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From crude glycerol and volatile fatty acids to biodiesel and other bioproducts using *Yarrowia lipolytica* NCYC 2904 as a cell factory

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Biodiesel production using microbial lipids derived from low-cost feedstocks could provide a sustainable alternative for the replacement of conventional fossil fuels. This work studied fed-batch and two-stage batch cultures for microbial lipids production by oleaginous yeast *Yarrowia lipolytica* NCYC 2904 from crude glycerol (a by-product of the biodiesel industry) and volatile fatty acids (obtained during the anaerobic fermentation of organic wastes). Fed-batch at a constant feeding rate of the medium with 100 g L^{-1} of glycerol and an initial dilution rate of 0.004 h^{-1}, followed by the pulse addition of an 18 g L^{-1} VFAs mixture at the end of the fed-batch phase was the best strategy to obtain the highest intracellular lipids accumulation per dry cell mass of 38% (w/w). Microbial lipids produced by *Y. lipolytica* NCYC 2904 (90% of unsaturated fatty acids) were successfully converted into biodiesel, whose main properties are within the international biodiesel standards EN 14214 and are similar to those of biodiesel produced from vegetable oils. Furthermore, a high amount of citric acid (28 g L^{-1}) and total polyols (mannitol and arabitol) of 3 g L^{-1} was produced during this bioprocess, which makes it even more interesting within an integrated biorefinery framework.

Introduction

Over the last few decades, biodiesel has been promoted as a renewable and environmentally preferable option to conventional fossil fuels. About 73% of biodiesel is produced from pure vegetable oils (31% palm oil, 24% soybean oil, and 14% rapeseed oil) and waste cooking oils (21%).[1] However, the use of these feedstocks has become controversial due to competition with food production and the diversion of land to biofuel production. Growing demand for crops of vegetable oils leads to an increase in the use of fertilizers and increases greenhouse gas emissions.[2] Microbial lipids, also known as single-cell oils, produced by oleaginous microorganisms, are considered a promising feedstock for sustainable biodiesel production owing to their composition of fatty acids similar to those of vegetable oils. Additionally, microbial lipids have some advantages over vegetable oils such as non-dependence on land and climate conditions, and can be produced from a wide spectrum of feedstocks with a short period of production.[3,4]

The oleaginous yeast *Yarrowia lipolytica* is considered a yeast model for microbial lipids production owing to its ability to accumulate high amounts of lipids from a wide range of substrates, making this yeast a sustainable source of lipids for the biodiesel industry.[5,6] Moreover, this non-conventional yeast has the remarkable ability to assimilate carbon sources from various agro-industrial wastes or by-products, including sugars from lignocellulosic biomass hydrolysates,[7] volatile fatty acids (VFAs) obtained by acidogenic fermentation of organic wastes,[8] crude glycerol from the biodiesel industry,[8] and fatty acids derived from olive mill wastewater,[9] animal fat,[10] and waste cooking oils.[11] Among the wide range of carbon sources utilized by *Y. lipolytica*, our previous work[8] demonstrated the feasibility of using crude glycerol and VFAs for lipids production. The main focus of that work was using VFAs from the beginning of the culture time and glycerol was employed as a co-substrate. Due to the availability of crude glycerol, its use as a principal substrate for high-cell density cultures and intracellular lipids accumulation is of utmost interest.

Crude glycerol is the main by-product of the biodiesel industry since about 10% (v/v) of crude glycerol is generated from the amount of biodiesel produced.[12] The global production of biodiesel is projected to reach 53.2 billion liters by 2026 (IEA, 2021),[13] implying that a large amount of crude glycerol will be available, providing opportunities for the development of innovative processes using this by-product. Volatile fatty acids are short-chain fatty acids (C2–C6) obtained during the acidogenic fermentation of a variety of wastes rich in organic matter, such as food waste. Among VFAs produced by acidogenic fermentation, 80% of VFAs correspond to acetic, propionic, and butyric acids.[8,14–18]

Besides being a yeast model for microbial lipids production, *Y. lipolytica* has an intense secretory activity and is considered

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a promising platform to produce a wide range of metabolites of industrial interest.[6] Citric acid is one of the most important organic acids produced by *Y. lipolytica*, with applications in the food and beverage industries as a flavoring and antioxidant agent, in the pharmaceutical industry as an excipient in formulations, and in the cosmetic, chemical, and detergent industries.[19] The global production of citric acid in 2022 reached 2.59 million tons and citric acid production by fermentation is expected to grow continually at an annual growth rate of 5%.[20,21] Other valuable compounds produced by *Y. lipolytica* include polyols like erythritol, mannitol, and arabitol, in response to osmotic stress.[22,23] Polyols can be used as a sucrose substitute in food formulations due to their sweetening properties or utilized in the pharmaceutical industry as an excipient in formulations. The global market of polyols reached EUR 5.15 billion in 2019, and an increase in polyol production at an annual growth rate of 6.1% until 2027 is predicted.[24]

Several studies have proven *Y. lipolytica* to be a cell factory for the production of valuable compounds; however, most of the practical applications of these compounds produced by *Y. lipolytica* remain in the exploratory stage. Few authors effectively produced biodiesel from lipids accumulated by yeast *Y. lipolytica*, and lignocellulosic biomass (sugarcane bagasse) and waste cooking oil were substrates used to produce lipids.[7,25,26] Therefore, the main aim of this work was to produce microbial lipids by using *Y. lipolytica* NCYC 2904 from low-cost substrates, crude glycerol, and VFAs, to be used as feedstock for biodiesel production. Fed-batch and two-stage batch cultures (TSC) of *Y. lipolytica* were carried out in a stirred tank lab-scale bioreactor for lipids production. Microbial lipids produced in fed-batch cultures were extracted, followed by conversion into biodiesel *via* alkaline catalytic transesterification. To assess the quality of biodiesel, a characterization by FTIR and an analysis of biodiesel properties in comparison to EU biodiesel standards were conducted. Citric acid and polyols production was also evaluated in all *Y. lipolytica* NCYC 2904 cultures.

