeast extract.

4. Conclusions

The results of this study show the promising potential biotechnological applications of *Rhodotorula glutinis* R4 as a key technology in microbial oil production. *R. glutinis* R4 demonstrates the ability to grow and accumulate lipids using 10% and 25% vinasse as substrates, exhibiting robust lipid production potential (49–88% *w*/*w*) under both conditions. Moreover, *R. glutinis* R4 demonstrates the capability to grow and accumulate lipids (40%, *w*/*w*) by utilizing two agro-industrial wastes, vinasse and glycerol, without the addition of supplementary growth components. Their remarkable resistance to impurities and inhibitors present in vinasse and crude glycerol, both agro-industrial wastes, underlines their adaptability and potential to efficiently utilize diverse substrates, prospering in hostile and challenging fermentation environments.

The results suggest the feasibility of formulating vinasse- and crude glycerol-based culture media, which offer an excellent low-cost strategy for the sustainable production of microbial oil by *R. glutinis* R4 on an industrial scale. Formulating a culture medium with 10% vinasse and crude glycerol reduces the cost of the medium by 100% (as the cost of both components is "zero"), while reducing water use by 10%. These results highlight the economic implications of selecting a suitable culture medium for lipid production in oleaginous yeasts, encouraging a synergistic approach that integrates the bioethanol and biodiesel industries, promoting a circular economy model. By optimizing this bioprocess, the utilization of waste for the generation of value-added products becomes feasible, mitigating environmental impact while improving resource efficiency.

On the other hand, the fatty acids profile of microbial oils synthesized by R4 from these substrates resembles those found in vegetable oils, rich in oleic (C18:1) and palmitic (C16:0) acids, suitable for biodiesel synthesis. Biodiesel derived from *R. glutinis* R4 meets international standards, ensuring quality and compatibility with diesel engines. This suggests a viable avenue for sustainable fuel production, aligning with global efforts towards renewable energy sources.

The integration of *R. glutinis* R4 into the bioethanol and biodiesel industries presents a synergistic approach, fostering a circular economy model. By optimizing this bioprocess, leveraging waste streams for value-added product generation becomes feasible, thus mitigating environmental impact while enhancing resource efficiency.

In summary, the multifaceted potential of *R. glutinis* R4 as a versatile strain for alternative oil sources not only underscores its significance in biotechnological applications but also underscores its role in advancing sustainable practices in oil production. Future research endeavors aimed at fine-tuning this bioprocess stand to amplify these benefits, propelling forward the paradigm of sustainable biofuel generation.

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