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Bioconversion of sugarcane molasses and waste glycerol on single cell oils for biodiesel by the red yeast *Rhodotorula glutinis* R4 from Antarctica

Pedro E. Sineli [a], D. Daniela Maza [b], Manuel J. Aybar [b,c,**], Lucía I.C. Figueroa [a], Silvana C. Viñarta [a,d,*]

[a] *Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), Tucumán, Argentina*
[b] *Instituto Superior de Investigaciones Biológicas (INSIBIO, CONICET-Universidad Nacional de Tucumán), Tucumán, Argentina*
[c] *Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina*
[d] *Cátedras de Química Bioinorgánica/Biología de los microorganismos, Facultad de Ciencias Exactas (FACEN) y Naturales, Universidad Nacional de Catamarca (UNCA), Catamarca, Argentina*

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ABSTRACT

In the context of the global energy crisis and the constant demand for biofuels, this work explored the biodiesel production from single-cell oils (SCO) produced by the Antarctic oleaginous yeast *Rhodotorula glutinis* R4 from low-cost local agro-industrial by-products (sugarcane molasses and waste glycerol). The lipid accumulation reached 46.8 % and 40.7 % at 120 h of culture using glycerol and molasses, respectively, which correspond to 5.72 and 8.68 g L^{-1} of final lipid concentration. *R. glutinis* R4 yielded 0.172 and 0.185 g of lipids per gram of substrate consumed grown in molasses or glycerol medium, respectively. These amounts being higher than the ones obtained in glucose medium (0.126 g g^{-1}). At 120 h of culture, lipid volumetric productivities were 0.048 and 0.072 g L^{-1} h^{-1} using glycerol and molasses, respectively, and 0.043 g L^{-1} h^{-1} in the glucose yeast extract (GYM) medium. Oleic acid is the predominant fatty acid in the oils from *R. glutinis* R4, reaching 67.5 % with molasses, thus indicating that it is adequate for biodiesel synthesis. This is the first study where SCO produced by *R. glutinis* R4 were converted into biodiesel by acid transesterification with an efficiency above 90 %. The biodiesel produced by *R. glutinis* R4 grown on culture media containing molasses or waste glycerol is fully compliant with the international standards for biodiesel. SCO obtained from *R. glutinis* R4 using molasses and waste glycerol can be effectively used as sources of triacylglycerols for biodiesel production.

1. Introduction

At present, various socio-economic, environmental, and health issues have arisen as a consequence of the increased dependence on fossil fuel reserves and their utilization to satisfy energy requirements in many countries [1]. In this context of global energy crisis, renewable energy sources such as biofuels constitute an environmentally friendly strategy.

Among biofuels, biodiesel has recently attracted much attention due to its environmental friendliness [2]. Biodiesel is an alternative fuel cleaner than petroleum-derived diesel. It is a biodegradable, non-toxic biofuel produced from renewable sources that reduces net carbon dioxide emissions [2]. Currently, biodiesel is produced mainly on an industrial scale from vegetable oils through transesterification reactions with methanol or with some short-chain alcohols [2]. Nevertheless, biodiesel production from vegetable sources has several disadvantages, the most relevant being its direct competition with the production of animal and human food, its negative environmental impact and its high production costs [3]. Single-cell oils (SCO) are neutral lipids produced by oleaginous microorganisms with fatty acids (FA) composition similar to vegetable oils. SCO have recently come to the fore as alternative non-food oil feedstock for biodiesel synthesis [4]. The composition and properties of SCO differ, depending on the microorganism (yeast, microalgae, filamentous fungi, or bacteria) and the substrate employed in the culture media [4–6]. Oleaginous microorganisms can accumulate more than 20 % (w/w) of their dry biomass in the form of neutral lipids, mainly as triacylglycerides (TAG), under specific culture conditions

* Corresponding author at: Laboratory of Fungal Biotechnology, PROIMI-CONICET (Planta Piloto de Procesos Industriales Microbiológicos), Av. Manuel Belgrano and Pje. Caseros, T4001MVB, San Miguel de Tucumán, Tucumán, Argentina (S.C. Viñarta).
** Co-Corresponding author at: INSIBIO (CONICET-UNT), Chacabuco 461, T4000ILI, San Miguel de Tucumán, Tucumán, Argentina (M.J. Aybar).
E-mail addresses: mjaybar@fbqf.unt.edu.ar (M.J. Aybar), scvinarta@hotmail.com, scvinarta@conicet.gov.ar (S.C. Viñarta).

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[4,7,8]. With respect to lipid production, oleaginous yeasts have shown certain advantages over microalgae and filamentous fungi, the most important being short duplication times, unicellular form, easy cultivation in large reactors, and the ability to grow on a variety of carbon sources including by-products and/or nutritional residues from agriculture and industry [3,4,6,7,9,10]. Examples of the agro-industrial wastes used as substrates of yeasts include sugarcane bagasse, corn stover and waste glycerol, among others [6,11].

Molasses is a major by-product of the sugarcane industry. It is a dark brown viscous liquid rich in nutrients, growth factors, and minerals, its main components being carbohydrates (mainly sucrose and reducing sugars; 40–60 %, w/w) and water (20 %, w/w) [2,12]. The composition of molasses varies depending on the sugar refining process and the plant variety. Molasses can be used as a carbon source for bioethanol production [12]. Additionally, it can also be used for microbial production of value-added compounds such as industrial enzymes, organic acids, biopolymers, oligosaccharides, and SCO [2,12,13].

On the other hand, glycerol is the main by-product of biodiesel production. In turn, glycerol is a direct metabolic precursor of triacylglycerol (TAG, the main component of SCO), and it has been reported that oleaginous yeasts can use it as their sole carbon source to produce SCO [14]. Considering that waste glycerol is a low-cost, widely available high-carbon content triol compound, its use for the formulation of culture media could be integrated into biorefineries and promote circular economy.

