de Vicente *et al. Microbial Cell Factories* (2025) 24:32
https://doi.org/10.1186/s12934-025-02647-7

Microbial Cell Factories

RESEARCH **Open Access**

Turning residues into valuable compounds: organic waste conversion into odd-chain fatty acids via the carboxylate platform by recombinant oleaginous yeast

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Abstract

Environmental concerns are rising the need to find cost-effective alternatives to fossil oils. In this sense, short-chain fatty acids (SCFAs) are proposed as carbon source for microbial oils production that can be converted into oleochemicals. This investigation took advantage of the outstanding traits of recombinant *Yarrowia lipolytica* strains to assess the conversion of SCFAs derived from real digestates into odd-chain fatty acids (OCFA). High yeast OCFAs content was aimed by using two engineered strains (*Y. lipolytica* JMY7780 and JMY7782). Batch and two-step batch fermentations were performed, reaching high lipid content (40.8% w/w) and lipid yield (0.07 g/g) with JMY7782, which overexpresses propionyl-CoA synthase. Fed-batch fermentation with an acetic acid pulse after 24 h was also carried out to promote SCFAs consumption and OCFAs production. In this case, SCFAs consumption rate increased and JMY7782 was able to accumulate up to 60.4% OCFAs of the total lipids produced from food waste-derived carbon sources.

Keywords Short-chain fatty acids, *Yarrowia lipolytica*, Lipids, Odd-chain fatty acids, Oleaginous yeasts

Introduction

Microbial oils are an alternative source for oleochemicals that can be further used to produce biofuels and bioproducts [5, 31]. Microbial oils share a similar fatty acid composition to plant oils and do not compete with the food industry. Furthermore, microorganisms have higher biomass, higher lipid production rates, shorter cultivation periods and smaller space requirements than plants [4, 9, 16].

Some oleaginous yeasts, accumulating over 20% of their biomass as lipids, are promising candidates for microbial oils production due to their capacity of growing and producing lipids from residual-based carbon sources such as short-chain fatty acids (SCFAs) [2, 25]. The lipid content that yeast accumulate depends on the selected yeast strain, the carbon source, and the culture conditions (pH, T, C/N ratio, etc.). Therefore, to achieve a better substrate to lipid conversion yield and a higher lipid productivity, process optimization is required [8, 33]. *Yarrowia lipolytica* is one of the most studied yeasts for lipids production. This yeast has the ability to accumulate lipids using both hydrophilic (sugars) and hydrophobic compounds

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that are metabolized through de novo and *ex novo* pathways, respectively [27]. These routes differ on substrate utilization and accumulation mechanisms. The de novo route utilizes hydrophilic substrates, such as glucose, to produce fatty acid precursors (acetyl-coA and malonyl-coA) involved in lipid biosynthesis. The *ex novo* pathway uses hydrophobic substrates, mainly free fatty acids. Fatty acids are further broken down into acyl-CoAs and acetyl-CoAs via β-oxidation for their utilization as precursors of lipid synthesis [34]. Compared to the de novo route, *ex-novo* enables simultaneous growth and lipid accumulation as culture conditions do not imply nutrient limitations [15].

With regard to potential carbon sources that can be used from wastes, SCFAs (acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acids) can be obtained through anaerobic fermentation. This bioprocess is a shortened version of the anaerobic digestion in which the methanogenesis step is inhibited resulting in SCFAs accumulation [18, 20]. SCFAs production cost imply less than 10% of the cost of glucose [10, 12]. Thus, research efforts are being conducted to optimize SCFAs production for an economically-viable microbial oils production [9, 16].

Some early reports showed that yeasts were inhibited when grown on SCFAs concentrations greater than 5 g/L [8, 9]. Yet, *Y. lipolytica* was shown to be able to tolerate up to 15 g SCFAs /L at different acids ratios [29]. This was also the case of the investigation conducted by Morales-Palomo et al., [23, 24] that demonstrated no detrimental effect on yeast growth when using 15 g/L of a SCFAs mixture, prevailing in acetic acid (up to 10 g/L) and reaching a lipid content as high as 43.4% (w/w).

The production of high added-value lipids, not conventionally available through traditional petrochemical processes [14], can also be accomplished in oleaginous yeasts by aiming at odd-chain fatty acids (OCFA) [6, 26]. OCFAs (including C15:0, C17:0, and C17:1) exhibit pharmacological properties with antifungal and anti-inflammatory effects alongside with many other industrial applications [1, 35]. Despite small amounts of C17:0 being detected in the FA profile of some oleaginous yeasts grown on propionic acid [6, 16] OCFA are not the major constituent of microbial oils [1, 26]. The reason behind the low production of OCFAs is that these FA are produced by the incorporation of propionyl-CoA instead of acetyl-CoA [26]. Propionate activation is the limiting step of OCFAs synthesis, and the balance of the precursors between acetyl-CoA and propionyl-CoA is crucial for OCFA accumulation [26]. Therefore, it could be hypothesized that propionic acid may have a key role for obtaining OCFAs. To cover the gap of knowledge related to the use of SCFAs as a carbon source for OCFAs production, this investigation assessed the ability of oleaginous yeast to accumulate total lipids and OCFAs from real anaerobic fermentation digestates derived from organic wastes. To this end, two different genetically modified *Y. lipolytica* strains were used. *Y. lipolytica* JMY7780 overexpresses a propionyl-CoA transferase (*RePCT*) while *Y. lipolytica* JMY7782 overexpresses a propionyl-CoA synthetase (*SeprpE*) to increase the precursor pool of OCFA. To evaluate their performance, yeast growth, SCFAs consumption, lipid and OCFAs production under different conditions and process configuration (batch, two-step batch, and a batch with an acetic acid pulse) were compared. It should be highlighted that limited research has been carried out using real digestate rich in SCFAs as carbon source for OCFAs accumulation. Therefore, this relevant study aims not only at attaining a more economically-feasible process but also contributing to circular economy by using real digestate for the production of highly valuable compounds such as OCFAs.

Materials and methods

Microorganisms and pre-culture preparation

Two different *Y. lipolytica* strains, JMY7780 (*Δphd1 Δmfe1 Δtgl4*+pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex *pTEF-RePCT- LEU2 ex*) and JMY7782 (*Δphd1 Δmfe1 Δtgl4*+pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex *pTEF-SePrpE-LEU2 ex*) were provided by the Biologie Intégrative du Métabolisme Lipidique (BIMLip) department, INRAE in France, and conserved at -80 °C in glycerol 30% v/v. These two strains were selected as they allowed the highest accumulation of OCFAs among all tested strains by Park et al., [25]. Isolated colonies were obtained in YPD agar plates (20 g/L peptone, 10 g/L yeast extract, 20 g/L agar and 20 g/L glucose), stored in a refrigerator and sub-cultured regularly.

For pre-culture, one colony of each yeast strain was inoculated in 50 mL YPD liquid medium (same composition as stated before without agar). Pre-culture was performed in 250-mL baffle Erlenmeyer flasks at 150 rpm and 27 °C for 24 h, with a pH of 6.8 until cells reached the late exponential phase.

Substrate preparation and fermentation conditions

Real digestate rich in SCFAs (4.2 g/L acetic acid; 3.4 g/L propionic acid; 1 g/L iso-butyric acid; 2.4 g/L butyric acid; 0.9 g/L iso-valeric acid; 1.4 g/L valeric acid; 0.3 g/L caproic acid) was obtained via anaerobic fermentation of protein-rich waste [19]. Before use, the digestate was sterilized using a 0.22 μm bottle filter, as it allows the sterilization of high volumes while not compromising the composition.

An acetic:propionic acid ratio of 2:1 has been previously determined as the best ratio to increase the OCFAs proportion in the total FA pool [25]. Thus, the real digestate was supplemented with acetic acid to reach the desired ratio, resulting in 15 g/L of total SCFAs. When needed, the digestate with 15 g/L was diluted with sterile water to obtain the digestate with 10 g/L of total SCFAs. The concentration of specific acids in all fermentation experiments is shown in Table 1. The microalgae origin of the digestate led to an initial C/N concentration lower than 15 [17]. In all cases, the fermentation pH was adjusted to 6.8 with NaOH (5 M).

