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RESEARCH ARTICLE

Phosphate limitation as crucial factor to enhance yeast lipid production from short-chain fatty acids

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Abstract

Microbial lipids for chemical synthesis are commonly obtained from sugar-based substrates which in most cases is not economically viable. As a low-cost carbon source, short-chain fatty acids (SCFAs) that can be obtained from food wastes offer an interesting alternative for achieving an affordable lipid production process. In this study, SCFAs were employed to accumulate lipids using *Yarrowia lipolytica* ACA DC 50109. For this purpose, different amounts of SCFAs, sulfate, phosphate and carbon: phosphate ratios were used in both synthetic and real SCFAs-rich media. Although sulfate limitation did not increase lipid accumulation, phosphate limitation was proved to be an optimal strategy for increasing lipid content and lipid yields in both synthetic and real media, reaching a lipid productivity up to 8.95 g/L h. Remarkably, the highest lipid yield (0.30 g/g) was achieved under phosphate absence condition (0 g/L). This fact demonstrated the suitability of using low-phosphate concentrations to boost lipid production from SCFAs.

INTRODUCTION

Oil-based chemistry has been set as an interesting substitute to replace petroleum in the synthesis of chemicals such as polymers, plasticizers and lubricants (Zeng et al., 2018). Currently, the oleochemical industry is mainly based on animal fats and vegetable oils (Murawski & Quirino, 2018). Nevertheless, their limited supply, high cost and competition with food necessities are some of the drawbacks that hinder the development of the oleochemical production from these materials (Schmidt et al., 2015). The fatty acid composition of microbial lipids produced in oleaginous microorganisms has high similarities with vegetable oils (Di Fidio et al., 2020) and is receiving considerable attention to serve as precursors of oleochemicals. Among other advantages, microorganisms require shorter incubation periods and lower volume surface than plants (Cho & Park, 2018).

Oleaginous yeasts present the capacity to produce over 50% w/w lipids per dry weight (DW) using different carbon sources (Chatterjee & Mohan, 2018). In this context, *Yarrowia lipolytica* is by far the most extensively studied oleaginous yeast due to its capacity to consume hydrophobic and hydrophilic feedstocks achieving high lipid yields. Furthermore, in the last years, there has been great progress in the knowledge of its genome and the availability of novel biological tools for its genetic modification (Abdel-Mawgoud & Stephanopoulos, 2020; Ramesh et al., 2020).

Studies on lipid production have traditionally used sugars as feedstock (Carsanba et al., 2020;

Juanssilfero et al., 2018; Tanimura et al., 2018; Yamada et al., 2017). However, given the high cost of these carbon sources (Patel et al., 2018), short-chain fatty acids (SCFAs) have been set as interesting alternatives to establish an economically viable production of microbial lipids. SCFAs are organic acids that can be obtained through anaerobic fermentation of organic wastes. Recent investigations have indeed shown that SCFAs are promising feedstocks for lipid obtention (Llamas, Tomás-Pejó, & González-Fernández, 2020; Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022) and could be, thereby, a solution against the high cost of sugar substrates while offering a novel route for organic waste valorization.

Nitrogen limitation has been extensively reported to promote lipid production after a metabolic shift from cell growth to lipid synthesis in yeast (Hapeta et al., 2020). Nevertheless, some organic wastes often exhibit an important amount of nitrogen, thus preventing yeasts from efficient lipid production (Wang et al., 2018). To circumvent this issue, the limitation of some other nutrients can be considered to trigger a shift toward the desired metabolism. For instance, the limitation of inorganic sulfate (SO_4^{2-}) and/or phosphate (PO_4^{3-}) has also been proposed to promote lipid production (Huang et al., 2018; Wu et al., 2011) from sugars. Although the effect of these two ions on yeast metabolism is well known when sugars are used as carbon source, the knowledge about cellular responses to SO_4^{2-} and PO_4^{3-} limitations and possible implications on lipid accumulation in yeast grown in presence of other substrates are still scarce. Similar to nitrogen, these anions are also present in the low-cost residues employed in the production of SCFAs (Ma et al., 2018; Montalvo et al., 2019), although their remaining amount after anaerobic fermentation tends to be low. This work was designed to unravel the interplay between nitrogen, SO_4^{2-} and PO_4^{3-} anions limitations to favour lipid accumulation in *Y. lipolytica* when using SCFAs as carbon sources. To elucidate the effect of SO_4^{2-} and PO_4^{3-} limitation, different concentrations of these two anions, as well as different ratios of carbon: phosphate (C:PO_4^{3-}), were evaluated. Yeast growth, SCFAs consumption and lipid production were studied on synthetic and real SCFAs-rich media.