Nowadays, an important global challenge is the adequate supply of sustainable alternative energies, including biofuels [1]. Therefore, SCO from yeasts represent a promising alternative feedstock for biofuel production, and a possible solution for a bio-based economy [15]. In this context, the purpose of this study was to evaluate the use of *R. glutinis* R4 as a producer of TAG for biodiesel supply using two local low-cost agro-industrial by-products (sugarcane molasses and biodiesel-derived waste glycerol) as carbon sources. The use of low-cost substrates contributes to a reduction in the cost of microbial lipid production which nowadays limits its commercialization [6].

Cold-adapted *Rhodotorula glutinis* R4 is a basidiomycete species isolated in Antarctica that produces large quantity of SCO under appropriate conditions by using glucose as a carbon source [3,4,9]. Moreover, the fatty acids composition of the SCO produced by *R. glutinis* R4 is similar to vegetable oils, with a high content of oleic and palmitic acids and suitable for biodiesel synthesis [4,9,16]. The use of glucose as a substrate is not economically profitable, and to reduce this expense oleaginous yeasts are a promising alternative when they are grown on low-cost raw-material (e.g. byproducts or agro-industrial wastes) [2]. Although some yeast strains have exhibited high potential concerning SCO production, only a few studies have shown lipid production by *Rhodotorula* spp. using molasses in fermentation media as a substrate [13]. SCO production by *R. glutinis* using sugarcane molasses remains poorly understood as yet.

The non-conventional red yeast *R. glutinis* R4 has demonstrated an outstanding ability to accumulate up to 50 % (w/w) of lipid content of its total dry weight by using glucose as carbon source, and showed interesting features suitable for biodiesel synthesis [4,16]. Up to date, the potential of *R. glutinis* R4 for the production of SCO from non-conventional substrates as molasses and waste glycerol has not been determined. The aim of this work was to comparatively evaluate the ability of *R. glutinis* R4 to grow, produce and accumulate intracellular lipids using low-cost local agro-industrial by-products (sugarcane molasses and waste glycerol) as carbon sources. In addition, SCO produced by *R. glutinis* R4 were converted into biodiesel by acid transesterification, and the properties of the biodiesel and the efficiency of the process were analyzed.

2. Materials and methods

2.1. Yeast strain, maintenance, and culture conditions

Rhodotorula glutinis R4 was isolated from soil samples collected during the 2011/12 Austral summer on Potter Caleta, 25 de Mayo Island, Antarctica (62°14′18′'S, 58°40′00′'W) and was characterized as an oleaginous yeast strain [4,9]. The yeast was cultured on solid-YM (in g L^{-1}: yeast extract 3, malt extract 3, peptone 5, dextrose 10, agar 20) plates at 25 °C and maintained at 4 °C [16].

Assays for lipid production were performed in GMY medium using glucose or non-conventional substrates as their sole carbon source. Two non-conventional substrates were used as carbon sources, waste-glycerol derived from biodiesel and sugarcane molasses. No pretreatment was performed by the authors on either industrial by-products. In both cases, substrates were supplied by industries. Waste glycerol is a by-product of vegetable oil-based biodiesel production and was provided by a local agroindustry. Its composition was 81.6 % glycerol, 0.01 % methanol, 10.4 % water, 6.2 % ash, and 1.8 % non-glycerol organic material. In the case of waste glycerol, it was pretreated by the supplier industry by gravity separation to eliminate solid particles. This method generates crude glycerol, which may still contain impurities such as methanol, oil, soap, salt, and other organic materials at ppm levels [17] and it can be used as substrate of microorganisms. Sugarcane molasses was supplied by “Ingenio La Corona”, a sugar and alcohol (ethanol) producer from Tucumán, Argentina. Its composition was 60 % reducing sugars and 20 % water, while the remaining 20 % contained nutrients, growth factors, and minerals. Molasses is a liquid by-product of the sugarcane industry and it can be added directly to the culture medium. In these assays, molasses and waste glycerol were used in liquid form. Stock solutions of each by-product supplied by the industries were added to a final concentration of 40 g L^{-1} required for assays to distilled water together with the other components of the medium (GMY).

In order to perform the growth and lipids production experiments, *R. glutinis* R4 was cultured for 120 h on 100 mL of nitrogen-limited GMY medium (in g L^{-1}: KH_2PO_4 8; $MgSO_4{\cdot}7H_2O$ 0.5; yeast extract 3; final pH 5.5) using 500 mL Erlenmeyer flasks in a rotatory shaker (250 rpm, 25 °C) under aerobic conditions [16]. Glucose, waste glycerol, and sugarcane molasses were used as carbon sources at a final concentration of 40 g L^{-1} in GMY medium in independent assays performed in triplicate in three different flasks. The sterilized media were inoculated (10 %, v/v) with a 24 h seed culture grown aerobically [4] on GMY medium with 40 g L^{-1} of glucose. In order to study growth and lipid production parameters, the culture samples were taken after 12 h of incubation and then, each 24 h in all cases, during 120 h of culture.

2.2. Analytical determinations

The biomass of 2 mL of culture broth was removed by centrifugation and washed twice with the same volume of distilled water. Biomass was dried at 105 °C to constant weight [9]. Total lipid extraction of yeast was carried out from lyophilized and pulverized biomass [9] with solvents (chloroform: methanol, 2:1; v:v) and constant stirring at 25 °C according to standard methodology [18]. Then, the samples were centrifuged at 14,000xg for 10 min at 4 °C, and the organic phase of the supernatant containing lipids was recovered and completely evaporated according to Maza et al. (2020) [16]. Lipids were gravimetrically determined overnight at 105 °C until constant weight [9,16]. Glucose and total reducing sugar from molasses were analyzed and determined by the DNS (3, 5-dinitrosalicylic acid) method (Miller, 1959) [18]. Residual glycerol was measured by HPLC (Gilson), a REZEX™ ROA-Organic Acid H^+ column (8 %) was used as stationary phase, and 5 mM H_2SO_4 was used as mobile phase, with a flow rate of 0.6 mL min^{-1} and at a temperature of 60 °C. The lipid content (%), lipids, and biomass yields coefficients ($Y_{X/S}$, $Y_{L/S}$, and $Y_{L/X}$; g g^{-1}), volumetric productivity of lipids (Q_L; g L^{-1}

h^{-1}), and biomass (Q_X; g L^{-1} h^{-1}) were also calculated [4].