Three different fermentation experiments were carried out. First, batch fermentations with SCFAs-rich digestates at 10 g/L (BATCH10) and 15 g/L (BATCH15) total SCFAs were performed. Secondly, to increase yeast biomass production rapidly in a nutrient-rich media followed by lipid accumulation, once the nitrogen concentration is depleted, two-step batch cultures were carried out with an overnight (18 h) growth phase on YPD media (20 g/L glucose), starting at an initial OD of 1. Cells were then transferred after centrifugation to a SCFAs-rich digestate with 10 g/L (G + BATCH10) or 15 g/L (G + BATCH15) of SCFAs. Both acetic and propionic acids have been shown to promote OCFAs by balancing acetyl-CoA and propionyl-CoA due to the *PHD1* deletion in the used strains [25]. Thus, a batch fermentation with 10 g/L of SCFAs with an acetic acid pulse (4 g/L) (BATCH10-AA) after 24 h was carried out.

Fermentations in 250 mL baffled shake flasks with 100 mL of media were incubated in a rotary shaker at 180 rpm and 28 °C with an initial optical density (OD) of 1 until all SCFAs were consumed or cultures surpassed 240 h (10 days). All experiments were performed in triplicates and samples were regularly taken for cell growth and carbon source consumption analysis. Lipid content and OCFAs were determined after 27 h, 72 h and at the end of the fermentation.

Analytical methods

Cell growth

Cell growth was followed by measuring the OD using a spectrophotometer (SPECTROstar omega) at 600 nm (OD600). To determine cell dry weight (CDW), 2 mL of culture were vacuum filtered using pre-dried and pre-weighted nitrocellulose 0.45 µm filters (Whatman). The filter was washed with MilliQ water before and after adding the culture, then dried for 10 min at 170 W in a microwave, left in a desiccator overnight and weighed.

SCFAs determination

SCFAs were analyzed by liquid chromatography (HPLC) using an Agilent 1260 HPLC_RID equipped with Cation H Refill Cartridge Microguard column (Biorad, Hercules, CA, USA) and an Aminex HPX-87H ion exclusion column (300 × 7.8 mm I.D.) (Biorad). 0.5 mL of sample was filtered using a 0.22 µm nylon filter (Branchia) and added to the HPLC vials for analysis. The mobile phase was 5 mM H_2SO_4 solution. Elution was conducted at a flow rate of 0.6 mL/min. The injected sample volume was 20 µL. The oven and detector temperatures were 25 °C and 35 °C, respectively. After 18 h of fermentation, glucose concentration was also analyzed by HPLC, using the same method as for SCFAs determination, exception made for the flow rate that was set at 0.5 mL/min.

Lipid content quantification

Lipid content was determined after 27 h, at the middle of the exponential phase (approx. 72 h) and when 95-100% of the SCFAs were consumed. Nile red (9-diethylamino-5H-benzo[a]phenoxazine) was used to quantify the amount of lipids following the protocol described in Zhao et al., [36]. Briefly, Nile Red was dissolved in acetone to prepare a solution at a concentration of 1 mg/mL. This solution was stored in the dark at 4 °C. 1 mL

Table 1 Summary of media composition and fermentation configuration used for each experiment. The table details the fermentation mode (batch or fed-batch), the acids compositions (C2- C6) and their respective concentrations, the total SCFAs concentration, and the use of glucose 20 g/L were applicable. (G + BATCH, indicates a two-step batch strategy, where both strains were cultured on glucose for 18 h, prior fermentation on SCFAs; BATCH10 + AA, indicates a fermentation on 10 g/L SCFAs, with a 4 g/L acetic acid pulse after 24 h)

Fermentation mode	Acid concentration (g/L)								Glucose (g/L)
	Acetic acid	Propionic acid	Iso-butyric acid	Butyric acid	Iso-valeric acid	Valeric acid	Caproic acid	Total	Total
1. BATCH10	4.2	2.2	0.5	1.6	0.6	0.9	0.3	10.3	
2. BATCH15	6.3	3.4	1.0	2.4	0.9	1.4	0.3	15.8	
3. G + BATCH10	4.2	2.2	0.5	1.6	0.6	0.9	0.3	10.3	20
4. G + BATCH15	6.3	3.4	1.0	2.4	0.9	1.4	0.3	15.8	20
5. BATCH10 + AA	8.2	2.2	0.5	1.6	0.6	0.9	0.3	10.3	

sample was used for Nile Red staining. Firstly, the sample was centrifuged at 14,200 rpm for 3 min. Then, the pellet was suspended in 3 mL of phosphate buffer solution (PBS) to reach an OD600 between 0.2 and 1. Cells were then heated at 50 °C in a rotary shaker for 20 min and left to cool down at room temperature for 10 min. 3 μL of Nile Red solution were added to the 3 mL mixture to reach a final concentration of 1 μg/mL. The mixture was left in the dark for 5 min before measuring the fluorescence intensity. Fluorescence intensity was determined at λex/em = 488/540 nm, ex/em slit = 10 nm (PerkinElmer® LS 55 Fluorescence Spectrometer). The total area was calculated with Origin (Pro), version 8.5 (OriginLab Corporation, Northampton, MA, USA) and used to obtain the quantum yield values of each sample, keeping in mind the absorbance and the emission index [23, 24].

Long chain fatty acids determination

5 mL samples were collected at 27, 72 and 144 h in all experiments to quantify the OCFAs. 10–20 mg of dried-cells were used for lipid extraction and converted into FA methyl esters (FAMEs), following Browse and colleagues´s (1986) procedure. Gas chromatography was used to analyze the FAMEs by employing a Varian 3900 instrument (Varian Inc. USA) equipped with a flame ionization detector and a Varian FactorFour vf-23 ms column, where the bleed specification at 260 °C was 3 pA (30 m, 0.25 mm, 0.25 μm). Comparison with commercial standards (FAME32 Supelco) was used to identify the FAMEs. Quantification was done using the internal standard, 100 μg of commercial dodecanoic acid (Sigma-Aldrich, USA).

Calculations and statistical analysis

Process performance was evaluated by calculating the SCFAs consumption rate, biomass growth and lipid yields. Consumption rate was calculated for the first 24 h and at the end of the fermentation time by considering the SCFAs consumed during that period ((Final SCFAs (g/L)—Initial SCFAs)/ time (h)). Biomass yield ($Y_{X/CS}$ (g/g)) considered the produced biomass ($_X$) and the total consumed carbon source. In the same way, lipid yield, $Y_{L/CS}$ (g/g), was calculated as grams of accumulated lipids divided by the grams of consumed CS (SCFAs). To assess the yeast growth (OD 600) and lipid production (% w/w), a one-way ANOVA analysis was carried out with a 95% confidence interval. Differences were considered significant at p-value < 0.05. A t-student test was also carried out when only two groups were compared, considering a p-value < 0.05 to be significant.

Results and discussion

Batch oleaginous fermentation from SCFAs

The two *Y. lipolytica* strains used in this study were genetically modified to express individual propionate activating enzymes for a higher conversion of SCFAs to OCFAs [25]. Growth, SCFAs consumption and OCFAs accumulation were evaluated under different batch fermentations in real digestates with different SCFAs concentration.

As shown in Fig. 1, as result of acetic acid consumption, both JMY7780 and JMY7782 grew to an OD of 6 after 24 h in media containing 10 g/L SCFAs (BATCH10). In this fermentation media, JMY7780 and JMY7782 reached a maximum OD of 20.2 and 21.8 (Fig. 1A and 1B), respectively, which corresponded to 3.6 g CDW/L and 5.0 CDW g/L, when all acids were consumed (Table 2). For the assay conducted at 15 g/L SCFAs (BATCH15), maximum OD was 8 and 6 for JMY7780 and JMY7782, respectively (Fig. 1A), reaching a CDW of 1.7 g/L for both JMY7780 and JMY7782 (Table 2). Contrary to what happened in BATCH10 where all SCFAs were consumed with a 0.07 g/Lh consumption rate, only acetic acid was completely metabolized in BATCH15 (Fig. 1E and 1F) due to the inhibitory effect of SCFAs at high concentrations on yeast growth [3, 11]. It is worth highlighting that despite the different SCFAs concentration, no lag phase was observed for BATCH10 and BATCH15 (Fig. 1C-F).

JMY7780 and JMY7782 strains reached a biomass yield of 0.35 ± 0.03 $Y_{X/CS}$ and 0.49 ± 0.07 $Y_{X/CS}$, respectively. It is worth highlighting that Llamas and colleagues [18] reported a biomass yield of 0.35 $Y_{X/CS}$ when using 10 g/L SCFAs as carbon source with *Y. lipolytica* ACA DC 50109. JMY7782 continued growing after SCFAs depletion probably due to lipid utilization for biomass formation, which resulted in the higher biomass yields. JMY7782 also displayed a faster consumption than the *Y. lipolytica* ACA DC 50109 strain, highlighting the metabolic changes of the recombinant strain for growth SCFAs consumption. JMY7782 overexpresses SeprpE that allows a more favorable acetyl-CoA pool than JMY7780, resulting in a higher growth.