EXPERIMENTAL PROCEDURES

Yeast strain and preinoculum preparation

The oleaginous yeast used in this study due to its ability to accumulate more than 20% w/w DW and consume SCFAs (Dourou et al., 2017) was *Y. lipolytica* ACA DC 50109 (from the Agricultural University of Athens' culture collection). The yeast was preserved under the conditions described in Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022. One colony of the strain was inoculated in YPD liquid medium (20 g/L peptone, 20 g/L glucose and 10 g/L yeast extract), with an initial pH of 7.0 (no adjustment was needed), in order to prepare a preinocula which was incubated overnight at 150 rpm and 27 °C in a rotary shaker until the late exponential growth phase was reached.

Fermentation media and conditions

The composition of synthetic media (SM) and real digestates (RDs) used in this study is shown in Table 1. The purity of the chemicals used for the preparation of the SM was ≥99.9%.

SM was prepared based on the profiles and concentrations showed on RD 1. To elucidate the effect of SO_4^{2-} limitation on lipid production and yeast growth, SM was composed of Delft media (0.5 g/L $(MgSO_4)_7H_2O$, 15 g/L SCFAs and 2 ml of trace metals solution) with different concentrations of $(NH_4)_2SO_4$: 0 g/L, 3.8 g/L, 5.6 g/L, 7.5 g/L, being 7.5 g/L the amount normally present in Delft media. These different concentrations corresponded to total SO_4^{2-} of 0 g/L, 2.7 g/L, 4.1 g/L and 5.4 g/L (nitrogen levels were adjusted with NH_4Cl). In the case of PO_4^{3-}, different amounts of KH_2PO_4 were added (0 g/L, 7.2 g/L, 10.8 g/L, 14.4 g/L, being 14.4 g/L the amount normally present in Delft media) in SM to achieve four different concentrations of total PO_4^{3-}: 0 g/L, 5.1 g/L, 7.6 g/L and 10.1 g/L.

The RD rich in SCFAs (caproic, valeric, butyric, propionic and acetic acid) were obtained by the centrifugation at 5000 rpm for 30 min (Heraeus, Megafuge 16, Thermo Scientific Fiberlite™ F15-6 × 100y) of the effluents attained by Greses et al. (2020) from

TABLE 1 Media composition.

Medium	SCFAs concentration[a] (g/L)					
	Acetic	Propionic	Butyric	Valeric	Caproic	Total
SM	2.6	1.2	6.3	2.4	2.6	15.0
RD 1	2.6	1.2	6.3	2.4	2.6	15.0
RD 2	1.7	0.8	4.2	1.6	1.7	10.0
RD 3	0.9	0.4	2.1	0.8	0.9	5.0

[a]The isobutyric and isovaleric values were negligible.

food wastes. After centrifugation, particle removal and sterilization were conducted by filtration using a 0.2 μm vacuum filter (PES membrane, Sterile, VWR North America).

RD with 15 g/L of total SCFAs was used to also prove the effect of PO_4^{3-} on lipid production and yeast growth. The same amounts of KH_2PO_4 mentioned above were added to RD 1, except for the 0 g/L which in this case was 1 g/L (value corresponding to the initial amount of KH_2PO_4 present in RD 1). For RD, total KH_2PO_4 was calculated considering the amount of PO_4^{3-} presented in media. RD 1 did not undergo any further modification, and it was diluted to reduce the amount of SCFAs presented in the medium to 10 g/L and 5 g/L of total SCFAs to obtain RD 2 and RD 3, respectively. Since the nitrogen and PO_4^{3-} content also decreased with the dilution, NH_4Cl and KH_2PO_4 were added to reach the initial nitrogen and PO_4^{3-} concentration exhibited by RD 1 (0.5 g N-NH_4^+/L and 1 g PO_4^{3-}/L).

RD 1, 2 and 3 had an initial PO_4^{3-} concentration of 1 g/L and 6, 4 and 2 g/L of carbon, respectively. Thus, C:PO_4^{3-} ratios in RD 1, 2 and 3 were 6, 4, and 2, respectively. Total carbon (g/L of carbon) was calculated considering the amount of carbons in each acid and the g/L of the corresponding acid added to the media.

RD 1, 2 and 3 were also supplemented with KH_2PO_4 to attain a final concentration of 10.1 g PO_4^{3-}/L to reduce the C:PO_4^{3-} ratios to 0.6, 0.4 and 0.2, respectively. Total PO_4^{3-}, SO_4^{2-} and C:PO_4^{3-} ratios were selected based on previous reports (Wang et al., 2018; Wu et al., 2010, 2011).

Fermentations were performed in triplicates in Baffled Erlenmeyer flasks of 250 ml. In total, 100 ml of medium at pH 6.0, previously adjusted using NaOH 3 M, was added to each flask. Initial optical density at 600 nm (OD_{600}) of 1 was used to inoculate each shake flask. This initial optical density was equivalent to 0.5 g dry weight cells/L. Fermentations were incubated at 170 rpm and 27 °C in a rotary shaker until 95–100% of the SCFAs were assimilated.