Experimental

Yeast strain and inoculum preparation

Wild-type *Yarrowia lipolytica* NCYC 2904, previously grown in a YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} glucose, and 20 g L^{-1} peptone), was maintained in a YPD medium and 20% (v/v) pure glycerol at – 80 °C. One cryo-stock (10^8 cells per mL) was inoculated in a YPD medium for the inoculum cultures and incubated in an orbital shaker at 27 °C and 170 rpm. After growing for 15 h, yeast cells (80 mL) were harvested through centrifugation and resuspended in the culture medium at an initial cell density of 0.5 g L^{-1}.

Bioreactor experiments

All experiments were performed in a 2 L stirred tank bioreactor (BIOSTAT® B Plus, Sartorius) at 27 °C with a specific airflow rate of 1 vvm and an agitation rate of 400 rpm. The culture pH was measured with a pH electrode (InPro3100/225/Pt100 type, Metter Toledo) and kept at 6.0 ± 0.5 by the addition of NaOH 2 M or HCl 2 M. An optical probe (InPro6860i/12/220/nA, Metter Toledo) was used to measure the dissolved oxygen concentration in the culture medium.

Y. lipolytica growth and metabolite production were evaluated in fed-batch and TSC cultures. Fed-batch experiments started with a batch culture with an initial working volume of 700 mL (about 50% of the final volume) of medium containing 20 g L^{-1} crude glycerol, 0.5 g L^{-1} corn steep liquor, and 0.5 g L^{-1} of ammonium sulfate (C/N mass ratio of 75). After 48 h of batch culture, a glycerol-concentrated medium (100 g L^{-1} crude glycerol, 2.5 g L^{-1} corn steep liquor, and 2.5 g L^{-1} of ammonium sulfate) was fed to the bioreactor by using a peristaltic pump using two constant feeding flow rates: (1) 4.96 mL h^{-1}, where the dilution rate ranged from 0.005 h^{-1} to 0.003 h^{-1} (FB-A); and (2) 3.10 mL h^{-1}, where the dilution rate ranged from 0.004 h^{-1} to 0.002 h^{-1} (FB-B). After 144 h (FB-A) or 202 h (FB-B) of the fed-batch phase, the culture mode was switched to the batch mode, which lasted for 72 h. In FB-B experiments, a pulse of an 18 g L^{-1} pure VFAs mixture (6 g L^{-1} acetate, 6 g L^{-1} propionate, and 6 g L^{-1} butyrate) was added to the culture at the end of the fed-batch phase (250 h of cultivation). The selection of the VFAs mixture (composition and concentration) was based on our previous studies with food-waste-derived VFAs[8] and synthetic VFAs.[28]

A TSC culture was also carried out in the bioreactor, filled with 1.3 L of culture media composed of 60 g L^{-1} crude glycerol, 1.5 g L^{-1} corn steep liquor, and 1.5 g L^{-1} of ammonium sulfate (C/N mass ratio of 75) – first stage. The initial concentration of crude glycerol corresponds to the glycerol consumed by the yeast culture in the FB-B experiments. After 192 h of cultivation, corresponding to the final part of the exponential growth phase, a pulse of an 18 g L^{-1} pure VFAs mixture (6 g L^{-1} acetate, 6 g L^{-1} propionate, and 6 g L^{-1} butyrate) was added to the medium – second stage.

Table 1 summarizes the principal experimental conditions of TSC and fed-batch cultures carried out in the stirred tank bioreactor.

Crude glycerol, provided by Prio Energy – Prio Biocombustíveis, SA, was composed (w/w) of 82% glycerol, 12% water, 4.7% NaCl, 0.5% organic matter (not glycerol) and less than 0.01% methanol.

The biomass yield ($Y_{X/S}$, g g^{-1}) was determined according to eqn (1), where X_f is the final biomass concentration, X_i is the initial biomass concentration, S_i is the initial substrate concentration and S_f is the substrate concentration at the end of the process.

$$Y_{X/S} = \frac{X_f - X_i}{S_i - S_f} \quad (1)$$

Analytical methods

Culture samples were taken at regular intervals to measure biomass, glycerol, VFAs, citric acid, and polyols concentration and analyze lipids content and long-chain fatty acid composition. The biomass concentration was quantified by using the optical density (OD) of cultures at 600 nm and converted to cell

Table 1 Summary of experimental conditions of two-stage batch (TSC) and fed-batch (FB) cultures carried out in a 2 L stirred tank bioreactor

	FB-A	FB-B	TSC
1st batch (h)	48	48	192
Feeding phase (h)	144	202	—
VFAs addition (h)	—	250	192
2nd batch (h)	72	72	72
Feeding rate (mL h^{-1})	4.96	3.10	—
Dilution rate (h^{-1})	0.005–0.003	0.004–0.002	—
Initial working volume (L)	0.7	0.7	1.3
Initial medium	20 g L^{-1} crude glycerol, 0.5 g L^{-1} corn steep liquor, and 0.5 g L^{-1} ammonium sulfate (C/N mass ratio of 75)		60 g L^{-1} crude glycerol, 1.5 g L^{-1} corn steep liquor, and 1.5 g L^{-1} ammonium sulfate (C/N mass ratio of 75)
Feeding medium	100 g L^{-1} crude glycerol, 2.5 g L^{-1} corn steep liquor, and 2.5 g L^{-1} ammonium sulfate		—

dry weight (g L^{-1}) by using a calibration curve (OD = 0.389 × cell dry weight (g L^{-1})).

Glycerol, VFAs (acetate, propionate, and butyrate), citric acid, and polyols (erythritol, mannitol, and arabitol) were quantified by high-performance liquid chromatography using an Aminex HPX-87H column (300 mm × 7.8 mm, 8 μm particle size) coupled to an ultra-violet (UV, λ = 210 nm) and a refractive index (RI) detector. The column was eluted with 5 mM sulfuric acid at 60 °C and a 0.5 mL min^{-1} flow rate.

