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Two-stage syngas fermentation into microbial oils and β-carotene with *Clostridium carboxidivorans* and engineered *Yarrowia lipolytica*

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ABSTRACT

An engineered *Yarrowia lipolytica* strain was able to simultaneously accumulate microbial oils and β-carotene from both acetic and butyric acids produced from syngas fermentation by *Clostridium carboxidivorans*, through the Wood-Ljungdahl pathway, with CO and CO_2 as carbon sources. It was first determined that *Y. lipolytica* showed no inhibition at concentrations < 16 g/L of a mixture of VFAs (Volatile Fatty Acids) containing acetic, butyric, and even hexanoic acids. The yeast was then grown in a bioreactor with a culture medium derived from acetogenic syngas (CO, CO_2, H_2) fermentation containing all three acids. *Y. lipolytica* consumed a total of 91 g/L VFAs, progressively supplied through eight successive additions of these acids to the reactor. A maximum lipid content of 36.18% g/g cell was achieved at 61 g/L VFAs consumed and with an airflow rate of 2.0 vvm. Besides, a total of 88.4 mg/g cell and 759 mg/L β-carotene content and concentration were achieved at about 80 g/L VFAs consumed and with a 2.0 vvm airflow rate. It was concluded that; a non-limiting air flow favors the metabolic pathway for β-carotene formation against lipid accumulation.

1. Introduction

Clostridium carboxidivorans is known to produce acetic acid as its primary fermentation product under optimal conditions. However, depending on aspects such as substrate concentration, pH fluctuations, or the presence of specific cofactors, it can alter its metabolic pathway to also produce butyric and hexanoic acids [1]. This metabolic flexibility offers the possibility to generate a mixture of VFAs. Some other studies also reported the production of alcohols with this strain if forced to perform solventogenesis, which is mainly achieved by reducing the pH of the medium down to sub-optimal growth levels [2]. This microorganism can grow chemoorganotrophically with different sugars, as well as autotrophically with gases such as CO_2/H_2, CO, and syngas through the Wood-Ljungdahl pathway [3]. This study seeks to maintain acidogenic conditions for *C. carboxidivorans* to produce VFAs using syngas as substrate. The selection of this organism for the gas bioconversion stage was driven by its ability to generate this diverse mixture of VFAs, providing an enriched substrate for the following steps.

On the other hand, microorganisms, such as specific yeasts, algae, or bacteria, that can store more than 20% lipids (microbial oils) in their biomass are considered oleaginous ones. It was first proposed, in the 1870 s, to use these oleaginous microorganisms to synthesize oils and fats [4]. However, because of the high cost of the feedstocks, the commercialization of microbial lipids remains still largely in development stage and cannot yet fully compete with petrochemical processes [5]. The cost of glucose as a raw material is around $400–500 per ton [6,7], while it is less than $100 per ton for volatile fatty acids [8], which is about five times cheaper. Therefore, looking for affordable, plentiful, and environmentally benign feedstocks is still vital for the synthesis of microbial lipids on a large scale.

Moreover, media based on VFAs have a particular advantage compared to typical biorefinery sugar substrates as they do not require complex, costly, hydrolytic pre-treatment [9]. Additionally, VFAs have the potential to be efficiently utilized for microbial lipid accumulation, compared to sugars, since they can directly be converted into acetyl-CoA, a crucial precursor for the synthesis of lipids in the

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cytoplasm via shorter metabolic pathways [10]. In addition to the above advantages, VFAs can be obtained by acetogenesis, which makes it an ideal and sustainable substrate because it can be bio-produced from gaseous mixtures or pollutants such as carbon dioxide and syngas. However, these acids can be toxic or even lethal, above a threshold concentration, for specific oleaginous yeasts, which is why some show better results than others. Therefore, it is essential to accurately identify the threshold concentration that a given yeast could tolerate, together with its optimal culture conditions, in order to reach reasonable lipid production.

The accumulation of carotenoids in oleaginous yeasts is a more recent field of research than that of lipids. Historically, carotenoids, particularly β-carotene, have been extracted from vegetable sources like carrots, tomatoes, grapes, and annatto. Chemical routes have also been explored, often with the drawback of producing hazardous waste [11]. Conversely, the method proposed in this research, utilizing a gas bioconversion process, represents a pioneering approach. Even though chemically produced carotenoids still dominate the market, this is progressively changing. Given the interest in environmentally-friendly and sustainable engineering approaches, increasing efforts are being put into the search for natural sources and carotenoids production techniques [12]. Certain oleaginous yeasts have the potential to produce carotenoids, such as β-carotene. The most studied native yeasts in this respect are *Rhodosporidium* spp., *Rhodotorula* spp., *Xanthophylomyces* spp., and *Sporobolomyces* spp. [13]. Advances in genetic engineering of the yeast *Y. lipolytica* have also converted it into a notable candidate to compete with these native yeasts, as also demonstrated in this manuscript.

The present research investigated the ability of *C. carboxidivorans* and the modified oleaginous yeast *Y. lipolytica* to jointly produce microbial oils (lipids) and β-carotene from a syngas mixture. A two-stage system was designed, where CO, CO_2, H_2, N_2 gas mixtures are first fed to an anaerobic reactor for their conversion into VFAs. The acids obtained in the first stage then become the substrates for the second stage, in a yeast-inoculated aerobic reactor, to finally produce the desired end products. The scarce, most comparable, configuration in the literature used acetic acid only, as single substrate, to produce a single end product, i.e., lipids [14,15]. The transformation of gases, especially syngas, into VFA mixtures and then directly into valuable compounds such as β-carotene, has not yet been documented, highlighting the novelty and potential game-changing nature of our methodology.

2. Material and methods

2.1. Gas fermentation stage

2.1.1. Strains and media

C. carboxidivorans P7 DSMZ 15243 was obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (Braunschweig, Germany). It was maintained on the medium described by the company from which the strain was purchased.

For batch cultures, *C. carboxidivorans* was grown in bottles with a total volume of 100 mL containing 40 mL medium with the following composition (per liter distilled water): yeast extract (YE), 1 g; NaCl, 2 g; NH_4Cl, 2.5 g; KCl, 0.25 g; KH_2PO_4, 0.25 g; $MgSO_4$ x $7H_2O$, 0.50 g; $CaCl_2$ x $2H_2O$, 0.1 g; cysteine−HCl, 0.6 g; Na-resazurin (0.1% w/v), 1 mL; trace elements solution, 10 mL; vitamins solution, 10 mL. The trace metals solution had the following composition (per liter distilled water): nitriloacetic acid, 3.50 g; $MgSO_4$ x $7H_2O$, 6.00 g; $MnSO_4$ x H_2O, 1.50 g; $(NH_4)_2Fe(SO_4)_2$ x $6H_2O$, 0.90 g; $CoCl_2$ x $6H_2O$, 0.40 g; $ZnSO_4$ x $7H_2O$, 0.38 g; $CuCl_2$ x 2 H_2O, 0.04 g; $NiCl_2$ x $6H_2O$, 0.05 g; Na_2SeO_4, 0.3 g; Na_2MoO_4 x $2H_2O$, 0.03 g; $FeSO_4$ x $7H_2O$, 0.2 g; $CoSO_4$ x 7 H_2O, 0.36 g; $CuSO_4$ x $5H_2O$, 0.02 g; $KAl(SO_4)_2$ x $12H_2O$, 0.04 g; H_3BO_3, 0.02 g. The vitamins solution had the following composition (per liter distilled water): biotin, 2.00 mg; folic acid, 2.00 mg; pyridoxine-HCl, 10.00 mg; thiamine-HCl, 5.00 mg; riboflavin, 5.00 mg; nicotinic acid, 5.00 mg; Ca-D-pantothenate, 5.00 mg; vitamin B_{12}, 0.10 mg; *p*-aminobenzoic acid, 5.00 mg; (±)-α-lipoic acid, 5.00 mg. The culture medium was flushed with pure N_2 for three minutes to ensure anaerobic conditions and with CO as substrate for another three minutes, and the pH of the culture medium was adjusted between 5.5 and 6.0. The bottles were then autoclaved for 20 min at 120 ºC. Then, they were placed in a thermostated room at 33 ºC and agitated at 150 rpm on an orbital shaker for 2–3 days.