Fermentation samples were periodically withdrawn to analyse SCFAs concentration and yeast growth. Lipid content in yeast cells was analysed at the end of the fermentation period.

Analytical methods

SCFAs measurement

SCFAs were quantified by liquid chromatography as described in Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022. In total, 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml/min and was used in isocratic mode for the elution. Temperatures for the detector and oven were set at 35°C and 50°C, respectively.

Media composition and yeast growth determination

The PO_4^{3-} present in both SM and RD media was calculated by the Vanadate-Molybdate colorimetric method (Tandon et al., 1968). A Spectroquant® Pharo 100 spectrophotometer was employed to measure cell growth at OD_{600}. Dry biomass was calculated using a previously established standard curve, which correlates the OD of the culture with the yeast biomass production (g/L).

The amount of SO_4^{2-} present in each medium was calculated according to the final concentrations to be achieved in the synthetic media, as indicated in Section 2.2.

Lipid determination

Lipid content was calculated by fluorimetrical means using the optimized protocol described in Morales-Palomo, Liras, et al., 2022. The data obtained from the area of each fluorescence curve were converted to quantum yield.

Lipid productivity was calculated using the following formulas:

Biomass productivity (g/L h) = (B1 – B2) / (t2– t1), where B1 and B2 are defined as the biomass in g/L at the beginning (t1) and at the end of the fermentation (t2) (expressed in h).

Lipid productivity (g/L h) = biomass productivity (g/L h) × lipid content (% w/w), where lipid content was obtained by means of the fluorescence protocol mentioned above.

Data analysis

Data assessment of cell growth (OD_{600}) and yeast lipid content (% w/w) was performed by using a parametric one-way ANOVA (confidence interval 95%). Differences were considered significant at p-value <0.05.

RESULTS AND DISCUSSION

Effect of SO_4^{2-} limitation in yeast growth and lipid production in synthetic SCFAs-rich media

SO_4^{2-} is a vital compound for microorganisms that is mainly incorporated into special amino acids and cellular cofactors such as biotin, cysteine, methionine, coenzyme A, thiamine and iron–sulfur clusters (Jayanthi & Thalla, 2019). SO_4^{2-} limitation has been shown to trigger lipid production by channelling excessive carbon from sugars toward lipid synthesis rather than to

cell growth (Wu et al., 2011). To evaluate the potential of SO_4^{2-} limitation for increasing lipid production in *Y. lipolytica* when using SCFAs as carbon sources, SM was prepared with four different concentrations of total SO_4^{2-} (Table 2), as described above (Section 2.2). In order to mimic the same C:N ratio presented in RD 1, the C:N ratio in SM was set at 12 (1.3 g N-NH_4^+/L). Under such low C:N ratio, the expected lipid accumulation would be low, considering the importance of high C:N ratios when using both sugar-based and SCFAs-rich media for oleaginous yeast fermentation (Amza et al., 2019; Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022; Wei et al., 2019).

Time courses for cell growth and SCFAs consumption at different SO_4^{2-} concentrations and C:N 12 are shown in Figure 1. No significant differences were observed in terms of lag phase ($p > 0.05$) (Figure 1A) independently of the SO_4^{2-} concentration. However, when 5.4 g SO_4^{2-}/L (no SO_4^{2-} limitation) were used, *Y. lipolytica* was able to consume all the SCFAs (Figure 1B), reaching a biomass of 9.5 g/L and a lipid content of 22.7% lipids w/w DW (Table 2). A significant decrease in cell growth down to 6.3, 6.1 and 4.8 g/L was observed with 4.1, 2.7 and 0 g SO_4^{2-}/L, respectively. Moreover, 1.5, 1.6 and 1.8 g/L of SCFAs remained in the media when SO_4^{2-} concentration was decreased to 4.1, 2.7 and 0 g SO_4^{2-}/L, respectively, indicating that SO_4^{2-} limitation affected the carbon source assimilation. Lipid content and lipid yields were also lower in those cases where the SCFAs were not completely consumed.