Y. lipolytica can grow well in the presence of acetic, propionic, and butyric acids [9, 11]. However, isobutyric, valeric, isovaleric and caproic acids can account for up to 30% of all the acids present in SCFAs mixtures produced in anaerobic fermentation [20]. In BATCH10, all the SCFAs were consumed in a 144 h-long fermentation by both strains (Fig. 1C and 1D). At this concentration, cell growth was arrested once acetic acid was consumed. By opposite, in BATCH15, only 50% of the SCFAs were consumed in 240 h and all acids remained in the media except for acetic acid (Fig. 1E and 1F). These results proved that independently of the concentration of SCFAs

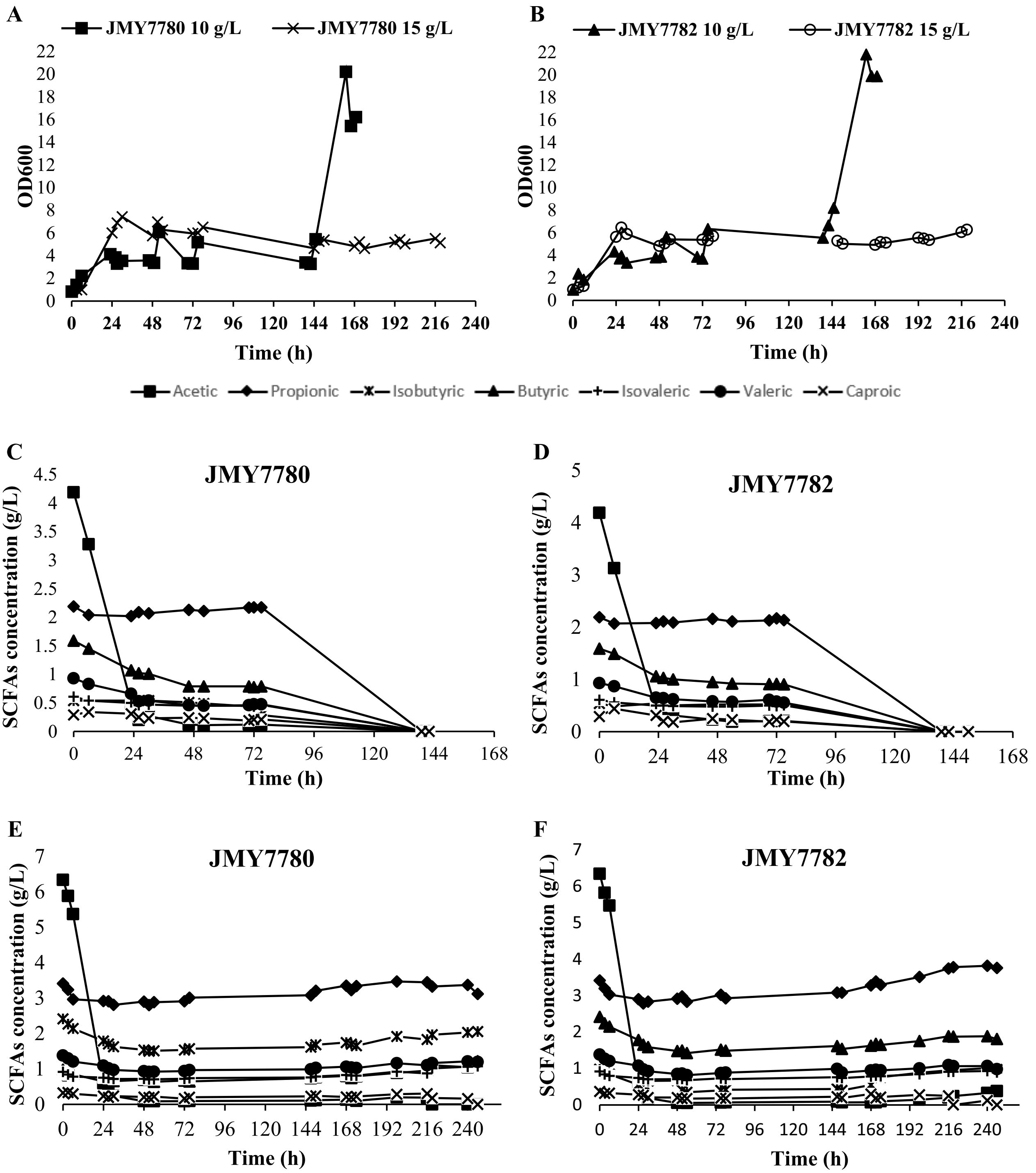
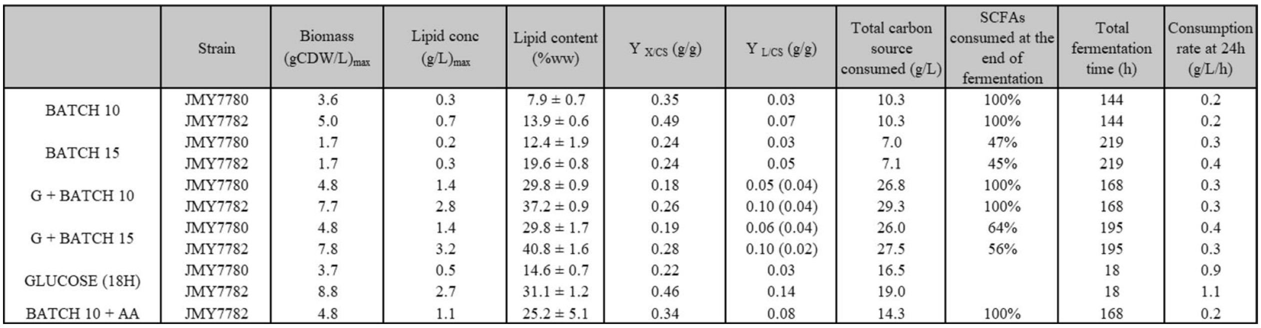


Fig. 1 Time courses for strains cell JMY7780 and JMY7782. Time course of both metabolically engineered *Y. lipolytica* strains in terms of growth (OD) (A, B) and SCFAs consumption in BATCH10 (C, D) with 10 g/L of SCFAs and BATCH15 (E, F) with 15 g/L of SCFAs derived from real microalgae fermentation digestate. The points herein indicated represent average values

in the media, both JMY7780 and JMY7782 showed a preference for acetic acid (Fig. 1C-F). Other strains, *Y. lipolytica* ACA DC 50109, *Y. lipolytica* NCYC 2904 and *Y. lipolytica* W29 have also showed a faster metabolization of acetic acid over the rest of the acids [20, 29].

Both strains (JMY7780 and JMY7782) reached the highest acetic acid consumption rates (0.16 g/Lh in

Table 2 Overview of experiments performance in terms of product yields and concentration, SCFAs consumption and rates and cell growth for both *Y. Lipolytica* strains under different fermentation conditions (batch, two-step batch and with acetic acid pulse). (G + BATCH, indicates a two-step batch strategy, were both strains were cultured on glucose for 18 h, prior fermentation on SCFAs; The lipid yield ($Y_{LC/S}$ (g/g)) for the two-step batch shows the total yield reach, but also the lipids accumulated during the SCFAs phase, in parenthesis. Glucose 20 g/L (18 h): results obtained during the first step of the two-step batch fermentation. BATCH10 + AA, indicates a fermentation on 10 g/L SCFAs, with a 4 g/L acetic acid pulse after 24 h)