Microbial lipids were measured by the colorimetric phospho-vanillin method in lyophilized cells according to Lopes *et al.* (2018). The results were expressed as lipids content (ratio of the lipids concentration obtained by using the calibration curve/concentration of biomass used to perform the method) and lipids concentration (lipids content × biomass concentration in the cultivation medium).

The fatty acids synthesized by *Y. lipolytica* were determined in lyophilized cells after extraction with chloroform, followed by methylation with a mixture of methanol and sulfuric acid (85 : 15, v/v) for conversion of fatty acids into their methyl esters (FAMEs), which were analyzed by gas chromatography.[11]

Biodiesel production and characterization

Microbial lipids were extracted from *Y. lipolytica* cells with an automatic Soxhlet extraction system (Soxtec™ 8000, FOSS, Denmark) using petroleum ether as the solvent at 70 °C for 11 h. The lipids extracted were converted into biodiesel *via* an alkaline catalytic transesterification method using pure NaOH as a catalyst and pure methanol as a solvent. The transesterification reaction was performed at an oil:methanol molar ratio of 1 : 6, 1% (w/w) of the catalyst, and the mixture was incubated at 65 °C for 4 h. After incubation, the mixture was centrifuged at 5000 rpm for 10 min and the upper layer containing biodiesel was separated. The yield of biodiesel was calculated according to eqn (2):

$$\text{Biodiesel yield } (\%) = \frac{\text{mass of biodiesel (g)}}{\text{mass of lipids (g)}} \times 100 \quad (2)$$

A Fourier transform infra-red (FTIR) spectrometer (ALPHA II, Bruker, USA) was used to analyze the structure of extracted lipids of *Y. lipolytica* and biodiesel produced. The absorbance spectra were recorded in a wavenumber range of 4000 cm^{-1} to 400 cm^{-1} using an average of 64 scans per sample and with a spectral resolution of 4 cm^{-1}.

The physical and chemical properties of biodiesel (kinematic viscosity, density, degree of unsaturation, oxidation stability, cold filter plugging point, and cetane number) were estimated using the BiodieselAnalyzer© Ver. 2.2 software (available on **http://brteam.org/analysis/**)[27] using the composition of fatty acids.

Statistical analysis

The statistical analysis was carried out using Statgraphics Centurion XVI 16.2.04 software (Statpoint Technologies, Inc, Virginia, USA). Tukey's test was used to identify significant differences between means, and differences were considered statistically significant at $p < 0.05$.

Results and discussion

Microbial lipids production by *Y. lipolytica* NCYC 2904

Yarrowia lipolytica has a high potential for microbial lipids production[8,28] and, consequently, is a promising precursor of great importance for biodiesel production. This work evaluated fed-batch and TSC cultures of *Y. lipolytica* for microbial lipids production from crude glycerol and VFAs.

The *Y. lipolytica* cell mass variation with time was similar in all the culture modes tested (Fig. 1). Although a similar biomass concentration was obtained, the biomass yield attained during the feeding phase in FB-A (0.33 g g^{-1} ± 0.01 g g^{-1}) was higher than that observed in FB-B (0.24 g g^{-1} ± 0.01 g g^{-1}) since more glycerol was consumed in the last case. Similar to this, a biomass yield for glycerol of 0.29 g g^{-1} ± 0.001 g g^{-1} was obtained in the TSC experiments. At the end of the glycerol feeding in the FB-A culture, approximately 15 g L^{-1} of glycerol remained in the culture medium, which was further consumed by *Y. lipolytica* in the batch phase (Fig. 1A). By contrast, in the fed-batch experiments with the lowest glycerol feeding (FB-B),