2.1.2. Continuous gas-fed bioreactor studies

Studies in Continuously Stirred Tank Reactors (CSTR) were carried out in 2 L BIOFLO 120 fermenters (Eppendorf, Germany). The reactors were filled with 1.5 L culture medium, with the following composition (per liter distilled water): yeast extract (YE), 2.30 g; NH_4Cl, 1.37 g; KCl, 0.125 g; KH_2PO_4, 1.175 g; K_2HPO_4, 5.07 g; $NaHCO_3$, 6.00 g; $MgSO_4$ x $7H_2O$, 0.46 g; $CaCl_2$ x $2H_2O$, 0.05 g; NaCl, 1 g; cysteine-HCl, 0.48 g; Na_2S x $9H_2O$, 0.54 g; Na-resazurin (0.1% w/v), 0.75 mL; trace elements solution, 5 mL; vitamins solution, 4 mL. To avoid any possible contamination, the bioreactor with the medium was autoclaved at 120 ºC for 20 min. In this case, cysteine-HCl and Na_2S were added after autoclaving to avoid potential undesired reactions at high temperatures. Then, the medium was flushed for about two hours with pure N_2 to ensure anaerobic conditions, and after that, N_2 was replaced by a gas mixture containing $CO:CO_2:H_2:N_2$ (30:10:20:40). For gas feed to the reactor, the gas flowrate was kept constant at 10 mL/min, using a GFC gas-mass flow controller from Aalborg company. The bioreactor was maintained at a constant temperature of 33 °C using the heating blanket system of the BIOFLO 120 fermenter and with continuous agitation at 250 rpm. It was inoculated with the actively growing culture explained in the previous section, and was seeded under aseptic conditions. The pH of the culture broth was maintained at a constant value of 6.2, suitable for acetogenesis, by adding either 1 M HCl or 1 M NaOH solutions with the aid of peristaltic pumps.

2.2. Lipids and carotenoids production stage

2.2.1. Engineered yeast strain and batch toxicity assays

The genetically modified *Y. lipolytica* species, labeled JMY6862 (ob-CHCTEFCTEF) and deposited at the French “Collection Nationale de Culture de Microorganismes – Institut Pasteur”, was constructed in accordance with the methods specified in Larroude and colleagues [16]. In essence, JMY6862 is an offspring of the "Obese" strain, JMY3501, which is referred to in Lazar Z et al. [17]. This modification entailed the step-by-step incorporation of three separate beta-carotenoid expression cassettes, as outlined in the 2017 publication by Celinska and colleagues [18]. The strain was first grown on Yeast Extract-Peptone-Dextrose (YPD) agar plates to perform batch experiments, for 2–3 days. The next step was to transfer a single colony to 100 mL YPD broth contained in 250 mL flasks and culture it in a liquid medium for 24 h at 30 °C, with a constant agitation speed of 150 rpm.

This culture was then used as inoculum to carry out the batch toxicity assays. These were carried out in 250 mL flasks, in a medium composed of 50 mL of (per liter distilled water): YE, 2.30 g; NH_4Cl, 0.275 g; KCl, 0.125 g; KH_2PO4, 1. 17 g; K_2HPO_4, 5.07 g; $NaHCO_3$, 6.00 g; $MgSO_4$ x 7 H_2O, 0.46 g; $CaCl_2$ x 2 H_2O, 0.05; NaCl, 1 g; cysteine-HCl, 0.48 g; Na_2S x 9 H_2O, 0.54 g. Different amounts of the mixture of acids were added to each bottle in order to assess the potential toxicity of the different acids on the yeast. The pH was adjusted to 6, and the bottles were autoclaved at 120 °C for 20 min. They were then inoculated to reach an initial optical density ($OD_{600\ nm}$) of 0.5. To achieve this density, the equivalent volume of 1 mL culture with an $OD_{600\ nm}$ of 0.5 was scaled up proportionally for 50 mL, ensuring that the overall inoculation density remained consistent. In order not to carry over nutrients from the YPD medium, the seed cultures were centrifuged at 4000 rpm for 10 min. The pellets were washed with a 0.9% NaCl solution and resuspended in the bottles with the autoclaved medium. These were kept in a thermostated room at 33 °C and shaken at 150 rpm on an orbital shaker.

2.2.2. Continuous air-fed bioreactor

Two air-fed bioreactor experiments were carried out with *Y. lipolytica*, grown in a medium rich in VFAs previously produced by *C. carboxidivorans* from syngas. The temperature was 30 °C, pH 6.0, volume 1 L, agitation speed 250 rpm, and C/N ratio of 25, a value determined based on our preliminary studies that indicated very good growth and VFA utilization rates of *Y. lipolytica* at this particular ratio. In this context, 'N' represents the total ammonium content remaining from the medium obtained after the previous anaerobic fermentation. The initial concentration of total VFAs was 11.9 g/L, with several additions of VFAs, later on, throughout each experiment. The difference between the two experiments lies in the airflow fed to the reactor, being 0.5 vvm in one case and 2.0 vvm in the other experiment. The bioreactors were inoculated with an $OD_{600\ nm}$ of 1 of *Y. lipolytica*, following the same cultivation and washing procedure as described for the batch experiments.

2.3. Analytical methods

2.3.1. Determination of biomass

Spectrophotometry was used to estimate the biomass concentrations and plot the different growth curves. A distilled water blank was used to measure the optical density of a 1 mL sample at a wavelength of 600 nm.

2.3.2. VFAs analyses

To quantify the amount of VFAs present in the culture media, high-performance liquid chromatography (HPLC) (HP1100, Agilent Co., USA) was used. The HPLC was equipped with a diode array detector and a refractive index detector maintained at 50 ºC. A 0.005 M sulfuric acid solution, with a flow rate of 0.80 mL/min, was used as the mobile phase. These detectors and methodology also facilitate the quantification of alcohol production in the event that production would occur.

To monitor acids production and consumption, 1 mL samples were regularly withdrawn from the batch or bioreactor experiments. Samples were centrifuged for 5 min at 7000 rpm, the supernatant was filtered through a 0.22 μm PTFE filter, and HPLC analyses were carried out by injecting 20 μL on the Agilent Hi-Plex H column (300 × 7.7 mm) at 45 ºC.

2.3.3. Determination of lipids content

For lipids (and carotenoid) analyses, samples were taken and centrifuged for 10 min at 4000 rpm, the supernatant was discarded, and 1 mL of distilled water was added. These samples were kept in the freezer until analysis.

From 10–30 mg of freeze-dried biomass, the accumulated total lipids are extracted with a solution of methanol and sulfuric acid (40:1). A gas chromatograph (GC) (Thermo Fisher) was used to quantify the fatty acid methyl esters (FAMEs) formed. It was equipped with a flame ionization detector (FID) and an Agilent vf-23 ms column (60 m × 0,25 mm × 0,25 μm). Helium was used as the carrier gas. The initial oven temperature was 120 ºC, which was held constant for 1 min and then increased by 25 ºC/min to 200 ºC and then increased by 4 ºC/min to 230 ºC and held for another minute. Identification of FAMEs was made by comparison with standard solutions. Besides, a standard internal method was used to quantify lipids by adding 25 mg of commercial C12:0 converted to FAME.