2.3. Saponifiable lipids extraction and conversion into biodiesel (FAME)

Cells from 40 mL of *R. glutinis* R4 cultures incubated for 120 h were collected by centrifugation, washed, and lyophilized [4]. The dry biomass was used to separate the saponifiable fraction of intracellular lipids following the method described by Pereyra et al. (2014) [19]. The dry biomass was treated with 5 mL of KOH 30 % (w/v) and 5 mL of ethanol 95 % (v/v) and incubated overnight at 70 °C. The unsaponifiable fraction was removed with hexane. In the remaining aqueous phase, pH was adjusted to 1.0 with pure HCl. Subsequently, the fatty acids were extracted twice with 10 mL of hexane. After hexane evaporation under reduced pressure, the fatty acids fraction of the saponifiable lipids was weighed.

In order to obtain the biodiesel (fatty acid methyl esters, FAME), the saponifiable lipids obtained were esterified with methanol, under acidic conditions, as described by Burja et al. (2007) [20]. Briefly, 20 mg of saponifiable lipids were transferred into a glass tube and 3 mL of methanol:HCl:chloroform (10:1:1, by vol.) was added and incubated at 90 °C for 2 h. Afterward, 1 mL of distilled water was added to the mixture, and the methyl esters were extracted three times with 2 mL of hexane:chloroform (4:1, by vol.). The water residue from the organic phase containing the methyl esters was removed by adding 0.5 g of anhydrous Na_2SO_4. The biodiesel yield (% per weight) was calculated (Eq. (1)) in relation to the weight of the yeast lipid [2]. In Eq. (1), "mass of biodiesel" represents the weight of biodiesel obtained, while "theoretical mass" alludes to the weight of the saponifiable lipids used in the transesterification process.

$$Biodiesel\ yield\ (\%) = (Mass\ of\ biodiesel / Theoretical\ mass) \times 100 \quad (1)$$

2.4. Determination of TAG and FAME by thin layer chromatography (TLC)

The presence of TAG in SCO samples and the FAME obtained by acid transesterification were qualitatively analyzed by thin layer chromatography (TLC) according to Álvarez et al. (2008) [21]. The samples were concentrated at 50 % of their original volume, subjected to TLC using plates silica gel 60 F254 Aluminum sheets (20 × 20 cm, Merck Millipore), and developed in a system solvent for TAG analysis consisting of hexane: ethyl acetate: acetic acid (90:10:1, by volume). Soybean oil was included as a control of vegetable TAG [16]. Commercial vegetable oil-based biodiesel was also used as a standard and positive control of FAME. TLC plates were stained with iodine vapor [21] and then photographed [16].

2.5. Fatty acids composition and estimation of biodiesel properties

In order to determine the relative composition of fatty acids, the FAME obtained from methanolysis reactions [4] were analyzed by GC-FID as described by Viñarta et al. (2020) [4]. A GC Agilent Technologies (Model 6890) equipped with an HP-5 capillary (30 m × 0.32 mm i.d., 0.25 μm) column and an automatic injector were used. The carrier gas was N_2 (flow rate: 15.0 mL min^{-1}). The injection temperature was 270 °C; the initial temperature was 40 °C, increasing to 190 °C at a rate of 23 °C min^{-1} and holding for 4 min, then increasing to 290 °C at a rate of 8 °C min^{-1} and holding for 5 min. The temperature of the detector was 300 °C. The fatty acids were identified by comparison with retention times to authentic reference standards [4].

The quality of the biodiesel produced by *R. glutinis* R4 was estimated using the FAME profile as described by Maza et al. (2020) [16]. Important physical properties such as cetane number (CN), saponification value (SV), iodine value (IV), degree of unsaturation (DU), long-chain saturation factor (LCSF), cold filter plugging point (CFPP), oxidative stability (OS), high heating value (HHV), kinematic viscosity (ν), and density (ρ) were estimated by previously reported empirical equations (Eqs. (2)–(11)) using the fatty acids composition [16,22,23].

$$SV = \sum 560 \times (\%FC/M) \quad (2)$$

$$IV = \sum 254\,DB \times (\%FC/M) \quad (3)$$

$$CN = 46.3 + 5458/SV - (0.255 \times IV) \quad (4)$$

$$DU(\%) = MUFA + (2 \times PUFA) \quad (5)$$

$$LCSF = (0.1 \times C16) + (0.5 \times C18) \quad (6)$$

$$CFPP = (3.417 \times LCSF) - 16.477 \quad (7)$$

$$OS = 117.9295/(wt\%C18:2 + wt\%\,C18:3) + 1.5905 \quad (8)$$

$$HHV = 49.43 - (0.41 \times SV) - (0.015 \times SV) \quad (9)$$

$$\nu = e^{\left[-12/2015 + 2.496 \times \ln\left(\sum M\right) - 0.178 \times \sum DB\right]} \quad (10)$$

$$\rho = 0.8463 + 4.9/\sum M + 0.0018 \times \sum DB \quad (11)$$

where M = molecular mass of each fatty acid component, DB = number of double bonds, FC = % of each fatty acid component, MUFA = weight % of monounsaturated fatty acids, and PUFA = weight % of polyunsaturated fatty acids.

2.6. Statistical analysis

The data were analyzed using the ANOVA parametric test and differences between culture conditions were detected using the Tukey HSD test at the 0.05 level. Statistical analysis were performed using Infostat statistical software.