Despite the decrease in cell growth and lipid production (g/L), no significant differences were observed in lipid content with 4.1, 2.7 and 0 g SO_4^{2-}/L ($p > 0.05$) being 16.7, 18.3 and 17.8% lipids w/w, respectively. However, the lipid content and lipid yield were 1.3-fold and 2.3-fold lower, respectively, when comparing the SO_4^{2-} limited media (4.1, 2.7 and 0 g SO_4^{2-}/L) with no SO_4^{2-} limitation (5.4 g SO_4^{2-}/L) (Table 2). These results differ from Wu et al., (2011), who observed that SO_4^{2-} limitation led to increased production of lipids up to 58.3% lipids w/w DW in *Rhodosporium toruloides*. It has been suggested that *R. toruloides* could have a similar mechanism to *Rhodosporillum rubrum* to inhibit cell growth and redirect the carbon flux toward lipid accumulation under SO_4^{2-} limitation, which could explain the higher accumulation of lipids with such nutrient limitation (Melnicki et al., 2016). Moreover, Wu et al., (2011) used glucose as carbon source, which is assimilated via de novo pathway (Amza et al., 2019), whereas SCFAs used in this study are probably mainly assimilated via *ex novo* pathway (Papanikolaou & Aggelis, 2011) which could result on a different effect of SO_4^{2-} limitation.

Independently of the SO_4^{2-} level, lipid yields (Table 2) (0.06 g/g — 0.14 g/g) were quite low when compared with those previously reported from sugars or even SCFAs. Wang et al. (2020) described a maximum lipid yield of 0.32 g/g, 0.34 g/g and 0.30 g/g when using glucose, xylose and glycerol, respectively. When using individual SCFAs such as acetic acid and butyric acid, 0.16 g/g and 0.14 g/g were achieved, respectively (Gao et al., 2020). Furthermore, a recent study reported lipid yields as high as 0.31 g/g and 0.33 g/g from synthetic SCFAs-rich media and real SFCAs-rich media,

TABLE 2 Lipid yield (w/w) and lipid content (% w/w) obtained at the end of the fermentation time with different SO_4^{2-} and PO_4^{3-} concentrations in SM and RD 1; nd: Non-detected. The points herein indicated represent average values and standard deviations.

Anions concentration (g/L)	Lipid content (% w/w)	Lipid yield (w/w)
g SO_4^{2-}/ L in SM[a]		
5.4	22.7 ± 0.5	0.14 ± 0.01
4.1	16.7 ± 1.4	0.07 ± 0.01
2.7	18.3 ± 1.3	0.08 ± 0.01
0	17.8 ± 1.1	0.06 ± 0.01
g PO_4^{3-}/L in SM[b]		
10.1	23.1 ± 0.5	0.14 ± 0.01
7.6	22.1 ± 0.5	0.14 ± 0.01
5.1	23.0 ± 0.7	0.14 ± 0.01
0	44.4 ± 0.9	0.30 ± 0.01
g PO_4^{3-}/L in RD 1[b]		
10.1	nd	nd
7.6	nd	nd
5.1	18.7 ± 1.1	0.16 ± 0.02
1	37.7 ± 0.7	0.25 ± 0.01

[a]The PO_4^{3-} concentration in all media was 10.1 g PO_4^{3-}/L.
[b]The SO_4^{2-} concentration in all media was 5.4 g SO_4^{2-}/L.

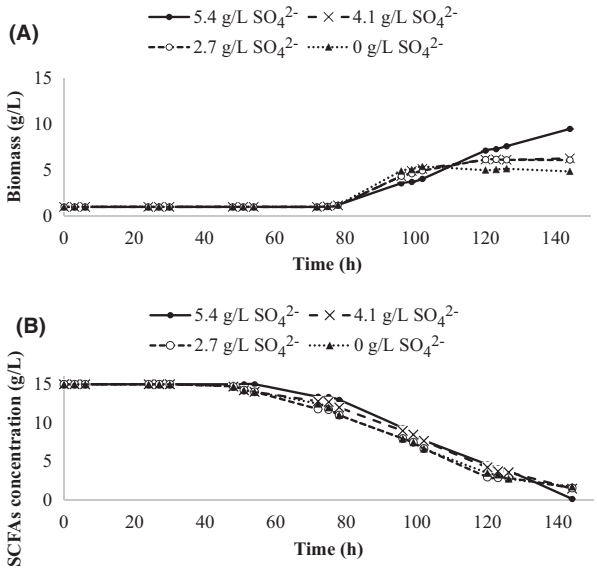

FIGURE 1 Time course of *Y. lipolytica* growth (A) and SCFAs consumption (B) in SM with different total SO_4^{2-} concentrations. The points herein indicated represent average values and standard deviations.

respectively (Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022). Based on these results, it could be concluded that SO_4^{2-} limitation was not an optimal strategy to increase lipid production in *Y. lipolytica* when using SCFAs as carbon sources.

Effect of PO_4^{3-} limitation in yeast growth and lipid production in synthetic SCFAs-rich media

Similar to SO_4^{2-} limitation, high yeast lipid content has been reported under PO_4^{3-} limitation (Bao et al., 2018; Nambou et al., 2014), even in nitrogen-rich media (Zhao et al., 2010). PO_4^{3-} is another crucial compound for several ubiquitous cofactors phosphorylated proteins and RNA and DNA synthesis. Thus, PO_4^{3-} limitation has key effects on cellular physiology and metabolism (Wang et al., 2018). To evaluate the potential of PO_4^{3-} limitation for increasing lipid production in *Y. lipolytica*, SM was prepared with four different concentrations of total PO_4^{3-}, as described above (Section 2.2). All these media also presented a C:N ratio of 12 (1.3 g N-NH_4^+/L), and thereby, low lipid content would be expected.