2.3.4. Determination of β-carotene

An extraction process is necessary as the β-carotene accumulates inside the cells. 250 microliters of culture medium, around 500 microliters of 1-millimeter diameter glass beads, and 1.2 milliliters of a 50:50 hexane and ethyl acetate solution with 0.01% butyl hydroxyl toluene were mixed. The mixture was then vortexed, incubated on ice, centrifuged, and the orange-colored supernatant was collected. This process was repeated until the supernatant is no longer discolored. The optical density of the supernatant was then measured at a wavelength of 448 nm using an Hitachi Model U-200 spectrophotometer. Then, the β-carotene content was determined with the help of a calibration curve plotted beforehand.

3. Results and Discussion

3.1. VFAs production from syngas by Clostridium carboxidivorans

The *C. carboxidivorans* strain was grown in a bioreactor. The main objectives of this experiment were to reach the optimal production of VFAs with this bacterium and the subsequent bioconversion of the produced acids into lipids with a modified *Yarrowia lipolytica* strain. The best conditions for the anaerobic bioreactor operation were decided based mainly on the study of Fernández-Naveira et al. [19]. In that sense, the pH value is a critical parameter, significantly affecting the products synthesized by acetogens [3,14,19]. It was decided to work with a constant pH of 6.2, favorable for the accumulation of acids rather than alcohols. Moreover, knowing that the presence/absence of certain trace metals also strongly influences the products obtained, it was decided to work in the absence of tungsten (W) to promote the production of acids but not alcohols [19,20].

Fig. 1 shows the optical density throughout the 230-hour fermentation. *C. carboxidivorans* grew efficiently, with no detectable lag phase and an exponential biomass growth during the first 40 h after inoculation, followed by a stationary phase until the end of fermentation as growth slowed down and subsequently remained roughly constant.

Regarding the products synthesized in this anaerobic fermentation, *C. carboxidivorans* can metabolize synthesis gas, via the Wood-Ljungdahl (WL) pathway, and is known to be able to produce ethanol and higher alcohols from such gases. However, in that pathway, gases must be converted first into 2–6 carbon fatty acids before being, eventually, converted into the corresponding C_2-C_6 alcohols [3,19,21]. Therefore, the experimental conditions described in Materials and Methods were established and allowed to successfully stop the metabolic pathway at the level of volatile fatty acids accumulation with no production of alcohols. This acetogenic species is the only one known so far to efficiently produce acids with up to 6 carbon atoms, which allows, in this research, to assess the effect of not only acetic acid but also longer chain fatty acids on oleaginous yeasts.

Fig. 2 shows the production of volatile fatty acids throughout the experiment. A typical acetogenic pattern of acids production can be observed. The first acid synthesized was acetic acid, and the onset of exponential accumulation of acetic acid could be detected already a few hours after inoculation. The maximum concentration of acetic acid, i.e., 6.3 g/L, was reached 160 h after inoculation, corresponding to a maximum $OD_{600\ nm} = 2.28$; thereafter, acetic acid production stopped.

Furthermore, comparing Fig. 1 and Fig. 2, it can be seen that acetic acid production is concomitant with bacterial growth. The presence of butyric acid and hexanoic acid was not observed until, respectively, 40 h and 207 h after inoculation, reaching their maximum concentrations after 230 h. The maximum concentrations of acids are summarized in Table 1, which also compares these values with those obtained in the

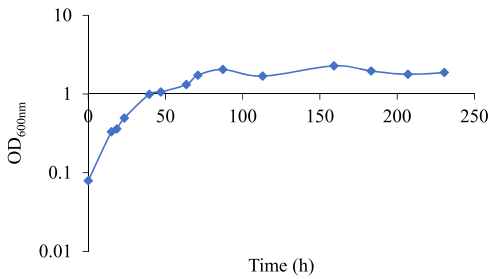

Fig. 1. Growth of *C. carboxidivorans* measured as $OD_{600\ nm}$ and represented on a logarithmic scale.

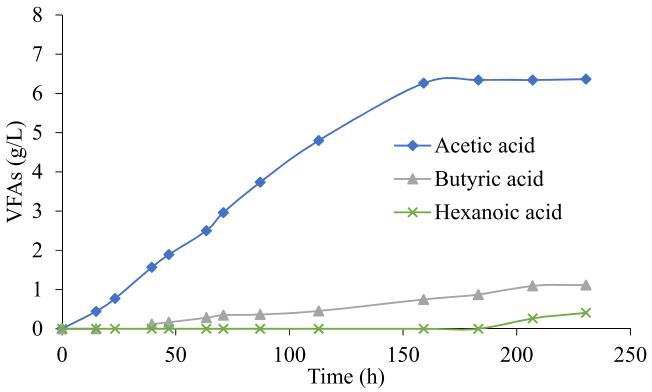

Fig. 2. VFAs production in the continuous gas-fed bioreactor by *C. carboxidivorans* at constant pH 6.2.

Table 1
Selective acid production during the HBE (Hexanol-Butanol-Ethanol) fermentation process at 33 ºC, pH 6.2, and 250 rpm.

Syngas feeding	Acids concentrations (g/L)	Acids production rates (g/L.day)	Ref.
$CO:CO_2:H_2:N_2$ (30:10:20:40)	Acetic acid: 10.051, Butyric acid: 1.119, Hexanoic acid: 0.133 Total: 11.303	Acetic acid: - , Butyric acid: - , Hexanoic acid: -	[19]
	Acetic acid: 6.368, Butyric acid: 1.117, Hexanoic acid: 0.408 Total: 7.893	Acetic acid: 1.08, Butyric acid: 0.1176, Hexanoic acid: 0.2064	This study

work of Fernández-Naveira et al., for the same pH conditions and in the absence of W in the medium [19]. The amount acetic acid obtained in the present research was somewhat lower than in the study reported by Fernández-Naveira and co-workers [19]. However, some experimental differences between both studies must be highlighted and it should be taken into account that, although most of the fermentation conditions are the same (i.e., same pH, temperature, agitation, absence of tungsten), the composition of the medium used for *C. carboxidivorans* was different. The reason is that a same culture medium, with identical composition, was designed for both *C. carboxidivorans* and *C. aceticum*, in order to allow accurate comparison of both organisms (non-published data) for production of acids and, subsequently, microbial oils. Moreover, one of the objectives of this experiment was to favor the production of (only) VFAs and avoid the production of alcohols, and it is worth noting that, at the end of the fermentation, no alcohols were produced except some negligible amount of ethanol (<0.2 g/L). All in all, it can be stated that the results obtained are truly promising.

The medium, which contains acetic, butyric, and hexanoic acid ratios of 0.81:0.14:0.05, was then assessed for its efficiency in the simultaneous production of lipids and carotenoids by *Y. lipolytica*.

3.2. Toxicity of engineered Y. lipolytica against VFAs

Volatile fatty acids, primarily acetic acid, are cheap and efficient substrates compared to other ones (e.g., sugars), for oleaginous yeasts. It has been proved that those yeasts can tolerate relatively low concentrations of organic acids and convert them into lipids [22]. However, higher concentrations can cause some inhibition or even become lethal if a threshold concentration is exceeded. This concentration threshold varies between different species and type of VFA. For example, it was observed that *Y. lipolytica* shows better resistance to high concentrations of acetic acid than to longer carbon chains, e.g., butyric or propionic acids [23].

Mimicking the VFAs present in the previous *C. carboxidivorans* bioreactor experiment, an assay was performed in order to determine whether the concentrations of C_2-C_6 acids obtained could generate a toxic effect on the yeast in the acetogenic medium. Table 2 lists the concentration of VFAs used in the experiment.

Throughout these *Y. lipolytica* experiments, both microbial growth and the consumption of acids were measured. Fig. 3 shows the yeast growth expressed in terms of optical density (600 nm) and the consumption of VFAs. It can be observed that the engineered yeast strain was able to grow efficiently in the acetogenic medium at all VFAs concentrations (Fig. 3 (A)). This proves that, with the initial concentrations of VFAs tested, *Y. lipolytica* did not suffer any inhibition.