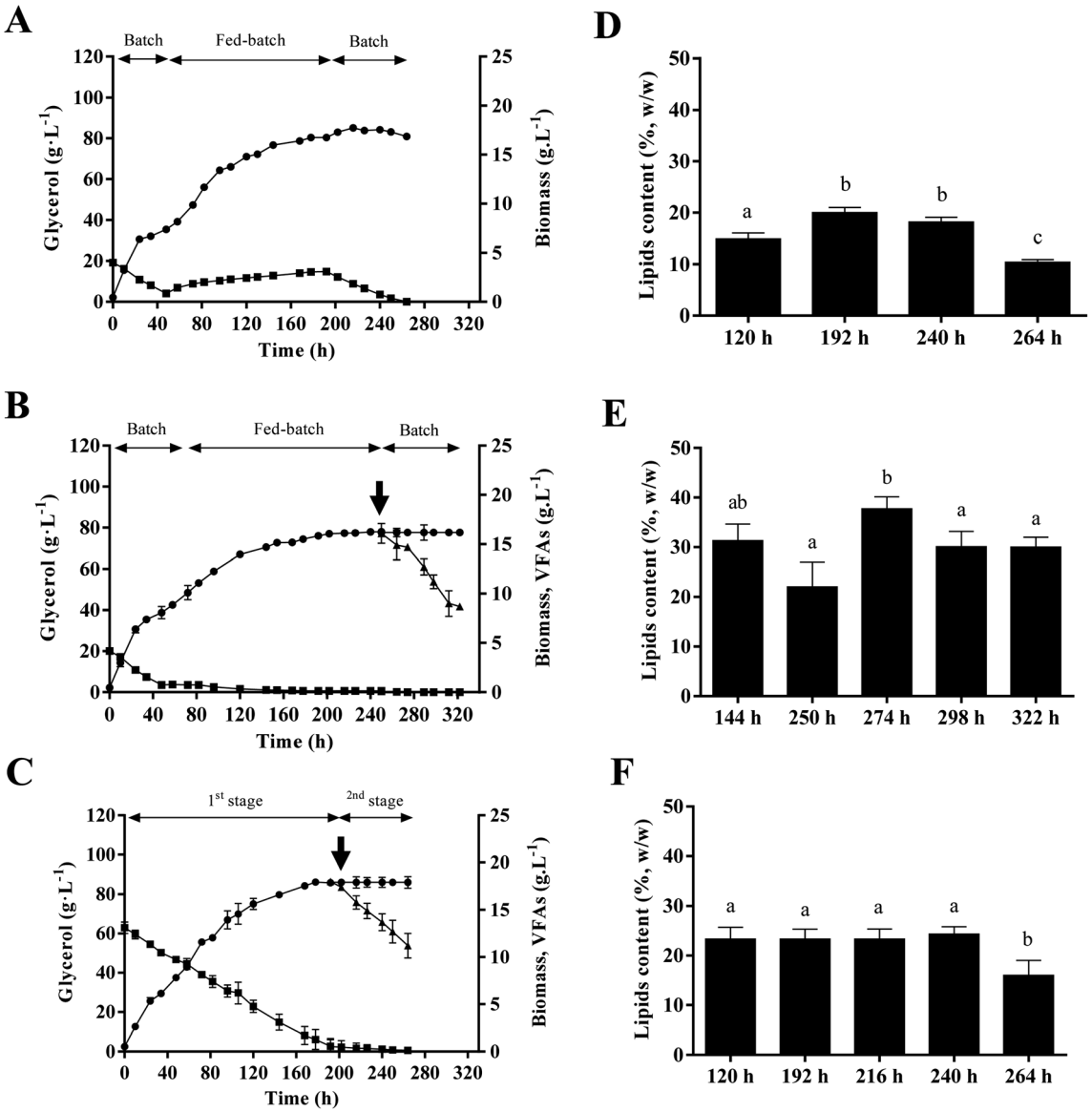

Fig. 1 Time course of biomass (●), glycerol (■), and total VFAs (▲) concentration obtained in *Y. lipolytica* cultures: FB-A (A), FB-B (B), and TSC (C). Microbial lipids content (%, w/w) of *Y. lipolytica* NCYC 2904 cells obtained in FB-A (D), FB-B (E), and TSC (F) cultures. The arrows indicate the addition of the VFAs mixture. The error bars represent the standard deviation of two independent replicates. Bars with the same letter are not statistically different ($p \geq 0.05$).

yeast cells consumed glycerol at the same rate as it was added and a residual concentration of glycerol remained in the medium (Fig. 1B). Crude glycerol took 192 h to be assimilated in the TSC culture, 58 h earlier than that observed in FB-B cultures. Moreover, it is worth noting that biomass yields reported herein are higher than others found in the literature with *Y. lipolytica* W29 (approximately 0.21 g g^{-1})[29] and A101 (0.21 g g^{-1})[30] strains growing in glycerol. A biomass yield of 0.32 g g^{-1} was obtained in *Y. lipolytica* W29 cultures using a mixture of VFAs or glucose and VFAs as substrates.[28] In our previous studies, despite the lower biomass concentration attained, a biomass yield of 0.48 g g^{-1} (mass of cells per mass of consumed substrate) was obtained for *Y. lipolytica* cultivated in 40 g L^{-1} crude glycerol under non-limiting oxygen conditions,[8] suggesting that the dissolved oxygen can be a limiting factor for biomass yield. Dissolved oxygen concentration remained close to zero during the feeding phases and the 1st phase of TSC. *Y. lipolytica* cells cultivated in a lower amount of dissolved oxygen could have a negative effect on cell growth since *Y. lipolytica* is a strictly aerobic yeast.[31,32]

Regardless of the operation mode that precedes VFAs addition, no significant differences were observed in VFAs uptake rates (0.11 g L^{-1} h^{-1} ± 0.01 g L^{-1} h^{-1} in FB-B and 0.10 g L^{-1} h^{-1} ± 0.04 g L^{-1} h^{-1} in TSC). Unlike what was observed in our previous work,[28] VFAs were not consumed by yeast after 72 h of cultivation (Fig. 1B and C). The total VFAs uptake rates obtained in this work are lower than those obtained in TSC (growth phase in glucose followed by an 18 g L^{-1} VFAs mixture).[28] Moreover, Pereira *et al.*[28] found that low dissolved oxygen concentration in

the culture medium had a negative effect on VFAs consumption by yeast, which could explain the low VFAs consumption observed in the current work. Despite the carbon sources being consumed, no cellular growth was observed in the batch phase (fed-batch experiments) and in 2nd stage of TSC (Fig. 1A–C). This could indicate that carbon sources were channeled toward cell maintenance or metabolite production instead of biomass production. A similar behavior was described in previous studies, in which a decrease in cell proliferation was observed after the addition of VFAs, being the carbon sources assimilated by yeast for lipids synthesis.[8,28]