3. Results and discussion

3.1. Growth and lipid production using alternative carbon sources

R. glutinis R4 was previously characterized as an oleaginous yeast by Viñarta et al. (2016) [9], and was found to be capable of growing and accumulating large amounts of lipids per gram of biomass (~50–60 %, w/w) using glucose as a carbon source in nitrogen-limited culture media [4,16]. In this work, *R. glutinis* R4 was cultivated for 120 h in a nitrogen-limited medium using biodiesel-derived waste glycerol and sugarcane molasses as the sole carbon source in order to evaluate the ability of the R4 strain to metabolize these agro-industrial by-products. A control of glucose as a carbon source was included in the assays. Under these conditions, *R. glutinis* R4 was able to grow in GMY liquid medium with all substrates evaluated (Fig. 1). Furthermore, glycerol and molasses delayed growth (Fig. 1), and maximum biomass values were achieved after 120 h of incubation, reaching 12.21 and 21.33 g/L, respectively (Table 1). In contrast, with glucose the maximum biomass value was obtained at 48 h of incubation.

With respect to the lipid production, *R. glutinis* R4 in a glucose-rich medium exhibited a considerable lipid concentration (<2 g L^{-1}) after 12 h of incubation, while in glycerol and molasses media almost no lipid production was observed after 12 h of culture (Fig. 2). In the latter two conditions, with the non-conventional carbon sources, lipid production was evident after 48 h (2.1 and 3.1 g/L, respectively) (Fig. 2). These differences can be attributed to the facilitated uptake of glucose when compared to other sugars. Glucose is taken up by a single constitutive facilitated diffusion system, whereas other sugars have different diffusion affinities [24]. Therefore, it could be suggested that the low assimilation rate of molasses and glycerol (Table 1) led to a delay and low initial lipid production (Fig. 2). However, after 96 and 120 h,

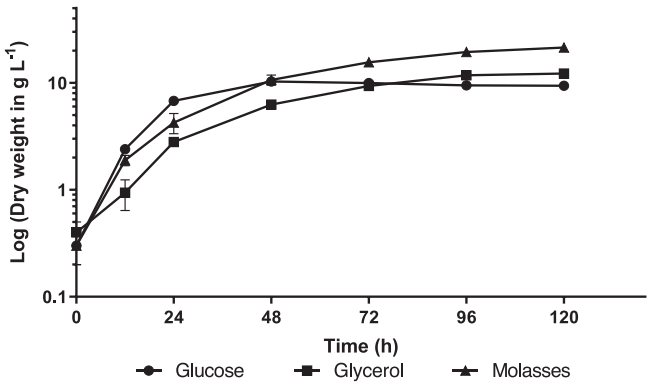

Fig. 1. Growth of *Rhodotorula glutinis* R4 during 120 h at 25 °C in GMY medium using 40 g/L of waste glycerol and sugarcane molasses as alternative carbon sources, and glucose as a conventional carbon source. Growth was represented at logarithmic scale (for details, see material and methods section).

cultures grown on molasses reached the highest values of lipid production compared to glucose and glycerol (Fig. 2, Table 1). In this work, *R. glutinis* R4 improved lipid production by 1.6-fold using molasses (8.7 g/L) in comparison to glucose (5.2 g/L) (Table 1). Similar observations were performed previously by Singh et al. (2020) for *R. toruloides* ATCC 204091, which reached 1.3-fold lipid production when grown on molasses medium rather than on glucose medium [25]. Furthermore, it should be noted that the impurities present in the waste glycerol and molasses did not inhibit the growth of *R. glutinis* R4 (Fig. 1), and biomass values at the endpoint (120 h of cultures) were higher than in the glucose medium, in particular with the use of molasses (Fig. 1, Table 1).

The parameters of biomass and lipid production including yields and productivity were analyzed after 96 and 120 h of culture (Table 1) due to the production of lipids by *R. glutinis* R4 began in the stationary phase and continue until the last experimental points. Furthermore, when *R. glutinis* R4 grew from agro-industrial by-products, it yielded higher biomass and lipid production at the end of the culture (Fig. 2, Table 1).

With respect to lipid accumulation, *R. glutinis* R4 was capable of accumulating 40.7 % (w/w) and 46.8 % (w/w) of dry biomass as lipid, which corresponds to a lipid yield of 8.7 and 5.7 g/L with molasses and glycerol, respectively, after 120 h of culture (Table 1). Glucose was used as control for lipid production and yeast accumulated 55.3 % (5.2 g/L) of lipids at the end of the culture (Fig. 2, Table 1). Results showed that *R. glutinis* R4 produces and accumulates lipids from two local by-products such as sugarcane molasses and waste glycerol and its yield was comparable to glucose (Table 1). These results are interesting since the amount of lipids yielded by *R. glutinis* R4 (40–47 %, w/w) from these industrial by-products (Table 1) are comparable and in some cases higher than those reported for oleaginous yeasts cultured from the same substrates (26.2–40.7 %, w/w) [2,26,27]. In this regard, Liang et al. (2021) observed that when *Rhodotorula mucilaginosa* LP-2, a yeast isolated from an activated sludge tank of a biodiesel production plant in Taiwan, was cultured using waste glycerol or molasses as carbon

Table 1
Biomass and lipid production parameters by *R. glutinis* R4 growth with 40 g/L of glucose, waste glycerol, and sugarcane molasses as carbon sources.