Conversely, in their study, Gao and colleagues reported that *Y. lipolytica* started to show some inhibition when acetic acid concentrations exceeded 5 g/L and butyric acid rose above 2.5 g/L, at initial pH 6.0 [23]. While it is true that in our experiments, the maximum concentration of butyric acid was not higher than 2.5 g/L, our assays exceeded the inhibition concentration of acetic acid reported by Gao et al. by more than 2.5-fold, without observing any evidence of inhibition. This may be explained by the fact that a genetically modified strain, that is able to tolerate a higher concentration threshold, was used in the present study. It is also important to note that the medium's acidity significantly affects the toxicity of VFAs, as reported in some study with *Y. lipolytica* grown in presence of 50 g/L acids, containing a mixture of acetic acid, propionic acid, and butyric acid in a 5:2:3 ratio, at different pHs [10]. It was shown that the yeast was able to produce a higher amount biomass, without any lag phase, under alkaline pH conditions; the optimum pH ranging between 7 and 9 [10].

Fig. 3 (B), (C), and (D) show the consumption of acetic, butyric, and hexanoic acids, respectively, for each flask. The consumption of hexanoic acid, plotted in Fig. 3 (D), suggests that the engineered *Y. lipolytica* species cannot metabolize this acid and, therefore, the concentration in each flask remained constant. For Fig. 3 (B) and (C), during the first 6 h of experiment, there was practically no consumption of any of the VFAs. About 24 h after inoculation, butyric acid in all the flasks was fully consumed, except in flasks 5 and 6. At the same time, only all the acetic acid in flasks 1 and 2 was consumed. Acetic and butyric acid consumption rates were approximately 0.48 and 0.19 g/L·h, respectively, calculated from flask 6, providing the most complete data for calculation. As expected, the consumption rate of acetic acid was higher than that of butyric acid, and more than double.

These consumption rates can be compared with results of similar studies involving acids and *Y. lipolytica* strains. For example, according to Pereira and colleagues, native strain *Y. lipolytica* W29 needed about 48 h to consume 6 g/L VFAs, composed of a mix of acetic, propionic, and butyric acids, with an approximate consumption rate of 0.125 g/L·h [24]. This result is significantly lower than that obtained in our study. Another example is that of Llamas et al. [22], who calculated an acetic acid consumption rate of 0.035 g/L·h, which is also much lower than the consumption rates obtained in this experiment. Naveira-Pazos and co-workers, using a native strain of *Y. lipolytica* and with an acetic: butyric:hexanoic acid ratio of 0.81:0.14:0.05, performed experiments with total acids concentrations ranging from 6 to 16 g/L, obtaining maximum acetic, butyric and hexanoic consumption rates of 0.66, 0.19 and 0.90 g/L·h, respectively [25]. The maximum consumption rate of butyric acid is identical to the one obtained in our experiment, and that

Table 2
Initial VFAs concentrations, in grams/liter, for each batch experiment.

Experiment	C/N	Total VFAs	Acetic acid	Butyric acid	Hexanoic acid
Flask 1	15	6	4.89	0.84	0.24
Flask 2	19	8	6.52	1.12	0.32
Flask 3	22	10	8.15	1.40	0.40
Flask 4	25	12	9.78	1.68	0.48
Flask 5	29	14	11.41	1.96	0.56
Flask 6	32	16	13.04	2.24	0.64

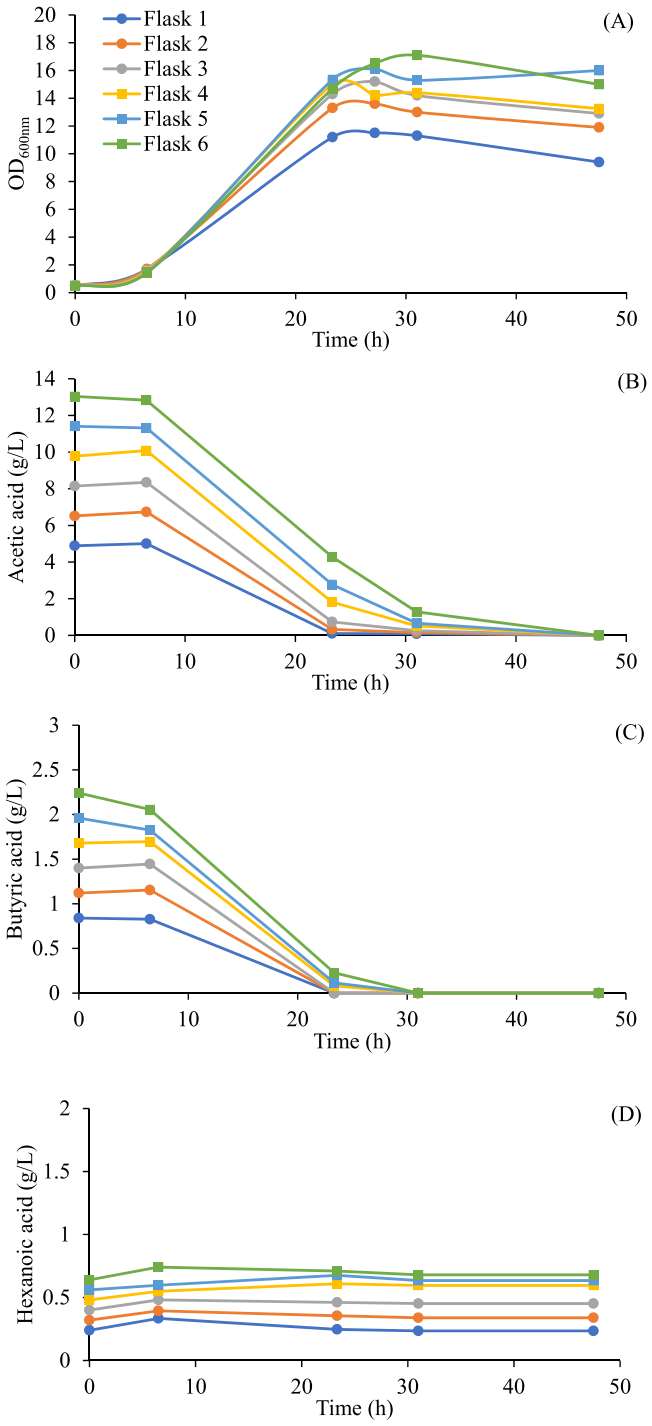

Fig. 3. (A) Growth of *Y. lipolytica* during the batch experiments, expressed in terms of optical density at 600 nm ($OD_{600\ nm}$); and consumption of (B) acetic acid, (C) butyric acid and (D) hexanoic acid.

of acetic acid is slightly higher.

In contrast, in our experiment with engineered *Y. lipolytica*, the modified yeast was unable to metabolize hexanoic acid. These results demonstrate how genetic engineering applied to *Y. lipolytica* can alter the assimilation capacity of VFAs. It should also be noted that in the toxicity experiment performed, the growth medium did not have nitrogen-limiting conditions. Nitrogen-limiting conditions favor lipid accumulation but disfavor biomass accumulation and therefore affect the substrate consumption rate [26].

3.3. *Y. lipolytica* bioreactor with *C. carboxidivorans* fermented medium from syngas

3.3.1. Growth and VFAs consumption

The VFA-rich medium produced in the *C. carboxidivorans* bioreactor fed syngas (section "*3.1. VFAs production from syngas by Clostridium carboxidivorans*"), was then used as culture medium for *Y. lipolytica*. Two experiments were performed, splitting the total volume of acetogenic medium into two bioreactors of 1 liter working volume each. Thanks to the toxicity experiments performed previously, it was possible to confirm the high degree of tolerance of *Y. lipolytica* to VFAs previously generated in the acetogenic fermentation medium. Given the findings from these toxicity experiments, it was decided to supply specific additional VFA concentrations that were deemed optimal for the yeast's growth and consumption. Table 3 shows the initial, added, and total concentrations of each VFA. It can be seen that no additional hexanoic acid was supplied because it was previously found that our engineered yeast is not able to consume it.