Although no differences were observed in *Y. lipolytica* growth, microbial lipids production had different profiles, depending on the culture mode (Fig. 1D–F). In the case of the FB-A culture, lipids content increased and a 1.4-fold improvement was achieved after 192 h compared to 120 h. The amount of microbial lipids at the end of the experiment was half that obtained at the end of the feeding phase (192 h) (Fig. 1D). In the FB-B culture, no statistical differences were observed in microbial lipids production during the feeding phase. A 1.7-fold improvement in lipids content was obtained after 24 h of VFAs addition (274 h of cultivation). VFAs addition without an extra nitrogen source led to an increase in the C/N ratio, which could favor microbial lipids production. Although lipids content attained at the end of the experiments was 1.3 times lower than that attained at 274 h, it is worth noting that no statistical differences were observed at the end of the feeding phase (250 h) and the end of the FB-B culture (Fig. 1E). According to Poontawee and Limtong,[33] microbial lipids synthesis was enhanced at the lowest feeding rate. Although no differences were observed in lipids content at the end of the feeding phase, a 2-fold improvement in maximum lipids productivity was obtained by decreasing the substrate feeding rate from 0.5 g h^{-1} L^{-1} (FB-A) to 0.4 g h^{-1} L^{-1} (FB-B). In the TSC culture, no statistical differences were observed in lipids content until 240 h of cultivation, and mobilization of lipids was observed after 72 h of VFAs addition. A 1.5-fold decrease in lipids content was observed at the end of the experiments (Fig. 1F). Microbial lipids accumulation by *Y. lipolytica* obtained at the end of 1st stage (192 h) was similar to that reached at the end of the feeding phase in the FB-B culture, suggesting that microbial lipids production was modeled using glycerol concentration and not cultivation mode since the same amount of glycerol was consumed by yeast in both experiments. While no differences in maximum lipids productivity were observed in TSC and FB-B cultures, higher productivity was attained in TSC cultures relative to FB-A cultures. A mobilization of synthesized microbial lipids was observed in the batch phase of fed-batch experiments and 2nd stage of the TCS culture, even though all glycerol was consumed by yeast (FB-A) and a significant amount of VFAs remained unconsumed in the medium (FB-B and TSC) (Fig. 1). Besides producing microbial lipids, *Y. lipolytica* cultivated under nitrogen-limited conditions is also triggered towards the production of low-molecular-weight metabolites (citric acid or polyols).[34] Microbial lipids mobilization and secretion of low-molecular-weight metabolites were observed for *Y. lipolytica* ACA YC 5030 [35], ACA-DC 50109,[36] and FMCC Y_{75} [37] in glycerol-based media under nitrogen-limited conditions. The maximum lipids content of 38% (w/w) achieved in FB-B cultures was 1.9- and 1.6-fold higher than that attained in FB-A and TSC cultures, respectively. Moreover, the maximum lipids concentration obtained in FB-B cultures (6.1 g L^{-1}) was 1.8- and 1.4-fold higher than that in FB-A and TSC cultures, respectively. To the best of our knowledge, no studies have tested similar strategies for microbial lipids production from crude glycerol and VFAs; however, the maximum amount of lipids production in fed-batch cultures tested herein is comparable to or even higher than that attained in *Y. lipolytica* fed-batch cultures with a pulse of glycerol.[34,38] Furthermore, a similar lipids content was reported in *Y. lipolytica* batch cultures in crude glycerol.[8] Yet, the highest lipids concentration was obtained in this study, since cellular density was increased in fed-batch cultures.

Other added-value metabolites produced by *Y. lipolytica*

Simultaneously with microbial lipids, considerable amounts of citric acid and polyols (mannitol and arabitol) were produced by *Y. lipolytica* (Fig. 2). Glycerol assimilated by yeast cells is first phosphorylated to glycerol-3-phosphate by glycerol kinase and then oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. Dihydroxyacetone phosphate is converted into glycerol-3-glyceraldehyde, which can be used in gluconeogenesis (production of hexoses and polyols) or in glycolysis (production of microbial lipids and organic acids). In glycolysis, glycerol-3-glyceraldehyde is converted into pyruvic acid and then acetyl-CoA, a precursor of a variety of metabolites. Under low nitrogen concentration conditions, microbial lipids and/or citric acid are produced.[6,39] The simultaneous production of microbial lipids and citric acid by *Y. lipolytica* from glycerol was already reported in fed-batch cultures with a pulse of glycerol.[38] Citric acid production was 2 times higher in fed-batch experiments than in TSC cultures. Moreover, citric acid production at the end of glycerol feeding in the FB-B culture (250 h) was 1.7-fold higher than that attained in the FB-A culture (192 h) (Fig. 2A). Karamerou *et al.*[40] applied different glycerol feeding rates for microbial lipids production by *Rhodotorula glutinis*, demonstrating that an increasing feeding rate resulted in low citric acid production. *Y. lipolytica* cells still produced citric acid from glycerol in the batch phase of the FB-A culture simultaneously with lipids mobilization (Fig. 1D and 2A). In turn, a continuous citric acid increase was observed in FB-B and TSC cultures until VFAs addition, the moment that yeast cells stop producing citric acid. In TSC, the maximum acid citric production occurred at 192 h of cultivation (corresponding to the time that all glycerol was consumed), remaining at this value after VFAs addition (Fig. 2B). A similar behavior occurred in FB-B cultures where the maximum citric acid production was observed at the end of the feeding phase (250 h) and no further citric acid production was observed after VFAs addition (Fig. 2B).

Y. lipolytica also produced polyols (mainly mannitol and arabitol) from crude glycerol, particularly in TSC experiments (Fig. 2C). Though some studies reported the synthesis of erythritol from crude glycerol,[22,37] no erythritol production was