Carbon source	Time (h)	Biomass (g/L)	Lipids (g/L)	Lipids (%)	$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)	Q_X (g/L h^{-1})	Q_L (g/L h^{-1})
Glucose	96	9.47 ± 0.19^{a}	5.60 ± 0.01^{a}	59.10 ± 1.17^{a}	0.232 ± 0.0013^{a}	0.138 ± 0.0025^{a}	0.098 ± 0.0005^{a}	0.058 ± 0.0010^{a}
	120	9.37 ± 0.27^{ab}	5.18 ± 0.39^{a}	55.28 ± 0.34^{a}	0.229 ± 0.0031^{a}	0.126 ± 0.0017^{ab}	0.078 ± 0.0010^{b}	0.043 ± 0.0027^{ab}
Glycerol	96	11.75 ± 0.57^{bc}	5.01 ± 0.63^{a}	42.66 ± 3.01^{ab}	0.443 ± 0.0210^{b}	0.191 ± 0.0143^{bc}	0.122 ± 0.0078^{bc}	0.052 ± 0.0033^{ab}
	120	12.21 ± 0.26^{c}	5.72 ± 0.74^{a}	46.86 ± 4.12^{ab}	0.369 ± 0.0079^{bc}	0.185 ± 0.0266^{bc}	0.102 ± 0.0022^{c}	0.048 ± 0.0061^{b}
Molasses	96	19.43 ± 0.75^{d}	7.78 ± 0.42^{b}	40.01 ± 1.06^{b}	0.393 ± 0.0150^{c}	0.169 ± 0.0043^{c}	0.202 ± 0.0078^{d}	0.081 ± 0.0022^{c}
	120	21.33 ± 0.67^{e}	8.68 ± 0.39^{b}	40.66 ± 0.87^{b}	0.412 ± 0.0057^{c}	0.172 ± 0.0038^{c}	0.178 ± 0.0055^{e}	0.072 ± 0.0023^{c}

R. glutinis R4 yeast were cultured in GMY at 25 °C for 120 h using 40 g/L of glucose, sugarcane molasses or waste-glycerol being the sole carbon source. Parameters of growth and lipid production were analyzed after 96 and 120 h of incubation. Data are the mean ± standard deviation (SD) of three independent experiments. The final concentration of biomass and lipids, lipids accumulation (%), lipid/substrate yield ($Y_{L/S}$), biomass/substrate yield ($Y_{X/S}$), and the volumetric productivities of lipids (Q_L) and biomass (Q_X) are shown. Within a column, values followed by the same letters do not differ significantly, Tukey's test. $\alpha = 0.05$.

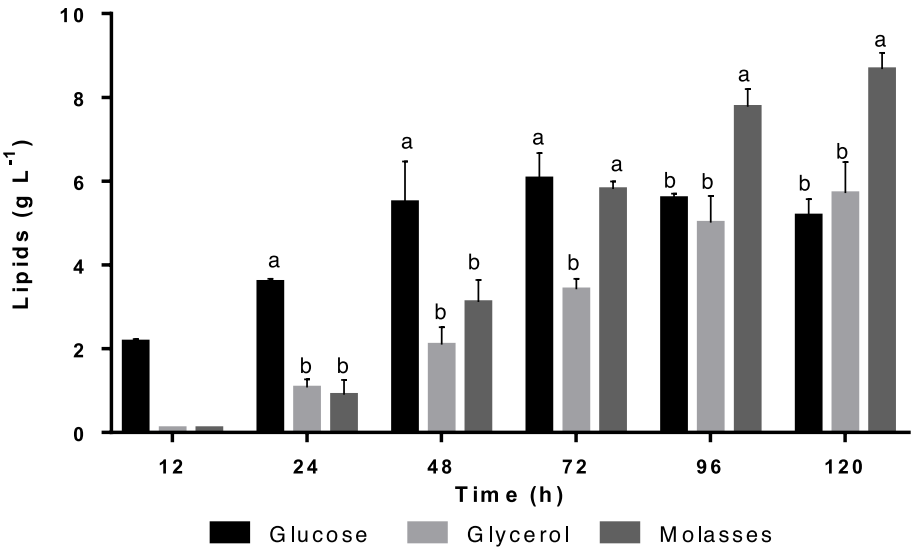

Fig. 2. Total lipids production by *Rhodotorula glutinis* R4 using different carbon sources. Yeasts were grown at 25 °C in GMY medium containing 40 g/L of waste glycerol or sugarcane molasses as alternative carbon sources, and glucose as a conventional carbon source. The data obtained at each time point were subjected to analysis of variance (one-way ANOVA), considering a significant probability level of $p \leq 0.05$ (Tukey's test). Values indicated by the same letters do not differ significantly.

sources, the percentage of lipids accumulated by LP-2 was 39.4 % and 26.2 %, respectively [27]. The lipid content accumulated by *R. glutinis* R4 was higher than LP-2 yeast strain in both conditions evaluated (40.6 % in molasses and 46.8 % in waste glycerol, see Table 1). These differences observed could be attributed to the complex composition of the wastes used to formulate the culture media and to the particular differences of the strains.

The results of lipid content and lipid production reached in this study by *R. glutinis* R4 (Table 1) from sugarcane molasses (40.7 %; 8.7 g/L; 120 h of incubation) are also higher than those reported for *Rhodotorula kratochvilovae* SY89 (38.25 %, 4.82 g/L, 144 h of incubation) by Jiru et al. (2018) [2].

Jiru et al. (2018) investigated the lipid production of *R. kratochvilovae* SY89 from sugarcane molasses (~50 g/L total sugars) in a stirred-tank bioreactor (0.8 L) for 168 h [2]. The highest lipid accumulation reached by the SY89 yeast strain was 38.25 % (lipid yield: 4.82 g/L) after 144 h of culture, while the highest biomass (13.25 g/L) was obtained at 120 h of incubation. According to the results for SCO production from sugarcane molasses by *R. glutinis* R4 (Table 1), the lipid yield (8.7 g/L) was significantly higher (almost twice) than that of the SY89 strain, while the lipid accumulation (40.7 %) was comparable, although both were achieved by *R. glutinis* R4 at shorter incubation times (120 h) than SY89 (144 h). Biomass produced by *R. glutinis* R4 from molasses (21.33 g/L) in this study (Table 1) was also significantly higher than to *R. kratochvilovae* SY89 (13.25 g/L) [2], thus demonstrating the outstanding performance of *R. glutinis* R4 to grow and accumulate lipids from molasses. The lipid content obtained using waste glycerol as substrate by *R. glutinis* R4 (46.7 %, w/w) was higher than to those previously reported by Chmielarz et al. (2021), who observed a lipid content of 37.3 % after 120 h of culture for *R. glutinis* CBS3044 but also similar to those observed for *Rhodotorula toruloides* CBS 14 (46.1 %) after 144 h of culture [26].