The first experiment, performed at an airflow rate of 0.5 vvm, took 100 h to consume all the VFAs, as seen in Fig. 4 (A). It should be noted that in this first experiment, a biomass amount of 0.5 was inoculated, in terms of $OD_{600\ nm}$. Still, the yeast failed to grow; therefore, after three days, it was inoculated again, this time with twice the previous amount biomass, i.e., $OD_{600\ nm} = 1$. The lag phase was approximately 60 h, after the new inoculation, considered as time t_0, and *Y. lipolytica* started to consume acetic and butyric acids at rates of 0.22 and 0.086 g/L·h, respectively. Because of this rapid consumption, it was decided to push the system further and supply an additional amount of VFAs to the bioreactor, equal to the initial one, except for hexanoic acid, at t = 100 h, as shown in Fig. 4 (A) and represented with an arrow. However, after the addition, acetic and butyric acid consumptions decreased by half, dropping to 0.12 and 0.047 g/L·h, respectively. The total VFAs consumption at the end of the experiment was 17.06 g/L.

In contrast, Fig. 4 (B) shows the second experiment, performed at an airflow rate of 2.0 vvm. In this case, inoculation was performed in order to immediately reach $OD_{600\ nm} = 1$. The lag phase was roughly similar as in the previous experiment, i.e., about 70 h. Then, *Y. lipolytica* started to consume acetic and butyric acids at rates of 0.25 and 0.083 g/L·h, respectively, that is with virtually no difference compared to the first reactor. Mimicking the conditions of the previous experiment, the same amount of initial VFAs was added when all the original substrates had been consumed, after t = 137 h. Contrary to the previous experiment, the consumption rates of both acids remained roughly identical to the initial values in this assay, reaching 0.25 and 0.075 g/L·h for, respectively, acetic and butyric acids. This suggested that the aeration rate could affect the rates of acids consumption by the yeast, observing a decrease in consumption capacity in the experiment with a constant airflow of 0.5 vvm, which was, however, nonexistent at an airflow rate of 2.0 vvm. Therefore, it was decided to continue progressively supplying acetic and butyric acids at concentrations similar to the initial one, as long as the yeast remained able to consume all the substrate.

Fig. 5 represents the total VFAs assimilation by *Y. lipolytica* until the yeast stopped consuming the substrates, reaching a total consumption of 90 g/L VFAs after 8 additions, each indicated with an arrow in the figure. The acid consumption rates remained virtually constant with each subsequent addition of VFAs. Still, from the seventh one, after 80 g/L already consumed, the yeast showed a decrease in the

Table 3
VFAs originating from anaerobic fermentation and amount added VFAs.

VFAs (g/L)	From anaerobic fermentation	Added	Total each
Acetic acid	6.3	3.7	10
Butyric acid	1.1	0.40	1.5
Hexanoic acid	0.4	0	0.4
Total	11.9		

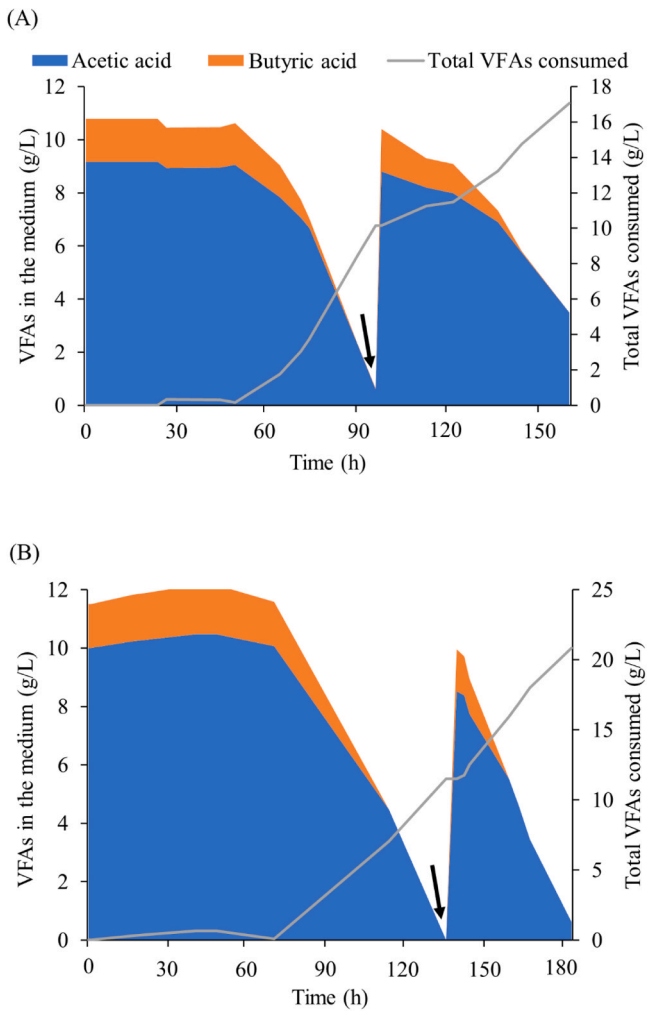

Fig. 4. VFAs in the culture medium and consumed by *Y. lipolytica*. (A) Experiment with an airflow rate of 0.5 vvm, (B) Experiment with an airflow rate of 2.0 vvm.

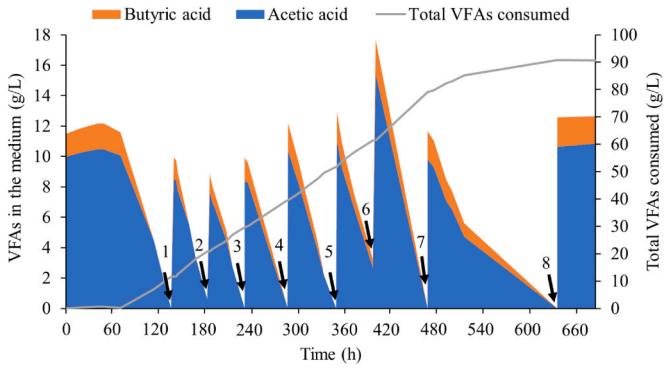

Fig. 5. VFAs in the culture medium consumed by *Y. lipolytica* in the 2.0 vvm airflow rate experiment.

consumption rate of almost 90% for acetic and butyric acid. After the eighth addition, the metabolism of *Y. lipolytica* seemed to have reached its limit, and it stopped metabolizing acids. This could also have been due to exhaustion of some nutrient in the medium or any other inhibitory condition.

The biomass concentration is represented, in terms of optical density, in Fig. 6, for both experiments. Comparing both curves indicates that in

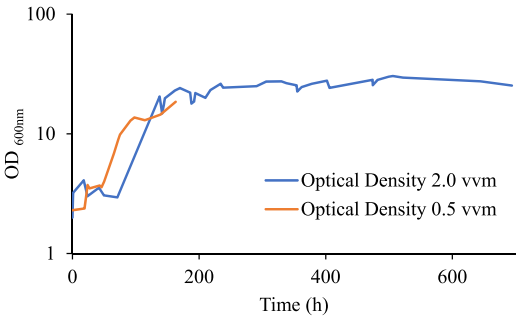

Fig. 6. Growth of *Y. lipolytica*, measured as $OD_{600\ nm}$, and represented on a logarithmic scale for both the 0.5 and the 2.0 vvm airflow rate experiments.

the first experiment, with an airflow rate of 0.5 vvm, somewhat faster growth was achieved, compared to the experiment with an airflow rate of 2.0 vvm. Besides, the maximum growth rate (μ_{max}) was slightly higher in that first experiment, i.e., 0.036 *vs* 0.030 h^{-1}, respectively. The reason could be that the first experiment with the airflow rate set at 0.5 vvm started with slightly higher biomass than the second one. This conclusion is drawn because it has been shown that the initial inoculum size (amount of inoculated biomass) can affect the growth rate of the microorganism. However, it does not affect other parameters, such as lipid accumulation [27].