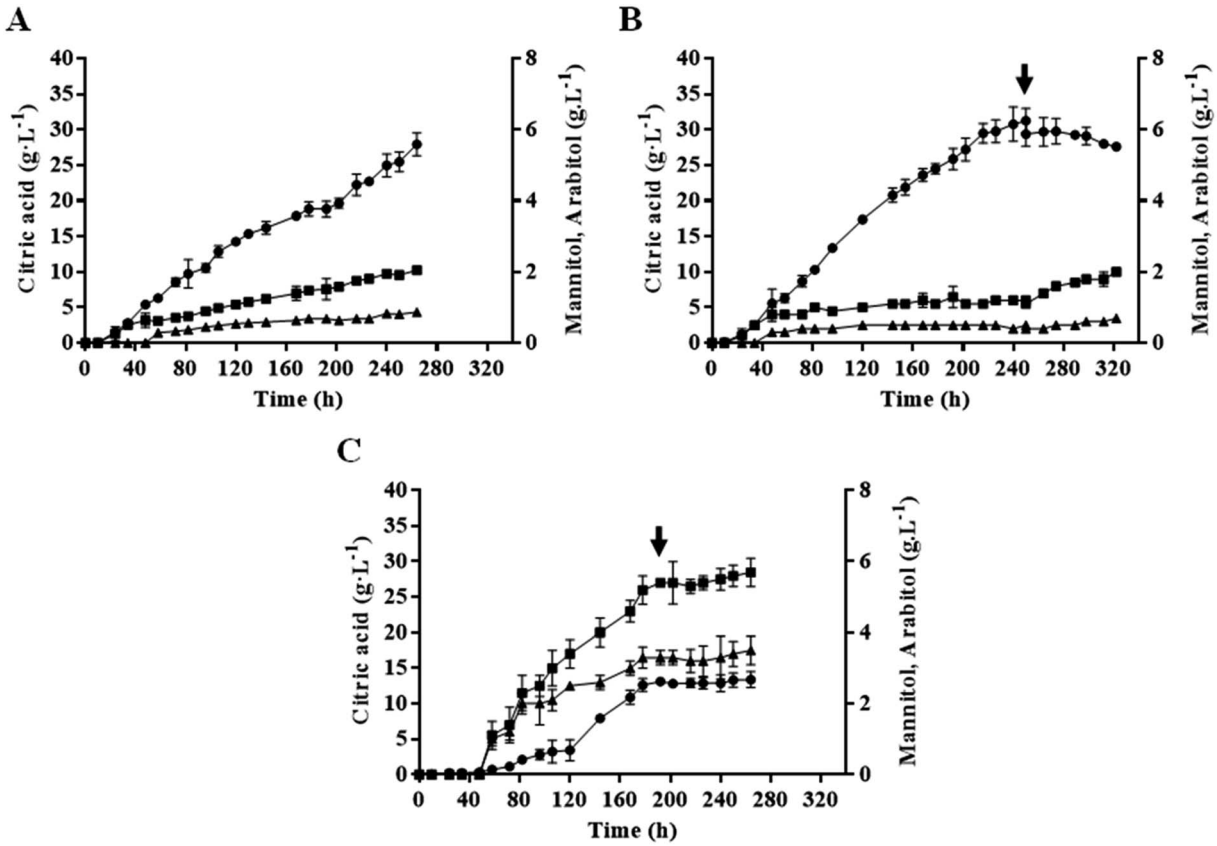

Fig. 2 Profiles of citric acid (●), mannitol (■), and arabitol (▲) concentration obtained in *Y. lipolytica* cultures: FB-A (A), FB-B (B), and TSC (C) cultures. The arrows indicate the addition of the VFAs mixture. The error bars represent the standard deviation of two independent replicates.

found in this work. Contrary to citric acid, the highest amount of mannitol and arabitol produced by *Y. lipolytica* was attained in TSC cultures. TSC cultures reached 2.8-fold higher mannitol concentration than fed-batch cultures. Additionally, arabitol concentration in TSC was 4- and 5-fold higher than that obtained in FB-A and FB-B cultures, respectively. The higher initial concentration of glycerol in the culture medium can increase osmotic pressure, which may explain the highest polyol production in TSC cultures. Mannitol was the main polyol in all cultures, whereas arabitol was produced in the lowest amounts. Although polyols production occurs mainly when cultures are carried out at low pH,[23] *Y. lipolytica* was able to produce polyols under the current work conditions. Similarly, it has been demonstrated that other *Y. lipolytica* strains cultivated under nitrogen limitation can produce polyols at medium pH values of 6.[34,35] Furthermore, mannitol was produced from VFAs, since a 1.7-fold increase in mannitol concentration was observed after VFAs addition in FB-B cultures (Fig. 2B). In TSC cultures, no polyols production was observed after VFAs addition, probably due to (a) metabolites other than those we measured being produced; (b) high substrate consumption for cellular maintenance; (c) the operation time, cell death is higher than cell proliferation and reproduction.

The current study shows the relevance of *Y. lipolytica* as a cell factory to convert crude glycerol and VFAs into bioproducts of industrial interest. *Y. lipolytica* has great potential for the production of a high amount of citric acid (which can be used in the food and beverage industry) along with lipids-rich biomass (used as feedstock for biodiesel production) and polyols (used as a sweetener), which reduces the production costs of each bioproduct, making these bioprocesses integrated within a biorefinery framework. Moreover, depending on the needs of the market, different routes may be selected by changing operating modes, such as fed-batch operation mode to produce citric acid or batch cultures to produce mannitol.

Biodiesel production

Microbial lipids produced at the end of *Y. lipolytica* cultures were mainly composed of unsaturated fatty acids (>84%), whose composition was similar to that of vegetable oils, traditionally used as raw feedstock for biodiesel production. Oleic acid (C18:1) was the main fatty acid produced, followed by palmitoleic acid (C16:1). Linoleic (C18:2), palmitic (C16:0), and stearic (C18:0) acids were also synthesized in low quantities (Table 2). As in other studies using VFAs, heptadecanoic acid (C17:1) was synthesized owing to the addition of propionate into the culture medium.[28] Moreover, a slight difference in the relative percentage of each fatty acid was observed between FB-B and batch cultures. While in FB-B cultures *Y. lipolytica* produced more linoleic acid, batch cultures led to a decrease in linoleic and oleic acids and an increase in palmitic acid contents.

Table 2 Fatty acids composition of microbial lipids produced by *Y. lipolytica* under different cultivation modes in a stirred tank bioreactor[a]

	Relative fatty acid content (%)					
Y. lipolytica culture	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2
FB-A	7.6 ± 0.1[a]	18.47 ± 0.01[a]	—	3.8 ± 0.1[ab]	62.6 ± 0.2[a]	7.51 ± 0.02[a]
FB-B	6.4 ± 0.5[a]	15.8 ± 0.8[a]	2.3 ± 0.3[a]	3.3 ± 0.5[a]	60.9 ± 0.5[a]	11.3 ± 0.2[b]
TSC	10 ± 1[b]	17 ± 2[a]	2.1 ± 0.2[a]	6 ± 1[b]	58 ± 2[b]	7.4 ± 0.8[a]

[a] Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Similar fatty acid profiles were found by using other *Y. lipolytica* strains grown in pure or crude glycerol,[34,35] VFAs,[41,42] or hydrophobic substrates.[10,43]

Y. lipolytica lipids produced in FB-B cultures (the condition where a higher amount of microbial lipids was obtained) were extracted and then subjected to an alkaline catalytic transesterification process resulting in 95% biodiesel yield (Fig. 3).