	Strain	Biomass $(gCDW/L)_{max}$	Lipid conc $(g/L)_{max}$	Lipid content (%ww)	$Y_{X/CS}$ (g/g)	$Y_{L/CS}$ (g/g)	Total carbon source consumed (g/L)	SCFAs consumed at the end of fermentation	Total fermentation time (h)	Consumption rate at 24h (g/L/h)
BATCH 10	JMY7780	3.6	0.3	7.9 ± 0.7	0.35	0.03	10.3	100%	144	0.2
	JMY7782	5.0	0.7	13.9 ± 0.6	0.49	0.07	10.3	100%	144	0.2
BATCH 15	JMY7780	1.7	0.2	12.4 ± 1.9	0.24	0.03	7.0	47%	219	0.3
	JMY7782	1.7	0.3	19.6 ± 0.8	0.24	0.05	7.1	45%	219	0.4
G + BATCH 10	JMY7780	4.8	1.4	29.8 ± 0.9	0.18	0.05 (0.04)	26.8	100%	168	0.3
	JMY7782	7.7	2.8	37.2 ± 0.9	0.26	0.10 (0.04)	29.3	100%	168	0.3
G + BATCH 15	JMY7780	4.8	1.4	29.8 ± 1.7	0.19	0.06 (0.04)	26.0	64%	195	0.4
	JMY7782	7.8	3.2	40.8 ± 1.6	0.28	0.10 (0.02)	27.5	56%	195	0.3
GLUCOSE (18H)	JMY7780	3.7	0.5	14.6 ± 0.7	0.22	0.03	16.5		18	0.9
	JMY7782	8.8	2.7	31.1 ± 1.2	0.46	0.14	19.0		18	1.1
BATCH 10 + AA	JMY7782	4.8	1.1	25.2 ± 5.1	0.34	0.08	14.3	100%	168	0.2

BATCH10 and 0.24 g/Lh in BATCH15) during the first 24 h. This agreed with other studies where acetic acid also exhibited higher consumption rates compared to propionic, butyric and iso-butyric acid [30]. The fastest consumption rate of acetic acid could be attributed to the different metabolic routes for each acid. Acetic acid is directly divided into acetyl-coA, facilitating its uptake [20] through the de novo pathway whereas the metabolism of other acids depends on their biochemical transformations into acetyl-CoA for their utilization in the *ex novo* pathway [12, 19]. Previous reports indicated that not only the SCFAs concentration needs to be considered but also the SCFAs profile [20, 23, 24]. In this sense, propionic acid consumption, necessary for OCFAs accumulation, may be linked to the presence of other SCFAs, such as acetic acid [20].

Previous studies reported inhibitory effects when *Y. lipolytica* was grown at SCFAs concentrations > 5 g/L [9, 11]. By contrast, Morales-Palomo., et al. [23] did not observe any inhibition when growing the *Y. lipolytica* ACA DC 50109 strain in presence of 15 g/L of SCFAs, achieving a lipid content of 43.3% w/w under a C:N ratio of 200.

As shown in Fig. 1C and D, propionic acid showed one of the slowest consumption rates together with isobutyric and isovaleric acids. The slow consumption of propionate can be caused by the higher sensitivity of the engineered strains to this acid. Previous research indicated that propionic acid follows a different metabolic pathway (with the presence of the enzyme propionyl-coA synthetase) that interferes in propionate activation and in the production of OCFAs [26]. For this reason, propionic acid consumption may differ from that of acetic acid. Additionally, the delayed consumption of propionic acid can be caused by the disruption of *PHD1* gene that can have a negative effect in growth and propionate use [25].

Genetic changes in both strains were not only evaluated in terms of growth or SCFAs consumption but also in terms of total lipid content. In BATCH10, JMY7780 reached a maximum lipid content of 7.8% w/w after 72 h (Fig. 2 and Table 2). However, in BATCH15, the total lipid content in JMY7780 was 12.3% w/w after 72 h and 12.4% w/w after 144 h (Fig. 2). JMY7782 also showed higher lipid content in BATCH15 (19.6% at 144 h) than in BATCH10 (13.9% at 72 h) but the lipid yield was lower, 0.5 g/g vs 0.7 g/g (Table 2).

These results agreed with those published by Park et al., [25] where JMY7780 was strongly affected by the presence of SCFAs, mainly propionic, obtaining lower OD, and slower growth rate at the end of the experiments when compared with the wild type. This strain expressing propionyl-CoA transferase, is capable of producing propionyl-CoA, in the presence of propionic acid and acetyl-CoA (Fig. 3), for its incorporation in the OCFAs synthesis. Therefore, this strain requires the presence of acetic acid, precursor of acetyl-coA (Fig. 3), which is the first acid to be consumed. The availability of acetic acid is required to restore proper growth of the RePCT strain [22]. In its absence, it led to lower lipid accumulation (% w/w), as showed by Park et al. [25]. The presence of acetate slightly restored JMY7780 growth as it increased the acetyl-CoA pool needed to grow while decreasing the toxicity associated with the released propionyl-CoA Park et al. [25]. When compared to the JMY7782 strain, with the recombinant expression of propionyl-CoA synthase (SeprpE), responsible for conversion of propionate into its activated form, propionyl-CoA needed for FA synthesis, mediated by the presence of ATP, JMY7782 performs better than the strain JMY7780.

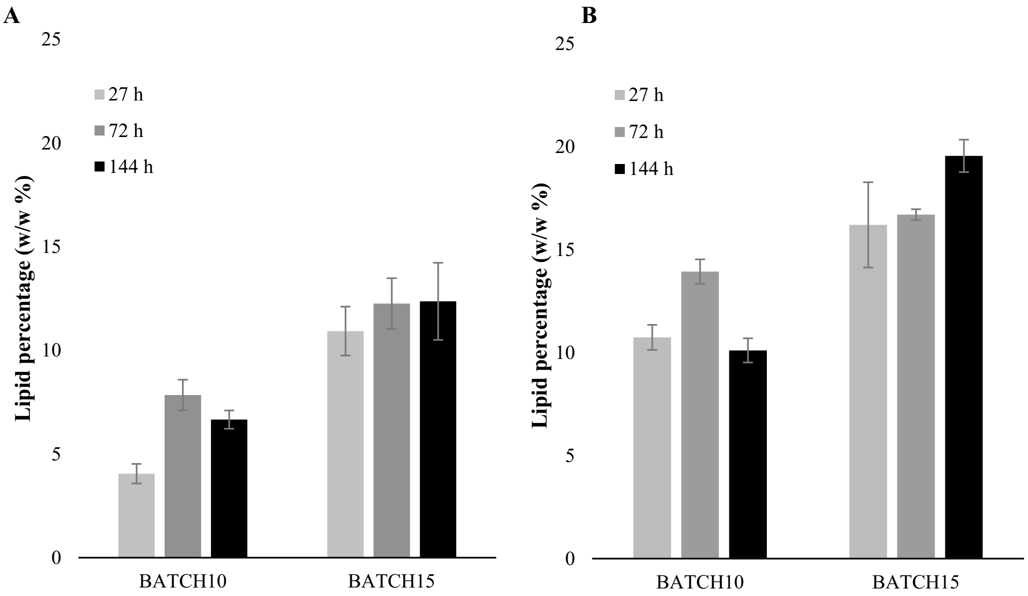


Fig. 2 JMY7780 **(A)** and JMY7782 **(B)** lipid content (w/w) (%) when cultivated with different carbon source (SCFAs) concentrations (10 g/L and 15 g/L) in batch. The bars herein indicated represent average values and standard deviations