Yarrowia lipolytica growth and SCFAs utilization at different total concentrations of PO_4^{3-} are shown in Figure 2. As expected, when using 10.1 g PO_4^{3-}/L (no limitation), the yeast presented a similar lag phase (54 h), biomass production (9.8 g/L), lipid content (23.1% w/w DW) and lipid yield (0.14 g/g) than those in the experiment without SO_4^{2-} limitation (Section 3.1), because in both cases the fermentation media had the same initial concentrations of SO_4^{2-} and PO_4^{3-}. Nevertheless, contrary to what was observed with SO_4^{2-} limitation, *Y. lipolytica* presented shorter lag phases when the amount of PO_4^{3-} in the medium was reduced (Figure 2A). When 7.6 g PO_4^{3-}/L and 5.1 g PO_4^{3-}/L were used, the biomass production decreased to 7.2 g/L and 8.7 g/L, respectively, when compared with 9.5 g/L achieved in the 10.1 g PO_4^{3-}/L medium. However, no significant lipid content differences reached with 10.1, 7.6 and 5.1 g PO_4^{3-}/L were observed (% w/w) ($p > 0.05$) (Table 2). This was because lipid production (g/L) also decreased as the amount of total PO_4^{3-} diminished. Likewise, no significant differences were observed between the lipid yields achieved under these conditions. It should be noted that, contrary to what was observed with the limitation of SO_4^{2-}, the carbon source was exhausted in all media (Figure 2B). These results are in good agreement with Hoarau et al. (2020) who concluded that the addition of KH_2PO_4 resulted in a noteworthy enhancement of the yeast biomass production, which was 1.4-fold higher than that obtained with crude distillery spent wash (low total PO_4^{3-} concentration). Moreover, these authors demonstrated that the supplementation of PO_4^{3-} did not affect either lipid content (% w/w) or lipid composition. Based on these results, it could be stated that PO_4^{3-} reduction from 10.1 to 7.6 and 5.1 g PO_4^{3-}/L did not affect lipid content.

The biomass achieved in the medium with the most PO_4^{3-} limiting condition (0 g PO_4^{3-}/L) was similar to that obtained with 10.1 g PO_4^{3-}/L (Figure 2A). However, the lipid content and lipid yield increased to 44.4% w/w lipids DW and 0.30 g/g when no PO_4^{3-} was