The structural characterization of *Y. lipolytica* lipids extracted from cells growing in the FB-B culture and biodiesel produced from microbial lipids was visualized using FTIR spectra (Fig. 4). Additionally, FTIR analysis of vegetable oil (commercial oil used for food purposes) and biodiesel produced from vegetable oil was carried out for comparison purposes. FTIR spectra of *Y. lipolytica* lipids and vegetable oil showed similar characteristics, although with small differences in spectral bands and absorbance (Fig. 4A and B). In *Y. lipolytica* lipids, the different lipid functional groups were represented by the strong peaks at 1457.5 cm^{-1} (C–H bond of the methyl group), 1744.1 cm^{-1} (stretching vibration of the ester carbonyl bond C=O), and 2855.3 cm^{-1} and 2925.4 cm^{-1} (asymmetric and symmetric stretching vibrations for C–H saturated carbon chains).[44] It should be noted that the FTIR spectrum of vegetable oil also showed the presence of bands at 1459.6 cm^{-1}, 1744.1 cm^{-1}, 2853.2 cm^{-1}, and 2923.3 cm^{-1}. Similar spectral peaks for microbial lipids extracted from *Y. lipolytica* as a potential feedstock for biodiesel production were also reported in other studies performed by Mitrea *et al.*,[45] Radha *et al.*,[44] and Vasaki *et al.*[7]

The FTIR spectrum of methyl esters in *Y. lipolytica* lipids has shown peaks at 2855.3 cm^{-1} and 2927.3 cm^{-1}, corresponding to sp^2 C–H and sp^3 C–H stretching, respectively. At 1740 cm^{-1}, the spectral peak represents the C=O stretch in the ester group as observed in the FTIR spectrum of *Y. lipolytica* lipids. The main change from the ester group to methyl ester occurred in the region of 400–1500 cm^{-1} (Fig. 4C). The broad and strong peak at 1096.8 cm^{-1} in the FTIR spectrum of microbial lipids (Fig. 4A) was split into two peaks at 1030.8 cm^{-1} and 1098.8 cm^{-1} in FTIR spectra of biodiesel (Fig. 4C), which represents C–C asymmetric vibrations in aliphatic esters. Another middling absorbance signal at 3007.9 cm^{-1} was also found. These results are in

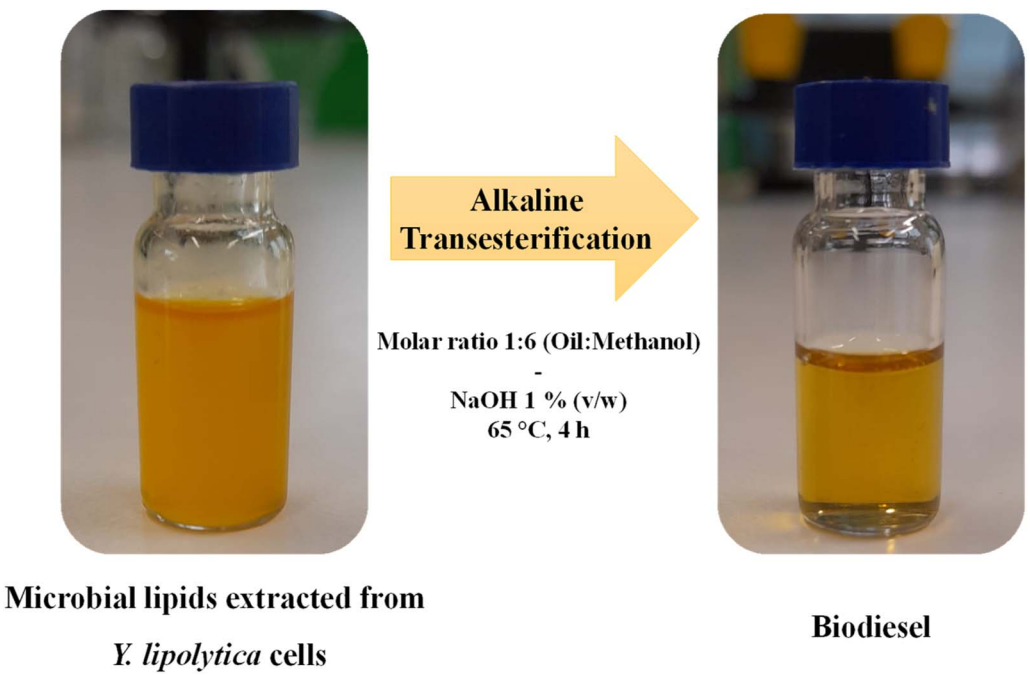

Fig. 3 Microbial lipids extracted from *Y. lipolytica* cells and biodiesel produced *via* alkaline catalytic transesterification of microbial lipids.