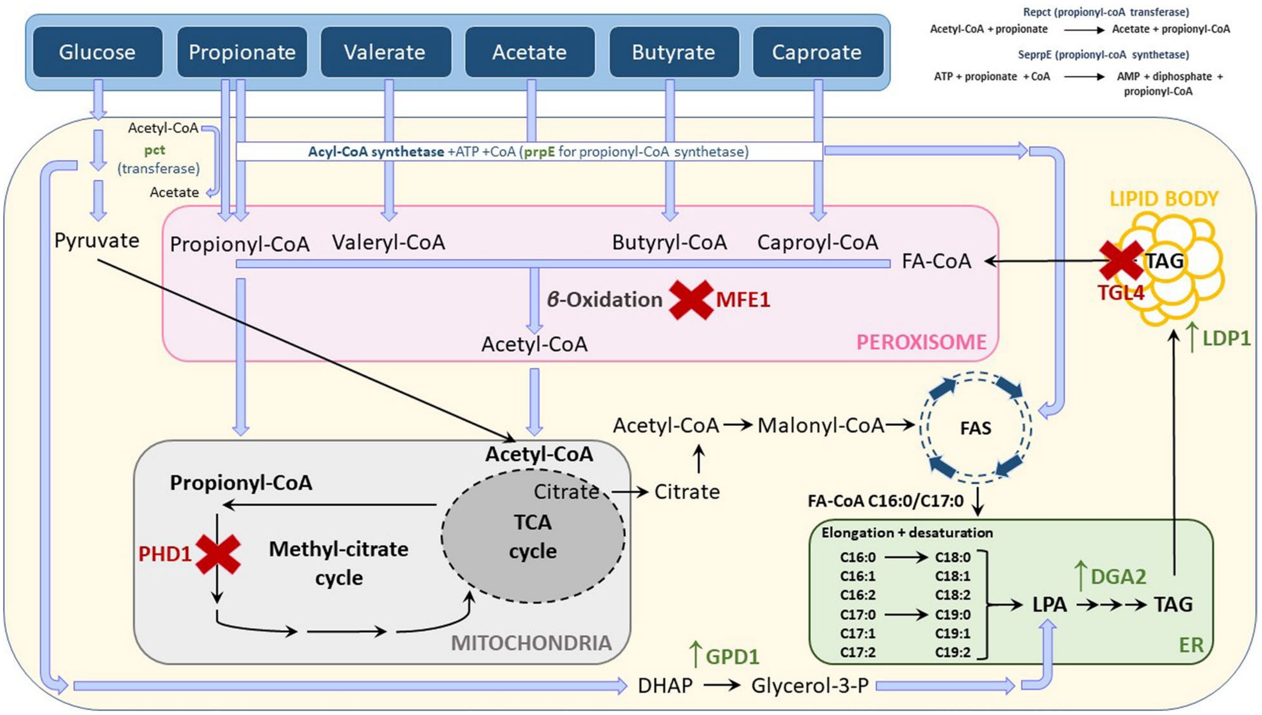


Fig. 3 Overview of lipids synthesis of the two engineered strain modification for OCFAs production in *Y. lipolytica* (JMY7780 and JMY7782). Engineered steps by gene overexpression are written in green. Inactivated steps are marked with a red cross, and the corresponding genes are also labeled in red. *DGA2*, acyl-CoA: diacylglycerol acyltransferase; *LDP1*, lipid droplet protein; *PHD1*, 2-methyl- citrate dehydratase; *TGL4*, triglyceride lipase; *MFE1*, multifunctional enzyme; TCA cycle, tricarboxylic acid cycle; TAG, triacylglycerol; FAS, fatty acid synthase; ER, endoplasmic reticulum. Enzymes in the blue box on the top-right indicate those enzymes involved in acetyl-CoA formation from longer acids (butyric and caproic acids). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.). This figure has been adapted from [25]

Two step-batch oleaginous fermentation from SCFAs

An initial growth phase on glucose was applied to rapidly increase cell biomass growth and activity, and ultimately promote SCFAs consumption and lipid production [30]. After 18 h of cell culture in 20 g/L glucose, 16.5 g/L and 19 g/L of glucose were consumed and 15–20 and 25–40 of OD were achieved by JMY7780 and JMY7782, respectively (data not shown). After this glucose phase, cells were transferred to a SCFAs-rich digestate with 10 g/L SCFAs (G + BATCH10) or 15 g/L SCFAs (G + BATCH15). Previous studies showed that after an initial growth in glucose, the addition of acetic acid mediated a lag phase of 20 h, with a decrease in CDW [9]. Strikingly, in this study, no lag phase was observed in the SCFAs phase in neither of the strains, in terms of initial consumption independently of cells pre-cultured with glucose or directly with SCFAs, both under 10 g/L and 15 g/L (Fig. 1 and Fig. 4).

In this two-step batch strategy, both strains needed 24 h more to exhaust all SCFAs in G + BATCH10 than in BATCH10 (Table 2, Fig. 1C, D and Fig. 4B, C), probably as a result of the different metabolic pathways employed by the yeast to assimilate glucose and acetic acid and the rest of SCFAs. During the glucose phase, all carbon source is metabolized via *de-novo,* as it is probably also the case for acetic acid. However, a metabolism shift towards the *ex novo* route could be needed to metabolize the rest of SCFAs [7, 34], which could result in a longer fermentation. Thus, once acetic acid is depleted a shift in the SCFAs uptake could be needed taking them longer to start consuming the rest of the acids. The OD remained stable until the end of the experiments. On the other hand, opposite to what was observed in BATCH15, JMY7780 and JMY7782 did not completely consume all SCFAs in G + BATCH15 (Table 2, Fig. 1E, F and Fig. 4D, E). Thus, the increased biomass from the glucose phase did not avoid SCFAs inhibition at concentrations of 15 g/L. This concentration was too challenging for JMY7780 and JMY7782, probably due to the genetic modifications that affected the acid tolerance when compared to other wild type strains. For example, *PHD1* inactivation blocks the TCA cycle, which would lead to a slower growth and a higher sensitivity to propionic acid [25]. Deletion of MFE1, a multifunctional enzyme, involved in β-oxidation would lead to a higher lipid accumulation but affect certain fatty acids consumption at high concentrations [21, 25]. In this sense, *PHD1* inactivation and *MFE1* deletion, could compromise *Y. lipolytica* acids tolerance by impairing stress response mechanisms and disrupting pH regulations intracellularly. This could lead to the accumulation of undissociated SCFAs within the cell, inducing a toxic response that could affect growth and lipid accumulation at high SCFAs concentrations [25].

In both G + BATCH10 and G + BATCH15, JMY7782 showed higher biomass yield (0.26 g/g and 0.28 g/g, respectively) than JMY7780 (0.18 g/g and 0.19 g/g, respectively). Pereira et al., [30] also observed a higher biomass yield when yeasts are first grown on glucose as result of the use of this carbon source for growth while also avoiding the slightly detrimental effect of SCFAs. In the same manner, glucose consumption rate in G + BATCH15 for JMY7782 was higher than on the SCFAs phase for the same fermentation (1.06 g/Lh vs 0.32 g/Lh, Table 2).

When yeast was previously grown in glucose, SCFAs consumption followed a similar trend than that attained for single batch fermentations. *Y. lipolytica* showed a preference for acetic acid, reaching the highest consumption rate (0.18 g/Lh) for this acid in both strains. Strikingly, when compared with the BATCH10, a faster acetic acid consumption rate was observed in two-step batch strategy (0.16 g/Lh vs 0.26 g/Lh for both strains in BATCH10 and G + BATCH10, respectively) (Table 2). The initial growth phase in glucose for 18 h, increased the yeast biomass concentration, promoting faster acetic acid consumption, in accordance with [28, 30]. On the other hand, a maximum of 168 h was needed to exhaust the rest of acids for BATCH10 and G + BATCH10 (Fig. 1C, D and Fig. 4B, C).

When comparing the results obtained in BATCH15 with those obtained under G + BATCH15, similar results were observed for both strains. Acetic acid was the only acid completely consumed. Valeric, butyric and caproic acids were partially consumed, whereas the other three acids (propionic, iso-butyric and isovaleric acids) were not consumed by any of the strains (Fig. 1E, F and Fig. 4D, E). These results agreed with other studies were acetic acid is the first one assimilated, followed by butyric acid [12, 30].

Under both two-step batch conditions, JMY7782 achieved higher lipid yield (0.096 g/g and 0.1 g/g, G + BATCH10 and G + BATCH15, respectively) than JMY7780 (0.053 g/g and 0.056 g/g for G + BATCH10 and G + BATCH15, respectively). Furthermore, lipid yields in the two-step batch strategy were higher than those achieved in BATCH10 and BATCH15 for both strains (Table 2).

Glucose utilization is easily metabolized by the yeast for acetyl-CoA accumulation, which is then used in the de novo FA synthesis [30, 34]. This two-stage can increase the total lipid production as a result of the initial growth on glucose. Lipid content under a two-step batch varied between 29.9% w/w and 40.8% w/w (Fig. 5). As in the single batch fermentations, JMY7782 showed the highest