The yields of lipids and biomass, as well as the volumetric productivities (Q_L and Q_X) after 96 and 120 h of culture are shown in Table 1. The values of lipid yield coefficient ($Y_{L/S}$) reached by *R. glutinis* R4 at 120 h of incubation were 0.185 and 0.172 g g^{-1} using glycerol and molasses respectively (Table 1). These values did not present significant differences among them, but were significantly higher than the 0.126 g g^{-1} obtained with glucose (Table 1), indicating for this time of culture a major conversion of glycerol and molasses in lipids in comparison to glucose (Table 1). These results can be due to the different growth phases experienced by the yeast strain in the medium with the different substrates. As observed at 96 and 120 h of culture in medium with glucose (Fig. 1), the yeast entered the dead phase, while when the yeast grew with glycerol and molasses, at 96 and 120 h, it was still in the log phase (Fig. 1). The efficiency to produce and accumulate lipids could be affected by the ability of lipid biosynthesis and biomass yield, which in turn depend on carbon source utilization [11].

The maximum value of lipid volumetric productivity (Q_L) at 120 h of culture was observed for *R. glutinis* R4 (0.072 g/L h^{-1}) in the molasses medium (Table 1). With the use of glycerol, lipid productivity was 0.048 g/L h^{-1} at 120 h of incubation (Table 1). Both values were higher than the value obtained with glucose, which was 0.043 g/L h^{-1} at the same time of incubation (Table 1). In molasses-containing medium the maximum values of lipid and biomass volumetric productivity (Q_L and Q_X) were observed (Table 1), thus demonstrating the excellent performance of *R. glutinis* R4 to grow and produce lipids from this low-cost industrial by-product.

Remarkably, results revealed that *Rhodotorula glutinis* R4 is a yeast strain capable of carrying out the "*de novo*" lipids accumulation using glucose, waste glycerol, and molasses as the sole carbon source in nitrogen-limited media. The use of both low-cost substrates could replace valuable or expensive compounds as a carbon source in the culture media to enhance cell growth and lipid production.

3.2. Determination of TAGs and conversion into their respective FAME

The presence of triglycerides in the SCO produced by *R. glutinis* R4 was qualitatively analyzed by thin layer chromatography (TLC), and it was demonstrated that *R. glutinis* R4 is able to synthesize lipids from different carbon sources (glucose, glycerol, and molasses) (Fig. 3). Subsequently, the SCO were converted into FAME by acid transesterification. All reactions showed transformation of SCO from *R. glutinis* R4 into FAME (biodiesel). Each biodiesel obtained was evaluated in a TLC and showed similar profiles to the commercial biodiesel used as control (Fig. 3). These results revealed the presence of TAG in SCO from *R. glutinis* R4 and their efficient conversion into biodiesel (FAME) by acid transesterification.

3.3. Fatty acids profile and estimation of biodiesel properties

The physical–chemical properties of biodiesel are controlled by the level of unsaturation, the length of the carbon chain, and the stability of fatty acids oxidation [28]. For this reason, an important criterion for deciding whether glycerol and/or molasses can be used as feedstock for biodiesel synthesis is to evaluate the composition of the fatty acids produced by *Rhodotorula glutinis* R4 under our experimental conditions. The conversion of the SCO from *R. glutinis* R4 into FAME, followed by GC-FID analysis, revealed the fatty acids profile (Table 2). Results showed that the SCO produced by *R. glutinis* R4 in a culture medium with glucose, waste glycerol, or sugarcane molasses after 120 h of incubation are constituted by long chain fatty acids with 16–18 carbon atoms (Table 2). In particular, *R. glutinis* R4 produces oleic acid (C18:1) as the largest FA component (49–68 %), followed by palmitic acid (C16:0; 15–22 %), and linoleic acid (C18:2; 9–20 %), which are suitable for the production of biodiesel; the sum of these three fatty acids represents 85–93 % of the FA profile of SCO from *R. glutinis* R4 (Table 2). In this regard, other researchers reported similar fatty acids profiles in oleaginous yeasts grown on molasses, glycerol and glucose [11,16,29,30]. On the other hand, when the fatty acids profile was compared with that of vegetable oils, *R. glutinis* R4 presented a distribution comparable to that observed in jatropha oil (Table 2), which is nowadays widely used for biodiesel production [28]. Overall, the results showed that the high relative percentage of SFA (saturated fatty acids) and MUFA (monounsaturated fatty acids) from *R. glutinis* R4 (greater than 80 %) and added to the low degree of PUFA (polyunsaturated fatty acids) makes the SCO from this yeast a suitable oil feedstock for biodiesel production (Table 2). Due to the fact that when oils are rich in polyunsaturated fatty acids, they become unsuitable due to their tendency to oxidation [31].

Furthermore, it was observed that SCO produced by *R. glutinis* R4 in a molasses-based culture medium exhibited a greater degree of monounsaturation (70 %) than those produced in glucose- or glycerol-based culture medium, which showed similar values to each other (50 %) (Table 2), demonstrating that the carbon source influences the fatty acids profile. Results indicated that the substrate used as a carbon source by *R. glutinis* R4 influenced the fatty acids composition (Table 2) as well as the growth and lipid synthesis (Table 1, Fig. 2). Similar results were also observed by Huang et al. (2009) for *Trichosporon fermentans* [5].