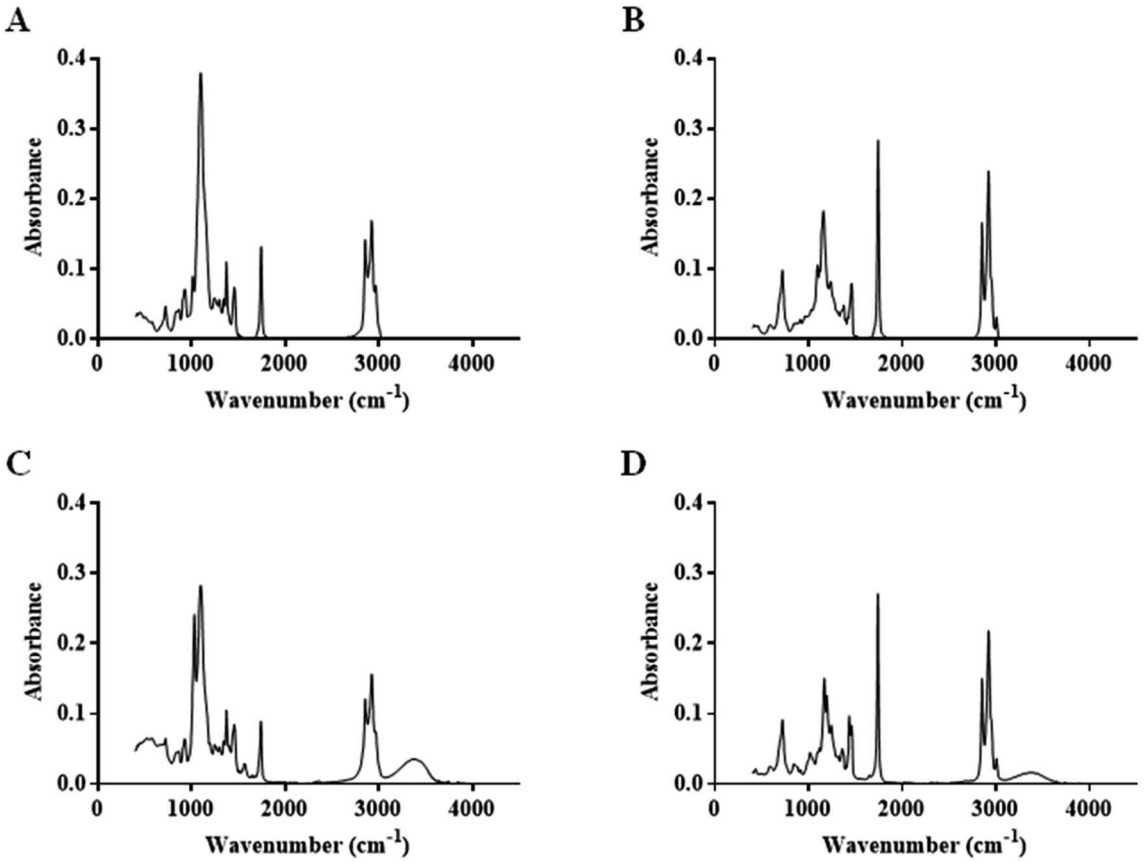

Fig. 4 FTIR spectrum of microbial lipids derived from *Y. lipolytica* cells in the FB-B culture (A) and FTIR spectrum of vegetable oil (B). FTIR spectrum of biodiesel obtained by the alkaline catalytic transesterification of *Y. lipolytica* lipids (C) and vegetable oil (D).

accordance with those of other studies that analyzed the functional groups corresponding to methyl groups in biodiesel samples for confirming the occurrence of fatty acid methyl esters.[46,47] Additionally, the spectral analysis of methyl esters derived from transesterified vegetable oil exhibited similar characteristics to those observed in biodiesel obtained by transesterification of *Y. lipolytica* lipids.

The potential of *Y. lipolytica* biodiesel to replace conventional fuel was evaluated to ensure that the biodiesel produced meets the criteria set up by international biodiesel standards EN 14214. The fatty acid composition of *Y. lipolytica* lipids was used to estimate several properties using the BiodieselAnalyzer© software. Table 3 shows the main parameters used to assess the biodiesel quality and compared it with those of biodiesel produced from soybean oil (one of the most common vegetable oils used as feedstock in biodiesel production) and the international biodiesel standard.

The properties of biodiesel produced from *Y. lipolytica* lipids agree with the specifications of the EU biodiesel standard EN 14214 and demonstrate the high quality of the biodiesel produced (Table 3). Furthermore, the biodiesel properties (density, kinematic viscosity, and cetane number) obtained from *Y. lipolytica* lipids showed high similarity to those of biodiesel produced from soybean oil. However, the value estimated for oxidation stability (property to evaluate storage behavior) is above the EU standard minimum thresholds and is higher than

Table 3 Properties of biodiesel obtained from *Y. lipolytica* lipids and soybean oil estimated by BiodieselAnalyzer© software and the EU biodiesel standard EN 14214[a]

Properties	*Biodiesel from Y. lipolytica* lipids	Biodiesel from soybean oil[48]	EU biodiesel standard EN 14214
Density (kg m^{-3})	856	882	860–900
Kinematic viscosity (mm^2 s^{-1})	3.71	4.37	3.5–5.0
Cetane number	53.31	51	47 min
Oxidation stability (h)	13.03	1.5	6 h min
Cold filter plugging point (°C)	−9.28	−6	NR*

[a] NR – not reported; NR* – cold filter plugging point limits depend on geography and time of the year; min – minimum; max – maximum; h – hours.

that obtained in biodiesel from vegetable oil. The cold filter plugging point estimated in this work is slightly lower than that obtained in biodiesel produced from soybean oil and lower than those obtained with *Y. lipolytica* lipids produced in other studies.[28,42] Although there is no specific recommendation in the EU standards, the lower the cold filter plugging point value, the better the cold flow properties of the biodiesel. Hence, *Y. lipolytica* lipids synthesized from crude glycerol and VFAs in FB-B cultures have the potential to be used as feedstock for the production of high-quality biodiesel.

Conclusions

The results obtained in this work are quite promising since they promote a model of circular economy, closing the loop from crude glycerol to biodiesel. Crude glycerol, the main by-product generated during biodiesel production, and VFAs were used as renewable and low-cost feedstocks for the production of microbial lipids and other metabolites of industrial interest (citric acid and polyols) by using the oleaginous yeast *Y. lipolytica* NCYC 2904. A fed-batch culture with a crude glycerol feed at the lowest feeding rate, followed by the addition of a VFAs mixture, resulted in a high amount of microbial lipids rich in oleic acid, which were used to produce biodiesel with a 95% conversion yield. The estimated biodiesel properties demonstrated that biodiesel obtained by transesterification of *Y. lipolytica* lipids can be used as high-quality biofuel. Nevertheless, *in situ* transesterification for the direct conversion of yeast biomass to biodiesel could be tested in future studies since the combination of lipids extraction and transesterification in a single step reduces the overall cost of this bioprocess.

Author contributions

Ana S. Pereira: investigation, methodology, formal analysis, writing-original draft. Marlene Lopes: conceptualization, supervision, validation, writing-review & editing. Isabel Belo: conceptualization, supervision, validation, writing-review & editing, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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