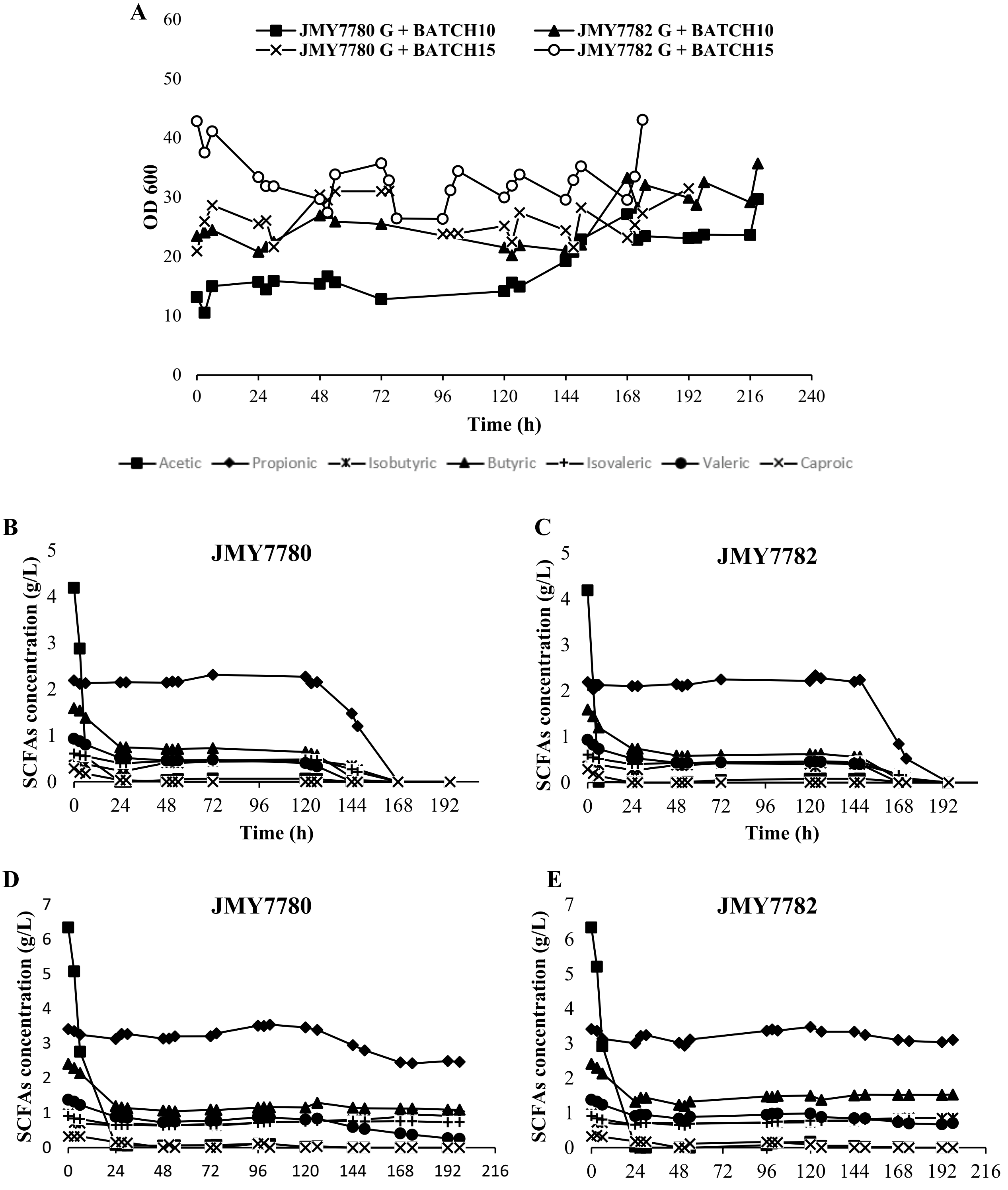


Fig. 4 Time courses for strains cell JMY7780 and JMY7782. Growth (OD) (**A**) and SCFAs consumption in G + BATCH10 (**B**, **C**) and G + BATCH15 (**D**,**E**). G + BATCH indicates growth on glucose for 18 h prior growth on SCFAs (10 g/L or 15 g/L). The points herein indicated represent average values

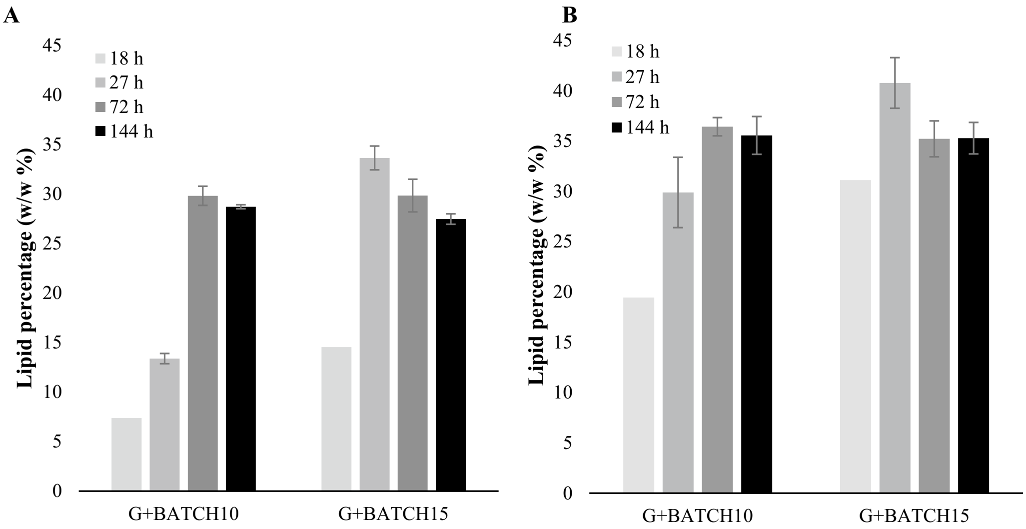


Fig. 5 JMY7780 **(A)** and JMY7782 **(B)** lipid content (w/w) (%) when cultivated in a two-step batch fermentation. G + BATCH indicates growth on glucose for 18 h prior growth on SCFAs (10 g/L and 15 g/L) at different time points. Light gray (•) indicate the lipid accumulated only in the glucose phase of the two-step batch. The bars herein indicated represent average values and standard deviations

lipid accumulation in two-step batch approach independently of the SCFAs concentration (Table 2).

In the two-step batch fermentation, due to pre-culturing in glucose, higher lipid concentrations than in batch were expected (Table 2). However, even though the total lipid yield was higher for the two-step batch, the lipid content during the SCFAs feeding phase, was lower to that obtained for the batch fermentations in which only SCFAs were used (Table 2). In the case of two-step batch fermentations, variations in lipid content were seen between the different time points. The lowest lipid content was reached at 27 h, except for G + BATCH15, (only considering the amount reached in the period fed with SCFAs), where only acetic acid was consumed. In G + BATCH15, the highest lipid content was reached at 27 h for both strains (Fig. 5). This could be explained by the SCFAs consumption profile, as no other acids besides acetic acid were significantly consumed after this point (Fig. 4D, E). After 27 h, lipids were probably used as a reservoir decreasing the intracellular lipid content from 33.6% w/w at 27 h to 27.5% w/w after 144 h for the JMY7780 strain, and 40.8% w/w to 35.3% w/w for the JMY7782 strain. When comparing the lipids produced in two-step batch experiments (considering only those produced in the phase in which yeasts were fed with SCFAs) to those in the batch experiments, lipid yield was very similar, showing no benefits of pre-culturing in glucose. When pre-cultured in glucose and then transferred to SCFAs, yeast have directed their metabolism towards growth, maintaining a high OD.

Odd-chain fatty acids production

Since the metabolic pathways for conversion of SCFAs into lipids are partly unknown, obtaining modified yeast capable of producing high OCFAs concentrations is still challenging. The JMY7782 strain did not only achieve the highest biomass and lipid content (Table 2), but also the highest percentage of OCFAs out of the total lipid (% w/w), reaching up to 50.7% when grown in G + BATCH10. Besides not showing big differences in terms of biomass and lipid yields when only considering the SCFAs phase, the two-step batch configuration allowed higher biomass concentration and total lipid content when compared to the batch fermentations as the result of the glucose phase (Table 2).

High OCFAs percentage were also obtained in BATCH15 and G + BATCH15, being 49.6% w/w and 49.9% w/w, respectively (Fig. 6C). Out of all OCFAs, C17:1 (heptadecenoic acid) was the most prevalent for every condition (Fig. 6A and B). For JMY7780, the highest % OCFAs were achieved in BATCH15 and G + BATCH15 (45.76% w/w and 45.90% w/w, respectively) (Fig. 6C). A slightly decrease in OCFAs was determined when transferring the yeast cells from glucose to SCFAs (G + BATCH) for JMY7780 but not for JMY7782 (Fig. 6C), probably as a result of their engineered background. The decrease was probably from the use of the glucose molecules (C6) and the metabolic changes in JMY7780 that resulted in the conversion of glucose into acetyl-CoA in favor of even-chain fatty acids (ECFAs) [25].