On the other hand, the results indicate that when sugarcane molasses and waste glycerol were used as the only carbon source, *R. glutinis* R4 produced SCO rich in oleic acid (C18:1) (Table 2). Notably, the highest oleic acid value was observed in the presence of molasses, 67.5 % (Table 2). Similar oleic contents were reported by Boviatsi et al. (2020) for *R. toruloides* and *R. kratochvilovae* growing on molasses media reaching up to 65.1 % [13]. Currently, high oleic acid vegetable oils are used in oleochemical production and are considered a suitable feedstock for the production of biolubricants due to their thermal and oxidative stability [13]. Some authors have indicated that the composition of the medium affects the fatty acid profile, which offers the opportunity to increase the oleic acid content in the microbial oil produced [13]. In this sense, microbial oils (SCO) from *R. glutinis* R4 rich in oleic acids could be

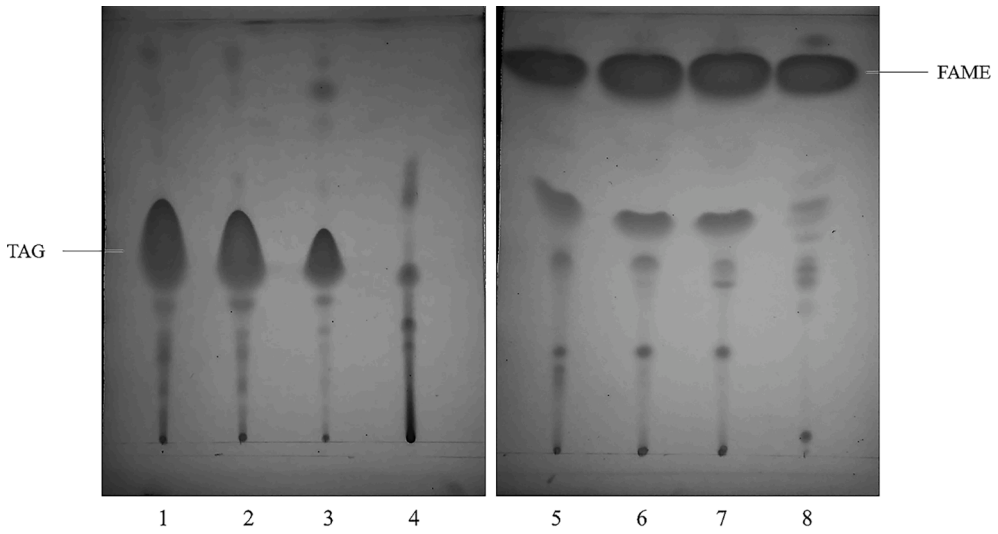

Fig. 3. Qualitative thin layer chromatography (TLC) analysis of triacylglycerides (TAG, left panel) present in SCO extracts of *Rhodotorula glutinis* R4 using alternative carbon sources in culture media, and their subsequent acid transesterification to biodiesel (FAME, right panel). Lanes: 1, SCO/glucose; 2, SCO/glycerol; 3, SCO/molasses; 4, SCO/soybean (positive control of TAG); 5, biodiesel/glucose; 6, biodiesel/glycerol; 7, biodiesel/molasses; 8, biodiesel/soybean (positive control).

Table 2
Fatty acids composition of SCO from *R. glutinis* R4 obtained from different carbon sources (glucose, waste glycerol, and molasses) in comparison with vegetable oils, and biodiesel physico-chemical properties tested according to ASTM D6751 and EN 14214 international biodiesel standards.

		Relative abundance of fatty acids (%, w/w)				
Fatty acids composition		*R. glutinis* R4 oils			Vegetables oils	
Fatty acids		Glucose	Glycerol	Molasses	Jatropha	Palm
Myristic acid	C14:0	0.980	1.480	0.787	0.02	0.04
Pentadecanoic acid	C15:0	0.197	0.223	0.203	ND	ND
Palmitic acid	C16:0	16.780	22.240	15.350	14.58	38.77
Palmitoleic acid	C16:1	1.420	2.870	2.650	0.77	ND
Margaric acid	C17:0	5.950	3.900	1.440	ND	ND
Stearic acid	C18:0	2.430	2.780	1.450	11.7	3.16
Oleic acid	C18:1	48.950	48.940	67.520	69.3	37.61
Linoleic acid	C18:2	19.603	15.370	9.720	3.31	8.78
Linolenic acid	C18:3	3.640	2.190	0.881	0.035	0.16
C16:0 + C18:1 + C18:2		85.370	86.552	92.597	87.19	85.16
SFA		26.340	30.630	19.230	26.3	41.97
MUFA		50.380	51.810	70.168	70.07	37.61
PUFA		23.280	17.560	10.603	3.34	8.94
Biodiesel properties	*ASTM D6751/EN 14,214*	*Glucose*	*Glycerol*	*Molasses*		
SV (mg KOH)	NS/NS	203.348	204.721	202.638		
IV (gI_2/100 g)	NS/≤120	90.983	80.699	83.383		
CN	≥47/≥51	52.669	54.803	54.474		
OS (h)	NS/≥6	7.656	9.307	13.712		
DU (% wt)	NS/NS	96.939	86.923	91.375		
LCSF (% wt)	NS/NS	2.893	3.616	2.260		
CFPP (°C)	NS/5 to −20	−7.388	−5.117	−9.377		
HHV MJ/Kg	NS/~35	39.465	39.445	39.506		
ν ($mm^2\ s^{-1}$)	1.9–6/3.5–5	3.831	3.848	3.923		
ρ (g/cm^3)	NS/0.8–0.90	0.876	0.875	0.875		
Reference		This study	This study	This study	[28]	[28]

R. glutinis R4 was cultured at 25 °C in GMY medium containing 40 g/L of each carbon source (see Material and Methods). Lipids were extracted from cell yeasts after 120 h of incubation and FAME composition was analyzed by GC-FID. ND: Not determined. SFA: Saturated Fatty Acids (%), MUFA: Mono Unsaturated Fatty Acids (%), PUFA: Poly Unsaturated Fatty Acids (%). NS: Not specified by international biodiesel standard, 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, υ: Kinematic Viscosity, ρ: Density, C18:3, Linolenic acid content.

considered a promising feedstock for sustainable oleochemical production. Furthermore, molasses and waste glycerol are two low-cost substrates appropriate for industrial production of high oleic acid microbial oils from *R. glutinis* R4.