Saenge and colleagues studied the effect of aeration on *R. glutinis*, and several of their results can be compared with our study [28]. For example, increasing the aeration rate from zero to 2.0 vvm also increased the growth and amount biomass produced by the yeast; but above 2.0 vvm, there was no difference anymore [28]. Based on those data, two distinct aeration rates were examined: 0.5 vvm, representing a lower oxygen availability scenario, and 2.0 vvm, near the threshold beyond which further growth benefits were not observed in the cited study. From our experimental results, it can be inferred that optimal growth occurs when oxygen in the medium meets or exceeds the yeast's oxygen demand. For instance, in the 0.5 vvm experiment, the growth rate declined as more acids were added and biomass increased, underscoring the yeast's increasing oxygen requirements. Conversely, with a consistent dissolved oxygen level in the culture medium, growth rate became limited as biomass rose.

3.3.2. Lipid production

Throughout the experiments, various samples were collected to evaluate *Y. lipolytica*'s ability to produce lipids; the results are shown in Table 4 and correspond to the first part of the experiment, when approximately the same amount VFAs was consumed in each assay.

The maximum values achieved in terms of lipid content and concentration were 18.42% g/g and 0.89 g/L, respectively. These amounts were achieved when the consumption of VFAs had reached a total of 17.6 g/L, corresponding to the first experiment, carried out at an airflow rate of 0.5 vvm. On the other hand, with an airflow rate of 2.0 vvm and 20.85 g/L VFAs consumed, *Y. lipolytica* produced 13.13% g/g and 0.62 g/L in terms of lipid content and concentration, respectively. Regarding biomass and lipid yields, the experimental yields obtained were higher in the experiment with the air feed maintained at 0.5 vvm. The highest yields obtained, in terms of biomass and lipids, were $Y_{(X/S)} = 0.304$ and $Y_{(L/S)} = 0.052$.

Fig. 7 shows the data of the second experiment, when the reactor was fed an air flowrate of 2.0 vvm. It shows data of lipids for different concentrations of total VFAs consumed by *Y. lipolytica*. The results indicate that, as the VFA consumption increases, the lipid content in the biomass also increases, reaching a maximum of 36% at 61 g/L VFAs consumed. However, at 79 g/L and 85 g/L, the lipid content started decreasing, to 27% and 22.9%, respectively. A similar behavior is observed for the lipid concentration, with a maximum value of 3.1 g/L, to later decrease to 2.4 g/L at about 91 g/L of VFAs consumed. The

Table 4
Different parameters obtained for the 0.5 and 2.0 vvm airflow rates experiments.

Airflow (vvm)	Time (h)	VFAs consumed (g/L)	Biomass (g/L)	Lipid content (%, g/g)	Lipid concentration (g/L)	Y ($_{X/S}$)	Y ($_{L/S}$)
0.5	114.5	11.26	3.19	15.90	0.55	0.283	0.045
	139	13.23	4.02	16.94	0.68	0.304	0.052
	163	17.06	4.84	18.42	0.89	0.284	0.052
2	137	11.51	3.45	11.48	0.40	0.300	0.034
	161	15.94	4.23	11.78	0.50	0.265	0.031
	185	20.85	4.73	13.12	0.62	0.227	0.030

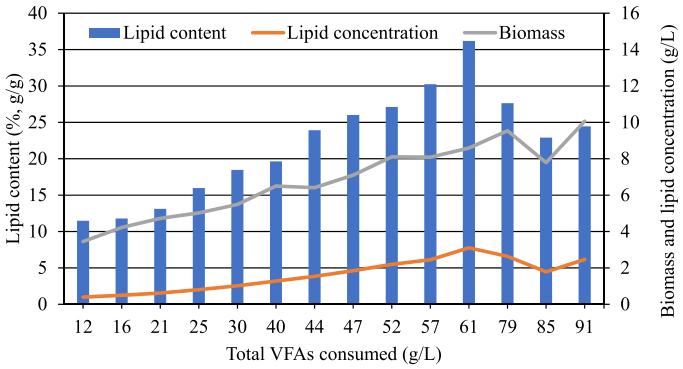

Fig. 7. Lipid content, lipid concentration, and biomass concentration as a function of different amounts of VFAs consumed by *Y. lipolytica* for the 2.0 vvm airflow rate experiment.

biomass results are also represented, being 10 g/L the maximum amount, reached after 91 g/L VFAs consumed. It can also be observed that if the difference between lipid concentration and biomass is compared for each value of VFAs consumed, this difference increases as *Y. lipolytica* consumes more VFAs (Fig. 7). Such difference, which becomes larger throughout the experiment, can be explained assuming that the amount biomass increases as the yeast consumes more acids but at a faster rate than the accumulation of intracellular lipids.

It can be deduced that increased VFAs consumption has a positive effect on lipid content, though there is an optimal consumption point after which the lipid content starts decreasing. This optimal point, in our experiment, was 61 g/L VFAs. Other studies have also analyzed the effect of VFA consumption on lipid production in *Y. lipolytica*. For example, Chai et al. reported that *Y. lipolytica* produced over 41.5% of its dry weight as lipids when grown on a 40 g/L mixture of 95% rapeseed oil and 5% acetic acid, i.e., one of the volatile fatty acids [29]. Another study, carried out by Llamas et al., proved how *Y. lipolytica* can metabolize a mixture of different acids such as acetic, propionic, butyric, isobutyric, valeric, isovaleric, and hexanoic acids, but the latter becoming more problematic at high concentrations of approximately 6.45 g/L [22]. In that study, a mixture of > 10.6 g/L VFAs was inhibitory, whereas if the VFAs are from a real digestate, the tolerance increased to 26.5 g/L VFAs [22]. Although that study focused on the growth of the microorganism and not on lipid production, it is worth mentioning because our experiment exceeded by as much as 3.5 more the amount of VFAs that *Y. lipolytica* managed to consume without showing signs of inhibition. It could be explained by the fact that, in our case, the strain used was genetically modified and also because the acids were added by pulses, which facilitated their metabolism without undesired inhibitory effects. For comparison of the lipid results obtained with a medium rich in acetic, butyric, and hexanoic acids, we must turn to the study performed by Naveira-Pazos and coworker [25]. For a total of 36.9 g/L VFAs consumed by the native strain *Y. lipolytica* W29, with an acetic:butyric:hexanoic acid ratio of 0.81:0.14:0.05, the concentration and lipid content were 0.30 g/L and 13% g/g, respectively [25]. Comparing these results with the present experiment, and for the same concentration of VFAs consumed (≈ 34 g/L), the lipid content is 18% and its concentration 1.2 g/L, which are both higher results, especially for the lipid concentration, highlighting a much higher amount of biomass in our experiment. On the other hand, the amount VFAs consumed in our experiment without experiencing any inhibition was also quite higher. These better results can be explained by the improved performance of this engineered strain, with increased acid tolerance and increased biomass accumulation. Another study reporting lipids accumulation from acetic acid, is that of Robles-Iglesias and colleagues [14]. Although it was performed under somewhat different experimental conditions and with different acetogenic and yeast strains, i.e., *A. woodii* and *R. toruloides*, it is worth comparing, as acetic acid also came from the acetogenic fermentation of syngas. In that case, a maximum lipid content of 18% g/g was achieved, with a total acetic acid consumption of 15.4 g/L [14]. These results, for the same amount acids consumed, exceed those obtained in our experiments with *Y. lipolytica* and an air flowrate of 2.0 vvm but are similar to those obtained with an air flowrate of 0.5 vvm (Table 4).