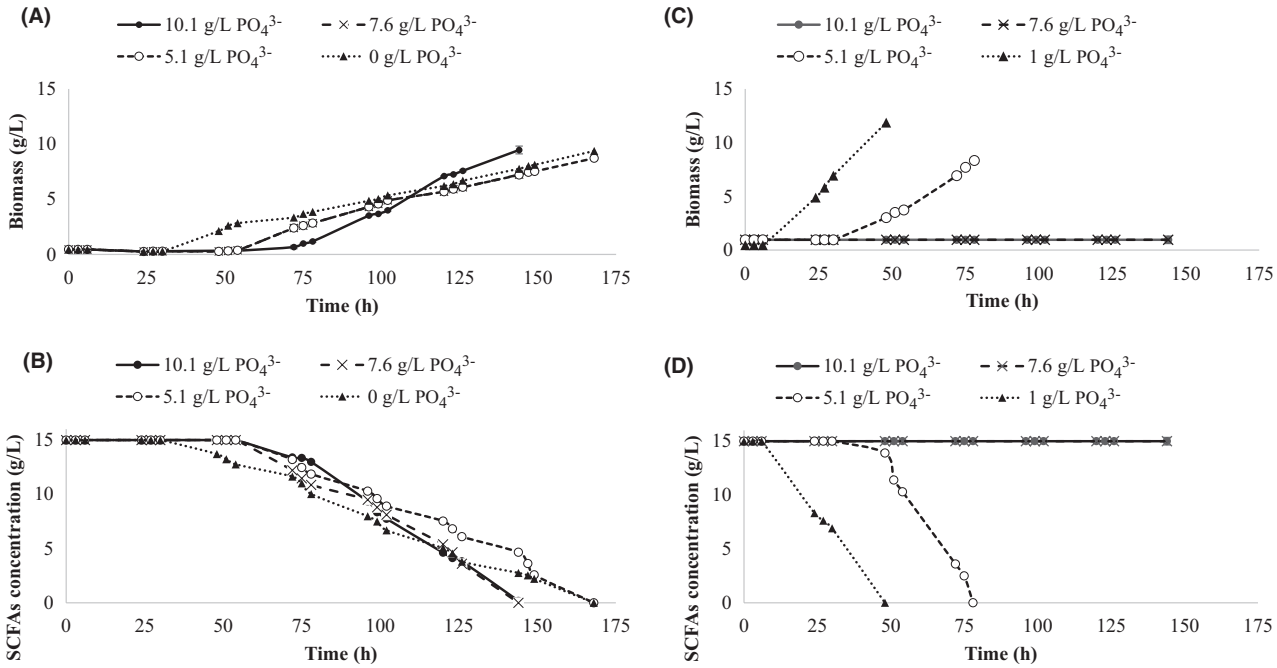

FIGURE 2 Time course of *Y. lipolytica* growth and SCFAs consumption in SM (A and B) and RD (C and D) with different total PO_4^{3-} concentrations. The points herein indicated represent average values and standard deviations.

present (Table 2), reaching a lipid productivity of 2.36 g/L h. In this case, the lipid yield was higher than those obtained using single SCFAs such as acetic and butyric acid as feedstocks and was similar to the lipid yields described from glycerol and sugars-based feedstocks (Gao et al., 2020). Indeed, with 0 g/L of PO_4^{3-}, the yeast used the carbon source more efficiently for lipid biosynthesis than for cell mass production. Lipid production under PO_4^{3-} limitation relies on a mechanism of regulation of diacylglycerol (DAG), NADPH supply and isocitrate dehydrogenation activity, where 817 and 585 and genes are down- and up-regulated, respectively (Wang et al., 2018). Under extreme PO_4^{3-} limitation, changes in metabolism can instantly restore intracellular PO_4^{3-} levels even before the PO_4^{3-} responsive gene-regulatory processes fully kick in (Gupta & Laxman, 2021). In this network of reactions, some of them will incorporate/consume PO_4^{3-}, while others will release it. A recent review also highlighted the fact that under this condition, cells can induce enzymes that release PO_4^{3-} from nucleotides as a last resort (Austin & Mayer, 2020). Considering this and the results attained in the present study, it can be inferred that *Y. lipolytica* ACA DC 50109 presented sufficient self-regulation methods to adapt to conditions with low concentration or even absence of PO_4^{3-}, switching its metabolism toward lipid production. Moreover, at 0 g PO_4^{3-}/L the yeast was capable of achieving a high lipid yield (0.30 g/g), which proved the suitability of using SCFAs-rich media as cost-effective alternatives to sugar-based substrates for lipid production in oleaginous yeasts.

Effect of PO_4^{3-} in yeast growth and lipid production in real digestate rich in SCFAs

SCFAs-rich digestate derived from food waste with 1 g PO_4^{3-}/L and a C:N ratio of 12 (1.3 g N-NH_4^+/L) was chosen to analyse the effect of PO_4^{3-} supplementation in real media (RD 1) (Table 1). As described in Section 2.2, PO_4^{3-} was also added to this medium to attain different total PO_4^{3-} concentrations (Table 2). When the medium without supplementation (1 g PO_4^{3-}/L) was used, *Y. lipolytica* barely presented a lag phase (24 h) (Figure 2C) and was able to consume all the SCFAs in 48 h (Figure 2D). Moreover, the yeast reached a biomass of 11.9 g/L and a lipid content of 37.7% w/w lipids DW (Table 2). It is worth mentioning that, although in this case the lipid content (% w/w) was 1.2-fold lower than that obtained when using the SM with 0 g/L PO_4^{3-}, the reduction in fermentation time led to an improvement in lipid productivity up to 3.8-fold higher (8.95 g/L h). This high growth has previously been reported in nutrient-rich environment characteristic of RD that improved yeast growth, even at high SCFAs concentrations (15 g/L) when compared with synthetic media with the same SCFAs profile and concentration (Morales-Palomo, González-Fernández, & Tomás-Pejó, 2022).

As can be observed in Figure 2C, the increase of PO_4^{3-} up to 5.1 g PO_4^{3-}/L led to a longer lag phase (48 h) and a decrease in biomass production (8.3 g/L) when compared with the medium with 1 g PO_4^{3-}/L. Even though all the carbon source was consumed in both cases (Figure 2D), lipid content was 2-fold lower in the case of 5.1 g PO_4^{3-}/L (18.7% w/w DW) when compared with 1 g PO_4^{3-}/L (37.7% w/w DW) (Table 2). Likewise, lipid yield also decreased from 0.25 g/g to 0.16 g/g when increasing PO_4^{3-} from 1 g PO_4^{3-}/L to 5.1 g PO_4^{3-}/L. When the amount of PO_4^{3-} was further increased up to 7.6 g PO_4^{3-}/L and 10.1 g PO_4^{3-}/L, yeast growth was completely restricted (Figure 2C). These results supported the aforementioned hypothesis and demonstrated the applicability of PO_4^{3-} limitation to enhance lipid production when *Y. lipolytica* grows in presence of SCFAs as carbon source. The lack of growth in presence of high PO_4^{3-} concentration differs from what was observed with 10.1 g PO_4^{3-}/L and glucose as carbon source, in which case, cell growth was not inhibited (Konzock & Norbeck, 2020). Thus, it can be inferred that high amounts of PO_4^{3-} could inhibit biomass production when using SCFAs as carbon sources, while its limitation could improve lipid production. These facts also highlight that the conditions favouring lipid production from sugar-based substrates should not similarly affect lipid production in yeasts that are thriving in SCFAs.