Additionally, the quality of the biodiesel produced from SCO of *R. glutinis* R4 was established. Various physico-chemical properties should be evaluated and compared with the specifications of the international standards, which should be similar to those of traditional biodiesel. These quality standards were established by many countries, such as the American Standards for Material Testing (ASTM 6751–3) and the standards established by the European Union (EN 14214). Some of these properties include saponification value (SV), iodine value (IV), cetane

number (CN), higher heating value (HHV), oxidation stability (OS), degree of unsaturation (DU), kinematic viscosity (υ), density (ρ), linolenic acid content (% C18:3), among others. For this purpose, fatty acids profiles were used to estimate the properties of biodiesel obtained from *R. glutinis* R4 under the different culture conditions tested and then compared with established international standards.

The calculated parameters for *R. glutinis* R4-produced biodiesel under the conditions analyzed in this work were within a narrow range established by international standards (Table 2). For example, CN values estimated for the yeast biodiesel (52.7–54.8) were higher than 47 and 51, the values recommended as the minimum CN by ASTM D6751 and EN14214 standards, respectively (Table 2). Cetane number is related to the combustion of biodiesel. Higher CN minimizes the formation of white smoke and guarantees good cold start properties [3,4].

The degree of unsaturation of oils influences fuel oxidation tendency and can be estimated by means of iodine value (IV). The IV assessed in this work for biodiesel from *R. glutinis* R4 were in the range of 80.7–90.9 gI_2/100 g, which are below the limit value of 120 gI_2/100 g established by EN14214 (Table 2). In addition, the OS values (7.7–13.7 h) indicated that the biodiesel produced from *R. glutinis* R4 SCO is stable (Table 2). This is an important parameter related to the stability and performance of biodiesel [3,4]. The density (ρ) and the kinematic viscosity (υ) values of the biodiesel from *R. glutinis* R4 SCO fall within the ranges established by international biodiesel standards (Table 2). Furthermore, the SCO presented a density comparable to that of jatropha and palm oils (0.86 mm^2/sec) [28] and the content of linolenic acid was less than 12 % in all conditions tested (Table 2), which is an important characteristic for biofuels used in Europe.

Briefly, fatty acid profiles and estimated physico-chemical properties indicate that the SCO produced by *R. glutinis* R4 can be potentially used as a source of triacylglycerols for biodiesel production. Besides, the results also highlight the potential of sugarcane molasses and waste glycerol as alternative and low-cost feedstocks for the growth and production of suitable microbial oils for biodiesel synthesis by *R. glutinis* R4.

3.4. Production of biodiesel by transesterification

With the aim of producing microbial biodiesel, SCO extracted from *R. glutinis* R4 were transesterified using methanol. Biodiesel yields of 90.56 ± 3.59 %, 92.05 ± 0.36 %, and 91.48 ± 0.61 % were obtained from the SCO produced by *R. glutinis* R4 using waste glycerol derived from biodiesel industry, sugarcane molasses, and glucose as carbon sources, respectively. This is the first report showing the conversion of SCO by *R. glutinis* R4 into biodiesel. The yields obtained in this work for *R. glutinis* R4, higher than 90 %, are better than those previously reported for other yeasts strains such as *R. kratochvilovae* SY89 (85.3 %) and *R. glutinis* (81.70 %), and even higher than those of biodiesel from microalgae such as *Chlorella protothecoides* (68–63 %) [2,32,33]. Results demonstrated that microbial biodiesel from SCO produced by *R. glutinis* R4 was obtained with an efficiency higher than 90 % by the acid transesterification process.

4. Conclusions

With the aim of contributing to the important challenge to supply sustainable alternative energy sources, such as biofuels, the purpose of the present study was to promote biodiesel production from microbial oils (SCO) produced by the Antarctic oleaginous yeast *R. glutinis* R4 from two local low-cost agro-industrial by-products (sugarcane molasses and biodiesel-derived waste glycerol) as feedstocks.

Results indicate that *R. glutinis* R4 was able to accumulate ~41 % and 47 % of SCO suitable for biodiesel synthesis when it was cultured in inexpensive molasses and waste glycerol-containing media as sole carbon sources, respectively. In addition, this work demonstrated that different carbon sources (glucose, sugarcane molasses, and waste glycerol) were metabolized differently by *R. glutinis* R4, driven to cell metabolism or lipid biosynthesis.

The composition of fatty acids from SCO synthesized by *R. glutinis* R4 from sugarcane molasses and waste glycerol confirmed it is a suitable source of TAG for biodiesel production. Moreover, this is the first study demonstrating that SCO of the Antarctic oleaginous yeast *R. glutinis* R4 can be converted into biodiesel by acidic transesterification with an efficiency higher than 91 %. Estimated biodiesel properties are in accordance with international biodiesel standards.

Results of this study thus open up new prospects for the utilization of *R. glutinis* R4 as a promising lipid producer for biodiesel synthesis and valorization of low-cost local agro-industrial by-products and promote a model of circular economy and environmental benefits.

CRediT authorship contribution statement

Pedro E. Sineli: Investigation, Validation, Writing – original draft. **D. Daniela Maza:** Investigation, Formal analysis, Validation, Writing – original draft. **Manuel J. Aybar:** Methodology, Visualization, Funding acquisition, Resources, Writing – review & editing. **Lucía I.C. Figueroa:** Funding acquisition, Resources. **Silvana C. Viñarta:** Investigation, Methodology, Data curation, Visualization, Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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