To sum up, the amount lipids accumulated in *Y. lipolytica* increases as more VFAs are consumed, up to a given high concentration, which was > 61 g/L VFAs in our experiment. Besides, aspects such as the experimental conditions, substrates, and yeast strain used can modify the amount lipids accumulated to a greater or lower extent.

3.3.3. Lipid profile

The lipid profile of several samples taken in both experiments is illustrated in Fig. 8 (A) and (B) for air flowrates of 0.5 vvm and 2.0 vvm, respectively, with a VFA consumption of 16–17 g/L. Both profiles are highly similar; the most noticeable difference being for the C16:0 compound, presenting a difference of 5% between both experiments, with a higher percentage at the air flowrate of 0.5 vvm. Fig. 8 (C) shows the lipid profile at 2.0 vvm but when the yeast had consumed 61 g/L VFAs, i.e., the point where the lipid content was highest (Fig. 7). Comparing this lipid profile with Fig. 8 (B), only slight differences are observed, again mainly for the C16:0 compound, whose concentration increased by 5% when the acid consumption was higher. In conclusion, the lipid profile remains roughly constant under all conditions, with only slight deviations and with the following trend in terms of abundance: C18:1 > C16:0 > C18:0 > C18:2 > C16:1 > Others.

However, as highlighted in Table 5, the lipid profiles of commercial vegetable oils are characterized by a wide range of variations in their individual compositions. Specifically, while the percentage of C18:1 in *Y. lipolytica* lies within the range of 48–51%, vegetable oils like soybean and canola exhibit C18:1 percentages of 27.5% and 61%, respectively. The differences, for instance in C18:2 content, could influence biodiesel properties. Specifically, the reduced C18:2 content in *Y. lipolytica* might result in biodiesel with better oxidative stability. This indicates that the lipid profile of *Y. lipolytica* is not only versatile but also potentially advantageous for certain biodiesel applications.

In any case, the lipid profile can vary considerably, even if the same microorganism is used, i.e., *Y. lipolytica*. For example, Naveira-Pazos and colleagues, feeding native *Y. lipolytica* W29 with the same VFAs as in our experiment, but using a bottle culture, obtained up to 49.7% of C18:2 when none of the samples extracted from both reactors exceeded 9% in the present research [25]. In short, *Y. lipolytica* is a powerful and promising tool to substitute vegetable oils used for biodiesel production

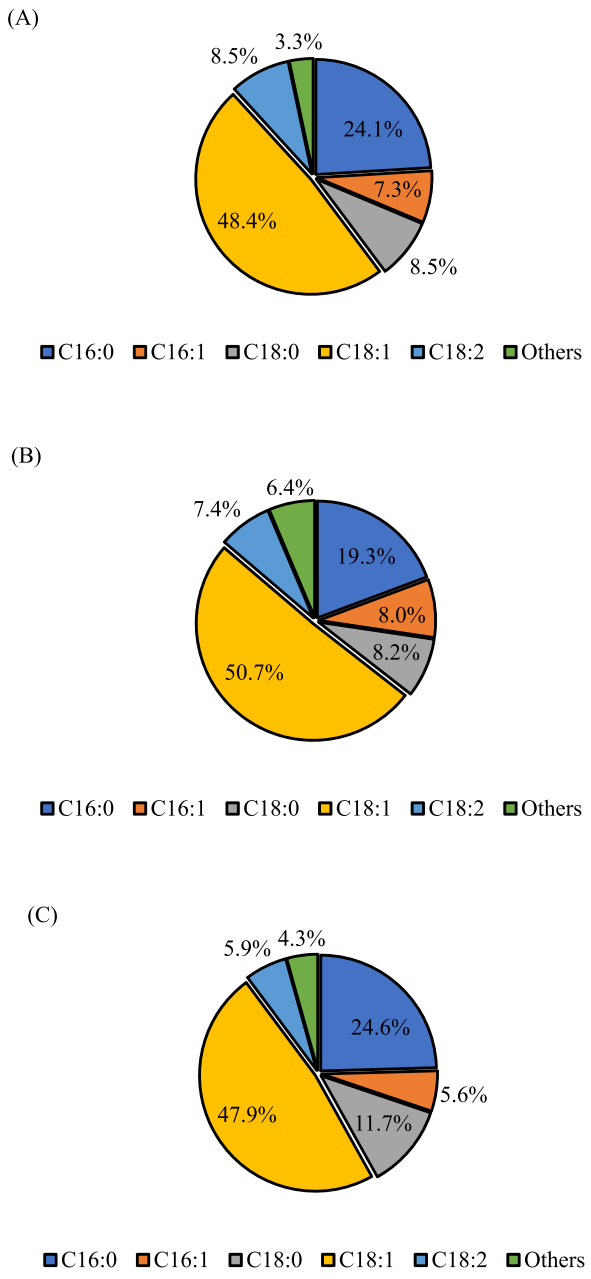

Fig. 8. Lipid profiles obtained for (A) 0.5 vvm airflow rate with 17 g/L VFAs consumed, (B) 2.0 vvm airflow rate with 16 g/L VFAs consumed, and (C) 2.0 vvm airflow rate with 61 g/L VFAs consumed.

Table 5
Typical lipid profile in biodiesel generation from different feedstocks compared to the average lipid profile of our study.

Lipid source	Lipid profile (%, g/g)						Ref.
	C16:0	C16:1	C18:0	C18:1	C18:2	Others	
Soybean	10.9	0.1	5.7	27.5	51.5	3	[30]
Corn	11.6	-	2.5	38.7	44.7	1.4	
Palm	18.7	1.6	0.9	56.1	21.1	-	
Rapeseed	2.8	0.3	1.3	64.4	22.3	7.3	[31]
Canola	4.1	0.3	1.8	61	21	8.8	[32]
Y. lipolytica	19–25	5–8	9–12	48–51	6–9	3–6	This study

and shows some flexibility allowing to obtain somewhat different lipid profiles depending on the experimental conditions.

3.3.4. *β-carotene production*

The engineered *Y. lipolytica* strain was also able to produce β-carotene from the VFA-rich medium generated from the fermentation of *C. carboxidivorans* using syngas. Fig. 9 shows the results of β-carotene produced as a function of VFAs consumed, for the bioreactors fed an airflow rate of 0.5 and 2.0 vvm. The best results in terms of β-carotene content and concentration were 46.3 mg/g cell and 195 mg/L, respectively, with a total consumption of VFAs of 18 ± 1.5 g/L and airflow of 2.0 vvm (Fig. 9). These results are higher than those obtained with an airflow rate of 0.5 vvm for the same amount VFAs consumed, i.e., 16.6 mg/g cell and 80.2 mg/L β-carotene content and concentration, respectively. It can also be observed how the amount of β-carotene increases with the concentration of acids consumed in the case of 2.0 vvm, but in the case of an airflow of 0.5 vvm, this difference in terms of increase is much lower. This can be attributed to several factors. Firstly, the higher airflow rate translates to increased oxygen availability, potentially leading *Y. lipolytica* to upregulate pathways linked with carotenoid biosynthesis as a protective response against heightened oxidative stress. This is reinforced by the fact that carotenoids are established protective compounds aiding cells in managing oxidative challenges. Furthermore, the metabolic dynamics at 2.0 vvm might undergo shifts due to this enhanced oxygen provision, possibly diverting metabolic flux from lipid accumulation towards carotenoid synthesis. This speculation aligns with our observation of lower lipid content at 2.0 vvm when juxtaposed with results from 0.5 vvm, under similar VFA consumptions. This could be because lipid and β-carotene biosynthesis share the same precursor, namely Acetyl-CoA [16]. Additionally, VFAs, in tandem with higher oxygen levels, might act synergistically, stimulating pronounced carotenoid synthesis. Lastly, the swift VFA consumption at an elevated airflow rate could exert a form of metabolic stress on *Y. lipolytica*, triggering an upsurge in protective carotenoid production. This interplay between airflow rate and VFA consumption underscores their crucial role in carotenoid biosynthesis in *Y. lipolytica*, hinting at the potential to modulate these parameters to optimize desired outcomes strategically.