Effect of C:PO_4^{3-} in yeast growth and lipid production in real media

To further evaluate whether the observed effect of high amounts of PO_4^{3-} on yeast growth was due to the PO_4^{3-} itself or to a low C:PO_4^{3-} ratio, three RD with different SCFAs concentrations (5, 10 and 15 g/L) (Table 1) and two different PO_4^{3-} concentration (1 g PO_4^{3-}/L and 10.1 g PO_4^{3-}/L) were prepared (Section 2.2). The C:N ratio was set at 12 in all cases.

As observed in Figure 3A, when the media without PO_4^{3-} supplementation (1 g PO_4^{3-}/L) was used, the increase of C:PO_4^{3-} ratio from 2 (with 5 g/L of SCFAs) to 6 (with 15 g/L of SCFAs) did not affect yeast growth and *Y. lipolytica* was able to assimilate all the SCFAs (Figure 3B). However, 15 g/L SCFAs in C:PO_4^{3-} ratio of 6 resulted in a longer time required to exhaust the SCFAs when compared to C:PO_4^{3-} ratio of 2. Nevertheless, the lipid content (% w/w) and the lipid yield (g/g) increased concomitantly with the increasing SCFAs concentration (Table 3). These results corroborated the hypothesis that low amounts of PO_4^{3-} and high concentrations of the carbon source, which translates into high C:PO_4^{3-} ratios, improved lipid

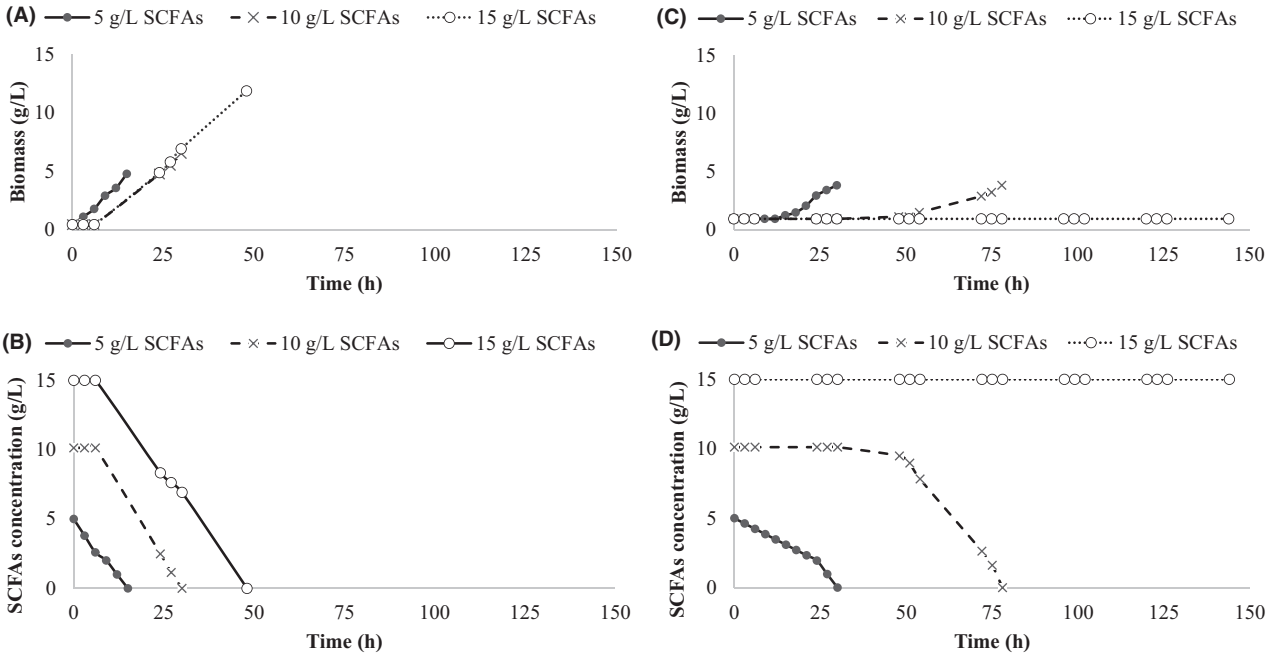

FIGURE 3 Time course of *Y. lipolytica* growth and SCFAs consumption in RD with 1 g PO_4^{3-}/L (A and C) and in RD with 10.1 g PO_4^{3-}/L (C and D) with different SCFAs concentrations and C:PO_4^{3-} ratios. The points herein indicated represent average values and standard deviations.