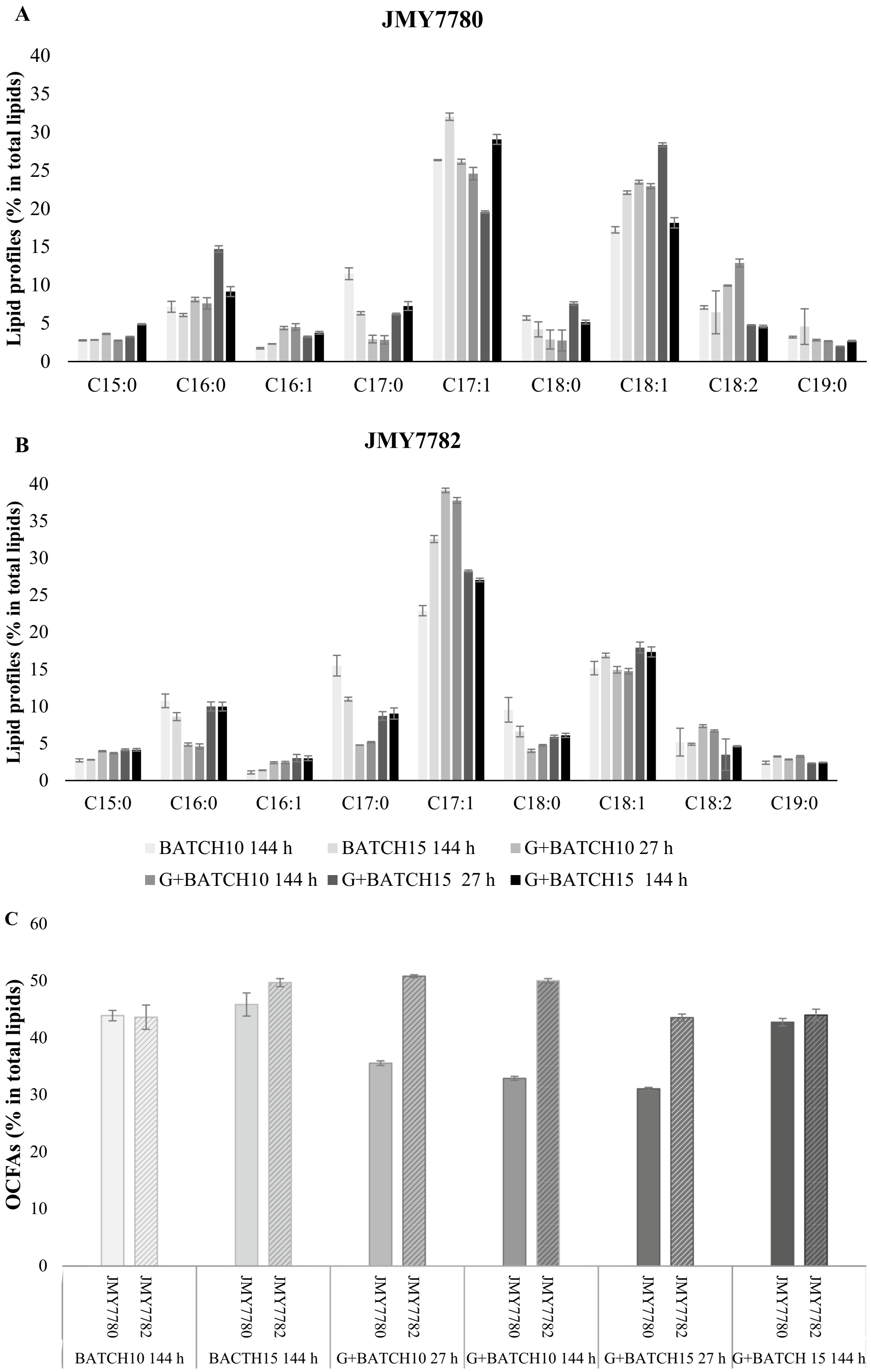


Fig. 6 Fatty acid distribution in different process strategies. (**A**) JMY7780; **(B)** JMY7782. (**C**) OCFAs in total lipid (%) at 27 h and 144 h for both strains under each SCFAs concentration. G + BATCH indicates previous growth on glucose for 18 h prior growth on SCFAs

Y. lipolytica CCY 29–26-36 achieved a lipid content of 8.9% w/w and 10.2% w/w of CDW on a 4 g/L propionic acid media and on a glucose media with 4 g/L propionic supplementation, respectively. This strain reached an OCFA content of 30% w/w and 15% w/w of the total lipids [13]. When *Y. lipolytica* JMY8438 strain was grown on a glucose:propionate:acetate ratio of 4:1:2 (% w/v), the % OCFA reached 60% [25]. Other approaches overexpressed the RePCT enzyme together with the deletion of the mitochondrial carrier Y1*crc1* involved in Carnitine/Acetyl-Carnitine shuttle in *Y. lipolytica* [22]. An OCFAs accumulation increase was reached with a total of 0.54 ± 0.14 g/L of OCFAs out of all the total lipids were accumulated, but only when grown on glucose (3 g/L) and propionic acid (0.5 g/L), in the absence of acetic acid [22]. However, the results under the present study indicate that the higher presence of acetic acid and other even-SCFAs, led to a slightly higher production of ECFAs instead of OCFAs for both BATCH10 and BATCH15 but also for G + BATCH10 and G + BATCH15. Besides this, up to 50.7% of OCFAs was achieved in G + BATCH10 for JMY7782. Indeed, most of the research studies available in literature normally ends up with low OCFA content, 7% of OCFAs [5, 12, 18]. For this reason, it can be concluded that the metabolic changes introduced in both strains together with the fermentation conditions (batch and two-step batch) induced OCFAs formation.

Furthermore, in this case, real digestate with a complete mix of SCFAs has been used, which reduces the use of synthetic sugars and extra nutrients, leading to a more economical process while attaining similar OCFAs percentages. Besides the slightly low lipid yields, it can be concluded that JMY7780 and JMY7782 are an interesting alternative for OCFAs production.

Supplementing acetate for faster SCFAs consumption

Due to their outstanding characteristics for producing OCFAs from real SCFAs-rich digestates, and its performance in terms of SCFAs consumption and lipid yields, the strain JMY7782 was used to further investigate the effect of the most easily consumed carboxylate, acetic acid. In this case, a pulse of acetic acid (4 g/L) was added to the medium with 10 g/L SCFAs (BATCH10 + AA) after exactly 24 h of fermentation when the acetic acid was totally exhausted to reach again the initial concentration. The effect of the acetic acid pulse on yeast growth, acid consumption and lipid and OCFAs accumulation was thus evaluated. This strategy aimed at OCFAs accumulation by balancing the acetyl-coA pool needed for growth and the propionyl-coA presence for OCFAs synthesis (a high pool compared with acetyl-CoA is needed). This strategy also intended a faster acid consumption in the presence of acetic acid.

According to Figs. 1 and 4, other acids such as butyric, valeric and caproic acids can also be consumed more rapidly in the presence of acetic acid since the consumption of the longer-chain SCFAs may rely on the presence of acetate (Figs. 1C–F and 4B–E). As it can also be seen in Figs. 1C–F and 4B–E, after acetic acid exhaustion, the consumption of the rest of acids was slowed down. Therefore, a quicker consumption of all the acids was expected when acetic acid was added again into the medium. The SCFAs consumption rate for JMY7782 increased from 0.019 g/Lh to 0.055 g/Lh for the first 48 h, without considering acetic acid, as the effect of this acid on the remaining was assessed (Fig. 7).

When comparing SCFAs consumption (Figs. 1D and 7A), butyric and valeric acids were consumed when acetic acid was added to the medium. When BATCH10 + AA was compared with BATCH10, butyric acid consumption rate was higher when acetic acid was added (0.023 g/Lh vs 0.017 g/Lh, respectively) and the same occurred for valeric acid (0.014 g/Lh vs 0.01 g/Lh, respectively). Propionic acid also had a higher consumption rate when using a pulse of acetic acid when compared with BATCH10 (0.004 g/Lh vs 0.001 g/Lh, respectively). These results pointed out that adding an acetate pulse, allowed a faster SCFAs consumption, maximizing carbon usage and avoiding the lost in the cultivation broth of unused acids as in BATCH15 and G + BATCH15.

The OD increased after 24 h in BATCH10 + AA (Fig. 7A) and the strain kept growing due to the extra carbon source availability. On the other hand, without the acetic acid addition (BATCH10), OD remained constant for the next 48 h (Fig. 1B). These results agreed with those from Pereira et al., [28], showing that a 6 g/L SCFAs (1:1:1 acetic, propionic, butyric acids) pulse enhanced yeast growth. However, Pereira et al., [28] observed that more than one pulse caused no biomass yield enhancement.