Fig. 10 shows β-carotene production throughout the experiment at different concentrations of VFAs consumed, with an airflow rate of 2.0 vvm. The maximum β-carotene content was 88.4 mg/g cell for a consumption of approximately 79 g/L VFAs. Besides, the best result in terms of concentration was 759.4 mg/L, also obtained at the same VFAs concentration. As was the case for the lipid production, an upward trend was observed, especially in the first half of the experiment. For example, from the first sample to the one corresponding to 40 g/L VFAs, the amount acids consumed increased by a factor 4 while the β-carotene content increased by a factor 6. Between 40 g/L VFAs consumed and

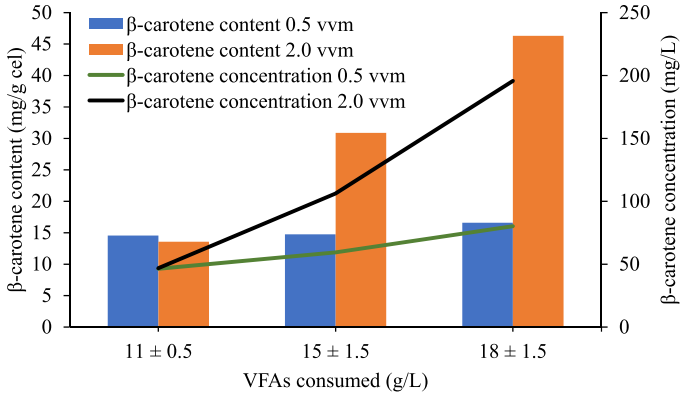

Fig. 9. β-carotene content and concentration as a function of different amounts of VFAs, for 0.5 vvm and 2.0 vvm airflow rates experiments.

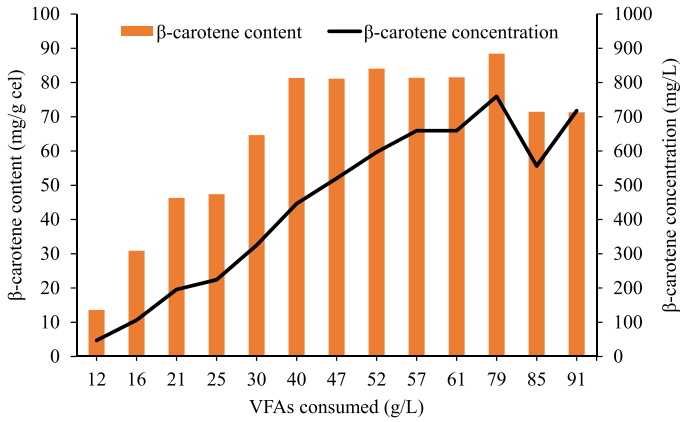

Fig. 10. β-carotene content and concentration as a function of different amounts of VFAs consumed by *Y. lipolytica*, for the 2.0 vvm airflow rate experiment.

80 g/L, the β-carotene content (mg/g cell) underwent a slight variation, remaining roughly constant or even decreasing somewhat from 80 g/L VFAs onwards. This behavior differs slightly from the one observed for the β-carotene concentration (mg/L); as the concentration increased linearly up to approximately 60 g/L VFAs consumed, and it then remained almost constant or with only some minor fluctuations (Fig. 10). As was the case for lipids, this occurs because, although hardly any more β-carotene accumulates inside the cell (between 40 and 60 g/L of VFAs consumed), the biomass keeps increasing, thus the β-carotene concentration (mg/L) tends to increase.

Larroude and co-workers used the same engineered strain in a reactor with glucose as substrate, at an airflow rate of 2.0 vvm, for the production of β-carotene. The result in terms of β-carotene content is highly similar to the one obtained in our experiment, 90 mg/g cell vs 88.4 mg/g cell [16]. This is an excellent result as the substrate used in our case can be up to more than 4 times cheaper than glucose [26]. Moreover, if the VFAs come from the anaerobic fermentation of syngas or greenhouse gases, such as CO_2, as in the present study, this will favor decarbonization and a circular economy. Regarding the concentration of β-carotene, the result obtained by Larroude and co-workers is relatively higher than in our experiment, 759.4 mg/L vs. 6500 mg/L [16], which is because the amount biomass obtained in that glucose experiment was also quite higher than here, with acids; 10 g/L in the present study vs 75 g/L with glucose.

In a nutshell, while carotenoids have traditionally been extracted from biomass, this approach is associated with significant challenges. It requires large areas of arable land, resulting in increased production costs, and is hampered by the instability and variability in composition of plant-derived carotenoids, as well as their dependence on unpredictable weather conditions [33]. Given these challenges, there is a pressing need for alternative, sustainable, and green sources of carotenoids. Microbial sources, and yeast in particular, represent a promising avenue for the future. Not only does this approach facilitate the acquisition of high-quality compounds in a cost-effective manner through optimized fermentation processes and downstream protocols, but yeasts also have the added advantage of being easily cultured on low-cost waste feedstocks. However, the success of this approach ultimately depends on both yield and production cost [34]. This study demonstrates the potential of *Y. lipolytica* for β-carotene production, paving the way for more sustainable and cost-effective carotenoid production processes in the future.

4. Conclusions

It is feasible to efficiently produce, simultaneously, microbial oils and β-carotene from VFAs, i.e., acetic, butyric, and hexanoic acids obtained from the anaerobic fermentation of syngas. The engineered *Y. lipolytica* strain could tolerate concentrations of the mixture of acids higher than 16 g/L. The lipid profile obtained in the experiments was similar as in vegetable oils and as used for the manufacture of biodiesel. Additionally, a non-limiting airflow rate favored the metabolic pathway yielding β-carotene.

Furthermore, *Y. lipolytica* was able to consume 91 g/L VFAs with a non-limiting airflow rate. However, the engineered strain was highly efficient in metabolizing acetic and butyric acids, but could not consume hexanoic acid, though its presence did not inhibit the strain. It was observed that, in order to achieve higher β-carotene concentrations, it is essential to work with experimental conditions that favor biomass accumulation. From 40 g/L VFAs consumed, *Y. lipolytica* was hardly able to accumulate more β-carotene intracellularly.

For the operational guidance, it is important to consider the airflow rate and the consumed VFA concentration. Our results suggest that a non-limiting airflow rate and consumed VFA concentrations up to 40 g/L favor β-carotene production, while beyond this point, biomass accumulation was still taking place but no additional intracellular β-carotene accumulation was observed. Therefore, for applications focusing on β-carotene production, it is recommended to operate under conditions that favor biomass accumulation up to 40 g/L VFAs consumed. For lipid production, our results indicate that it is advisable to operate at around 60 g/L VFAs consumed, with this specific organism and conditions.

Overall, our study demonstrates the potential of anaerobic fermentation of syngas with *C. carboxidivorans* as a viable method for efficiently producing lipids and β-carotene and the potential of the engineered strain *Y. lipolytica* to tolerate high concentrations of the mixture of acids and efficiently catalyze the bioconversion process.

CrediT authorship contribution statement

Raúl Robles: Investigation, Data curation, Methodology, Writing – original and revised drafts; **Cecilia Naveira**: Investigation, Data curation, Methodology, Writing – original and revised drafts; **Jean-Marc Nicaud**: Writing – original and revised drafts; **María C. Veiga**: Supervision, Resources, Reviewing drafts; **Christian Kennes**: Conceptualization, Funding acquisition, Supervision, Project administration, Methodology, Data curation Reviewing drafts.

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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