TABLE 3 Lipid content (% w/w) and lipid yields obtained at the end of the fermentation time with different SCFAs concentrations and C:PO_4^{3-} ratios in RD; nd, non-detected. The points herein indicated represent average values and standard deviations.

	C:PO_4^{3-} ratio[a]					
	2	**4**	**6**	**0.2**	**0.4**	**0.6**
Lipid content (% w/w)	8.9 ±0.5	14.1 ±0.7	38.1 ±0.6	9.1 ±0.5	9.2 ±0.9	nd
Lipid yield (w/w)	0.08 ±0.01	0.09 ±0.01	0.25 ±0.01	0.07 ±0.01	0.04 ±0.01	nd

[a]The SO_4^{2-} concentration in all media was 5.4 g SO_4^{2-}/L.

production. The decrease in C:PO_4^{3-} (by supplementing PO_4^{3-} in the media) significantly affected yeast growth (Figure 3C). In the medium with a C:PO_4^{3-} ratio of 0.2 containing 5 g/L of SCFAs, the yeast showed a short lag phase of 6 h and the biomass and lipid content decreased down to 3.8 g/L and 9.1% lipids w/w DW, respectively (Table 3). The lag phase was prolonged to 30 h in the medium with a C:PO_4^{3-} ratio of 0.4 with 10 g/L of SCFAs. In this case, biomass and lipid content of 3.8 g/L and 9.2% lipids w/w DW were attained, respectively. Nevertheless, no significant lipid yield differences were observed (g/g) ($p > 0.05$) when comparing C: PO_4^{3-} 0.2 and 0.4. It should be noted that in both cases all the carbon source was exhausted (Figure 3D). Similar results were observed by Wu et al. (2010), who also observed a decrease in lipid content and yeast growth (% w/w) when decreasing C:PO_4^{3-} ratio in the medium culturing *R. toruloides* with glucose. When a C:PO_4^{3-} ratio of 0.6 (corresponding to 10.1 g/L PO_4^{3-}) in presence of 15 g/L of SCFAs was used, the yeast was not able to grow, which could suggest that high PO_4^{3-} concentrations may be inhibitory for yeast. Although it was expected that a concentration of 15 g/L of SCFAs would inhibit yeast growth (Fontanille et al., 2012; Gao et al., 2017), results showed no inhibitory effect when media without PO_4^{3-} supplementation were used (Figure 3A,B). It is worth mentioning that in none of the previously mentioned investigations, neither the amount of total PO_4^{3-} nor the C:PO_4^{3-} ratio was considered as a possible limiting factor. These results confirm that yeast's growth and lipid production are not only affected by total SCFAs concentration but by C:PO_4^{3-}. Specifically, the combination of high amounts of SCFAs and low C:PO_4^{3-} ratio (high PO_4^{3-} concentration) could inhibit yeast growth. In the same line, the knowledge generated herein provides a new strategy to enhance lipid production by properly tuning the amount of PO_4^{3-} when using SCFAs as carbon sources. It should be noted that PO_4^{3-} concentration in effluents derived from anaerobic fermentation of food wastes tends to be low, contrary to what happens with nitrogen content (Huang et al., 2018). In this sense, it would be much feasible to achieve high

C:PO_4^{3-} ratios than high C:N ratios without the need to tune the medium. Thus, it would be interesting for future research to elucidate the effect that different substrates can have on yeast growth and metabolism under PO_4^{3-} limitation conditions.

CONCLUSION

The limitation of SO_4^{2-} was not a suitable approach to increase lipid production in *Y. lipolytica* ACA DC 50109 using SCFAs as carbon source. Alternatively, PO_4^{3-} limitation was proven to be a promising strategy to improve lipid production, even in media with low C:N ratios as waste-derived digestates, achieving lipid yields similar to or even higher than those obtained when using sugar-based media and single SCFAs as substrates. These results provide a new insight into the potential of using cheap effluents with high SCFAs and nitrogen content as interesting alternatives to improve the economic viability of lipid production process.

AUTHOR CONTRIBUTIONS

Sergio Morales-Palomo: Conceptualization (equal); formal analysis (equal); investigation (lead); writing – original draft (lead). **Elia Tomás-Pejó:** Conceptualization (equal); funding acquisition (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Cristina González-Fernández:** Conceptualization (equal); funding acquisition (equal); resources (equal); supervision (equal); writing – review and editing (equal).

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