At 27 h (3 h after the acetic acid pulse), lipid content obtained in BATCH10 + AA was very similar to that obtained in BATCH10 (10.3% vs 10.5% w/w, respectively). At 48 h, all the added acetic acid was consumed and the concentration of remaining SCFA (g/L) was lower than in the BATCH10 (3.6 g/L and 4.7 g/L, respectively). Once again, acetic acid was the first acid to be consumed. Consumption of the rest of the acids stopped after acetic exhaustion for a 48-h long lag phase (Fig. 7A). After this time, consumption continued until SCFAs exhaustion at 168 h (Fig. 7A). These results agreed with those from Llamas et al., [20], that showed a codependence consumption of acids. Individual SCFAs consumption may not just depend on the specific acid but its utilization can be linked to the presence of other acids. As it can be seen in Figs. 1, 4 and 7A, the consumption of caproic acid may be linked to the presence of acetic acid.

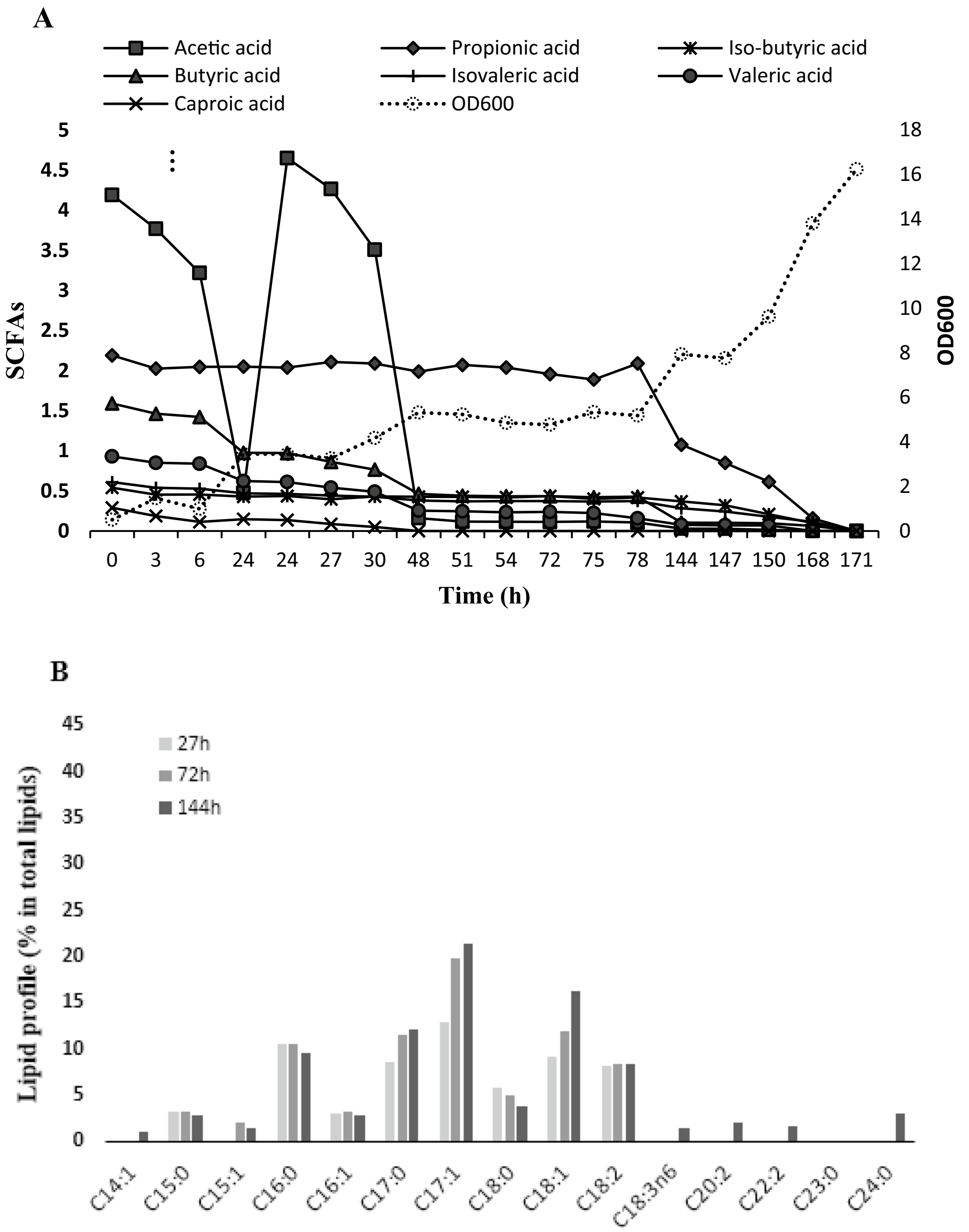


Fig. 7 **A** Time course for OD and SCFAs individual consumption (g/L) for *Y. lipolytica* JMY7782 during batch culture under 10 g/L SCFAs mixture, with addition of acetic acid (4 g/L) after 24 h. **B** Lipid profile at 27, 72 and 144 h for JMY7782 when grown at 10 g/L of SCFAs, followed by an acetic acid pulse (4 g/L) after 24 h

Lipid accumulation also increased at 144 h in BATCH10 + AA (25.2% w/w) when compared with BATCH10 (10.1% w/w) (Fig. 2B and Table 2). However, when comparing both FA profiles, similar percentages of C17:0 and C17:1 were accumulated with a lower concentration of C18 and C18:1 at 144 h (Figs. 6B and 7B).

The OCFAs content at 27, 72 and 144 h for the BATCH10 + AA experiment were 60.4, 56.1 and 46.4% w/w out of the total lipids, respectively. This demonstrated higher OCFA production in BATCH10 + AA (60.4%) than in BATCH10 (43.5%). Therefore, an acetic acid pulse was beneficial for the process performance as it increases the acetyl-CoA pool [25, 30]. Chalabi et al., [3] also achieved high OCFAs concentrations up to 67% OCFAs, when grown on synthetic media (20 g/L of sodium acetate and 5 g/L of sodium propionate), with *Y. lipolytica* JMY9178 strain overexpressing the *YlOLE1* and *YlDGA2* genes. Therefore, the balance between acetyl-CoA and propionyl-CoA is needed for OCFAs accumulation [25, 32]. Thus, acetate presence has been shown to be crucial for growth and to produce higher quantities of OCFAs as it is necessary for obtaining malonyl-CoA involved in fatty acid synthesis [25].

Contrary to what it was expected, the knowledge generated up to now would lead to the conclusion that the increase in acetyl-CoA would increment ECFAs at the expense of OCFAs for wild-type strains. However, the present research revealed that an acetic acid pulse allowed a higher OCFAs concentration, as a result of the metabolic changes carried out in the strain JMY7782. Future research on this topic should be focused on further optimization of the fermentation conditions. Furthermore, targeting genes involved in propionyl-CoA and other precursors of OCFAs synthesis like malonyl-CoA, may be crucial for developing novel strains capable of tolerating high propionic acid concentrations and achieving high concentrations both lipids and odd-chain fatty acids.

Conclusions

This investigation showed the potential of two genetically engineered strains, JMY7780 and JMY7782 for lipid and OCFAs accumulation, when utilizing real SCFAs-rich digestate. Lower SCFAs concentrations (10 g/L) allowed higher biomass and similar lipid yields than 15 g/L. However, 15 g/L exhibited the highest OCFAs content under batch and two-step batch fermentation, mainly for JMY7782. Two-step batch enhanced yeast metabolism, increasing the lipid content. Out of SCFAs, *Y. lipolytica* preferred acetic acid. Indeed, when reintroduced in the media as a pulse, OCFAs production was even higher. This result supports the fact that SCFAs consumption may rely on acetic acid presence.

Acknowledgements
This work has been supported by the Spanish Ministry of Economy and Competitiveness through the grand RYC2019-027773-I, BIOMIO (PID2020-119403RB-C21) supported by MCIN/AEI/https://doi.org/10.13039/501100011033 and the Ministry of Science and innovation for the predoctoral contract for the formation of future doctors (FPI 2021) (PID2020-119403RB-C21).

Author contribution
M.dV: Investigation, data curation, writing-original draft. E.TP: conceptualization, writing—review and editing, funding acquisition. JM.N: conceptualization and resources. C.GF: conceptualization, writing—review and editing, supervision, funding acquisition.

Availability of data and materials
The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/records/13928746 with DOI https://doi.org/https://doi.org/10.5281/zenodo.13928746

Declarations

Competing interests
The authors declare no competing interests.

Received: 24 October 2024 Accepted: 8 January 2025

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