Environmental Technology & Innovation 36 (2024) 103767

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Phosphate chelation over calcium impacts yeast growth and lipid production from short-chain fatty acids-rich media

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ARTICLE INFO

Keywords:
Phosphate
Calcium
Chelation
Lipids
Yarrowia lipolytica
Short-chain fatty acids

ABSTRACT

Some oleaginous yeasts have the ability to produce microbial oils from alternative carbon sources, such as short-chain fatty acids (SCFAs). Nevertheless, there is still a lack of information about the possible effects that media nutrients have on yeast metabolisms when using SCFAs. For instance, inorganic phosphate (PO_4^{3-}) has been reported to promote yeast growth in literature but its chelating effect over other elements such as calcium (Ca^{2+}) is often not considered in fermentation processes while limitation of nitrogen is probably the most studied. Attending at the need to better understand the role of PO_4^{3-}, this work assessed the lipid production capacity of *Yarrowia lipolytica* ACA DC 50109, both in synthetic and real SCFAs-rich media, at different SCFAs concentrations and PO_4^{3-}:Ca^{2+} ratios. Reducing PO_4^{3-}:Ca^{2+} ratio was identified to be an important factor to improve yeast growth, reaching the highest lipid content (52.7 ± 0.9 % w/w) and lipid yield (0.31 ± 0.01 w/w) in media without PO_4^{3-}. These results demonstrated the importance of Ca^{2+} availability in the medium and nutrients interactions in yeast growth that are often underestimated.

1. Introduction

Significant global climate changes and environmental problems have boosted the replacement of petroleum-based compounds with green and renewable bioproducts. Animal fats and vegetable oils are recognized as environmentally-friendly alternatives for oleochemicals that can substitute petroderivatives in the chemical industry. Nonetheless, the development of the oleochemistry sector using animal fats and vegetable oils has been hampered due to their competition with food demands, limited availability, and the requirement of extensive cultivation areas and seasonal dependency for plant growth (Patel et al., 2020). Within this scenario, given their similarity in fatty acid composition to vegetable oils (Xue et al., 2018), microbial lipids have emerged as a promising and sustainable source of oleochemicals.

Oleaginous yeasts have the ability to accumulate over 60 % g lipids/g biomass (% w/w) using sugars and other carbon sources derived from organic waste, such as glycerol and short-chain fatty acids (SCFAs) (Di Fidio et al., 2019; Tomás-Pejó et al., 2021). Among all the oleaginous yeasts, *Yarrowia lipolytica* is the most widely studied due to the available knowledge about its genome and numerous alternatives for its genetic modification (Abdel-Mawgoud and Stephanopoulos, 2020; Ramesh et al., 2020).

Sugar-based feedstocks have been traditionally used as substrates for microbial fermentation, as they are easily assimilated by most

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https://doi.org/10.1016/j.eti.2024.103767
Received 17 May 2024; Received in revised form 24 July 2024; Accepted 25 July 2024
Available online 26 July 2024

Table 1
Composition of the different fermentation media used in this study.

Media	SCFAs concentration (g/L)*					Total SCFAs (g/L)	PO_4^{3-} (g/L)	Ca^{2+} (g/L)
	Acetic	Propionic	Butyric	Valeric	Hexanoic			
SM 1	2.6	15	6.3	2.4	2.6	15	0	0.03
SM 2	3.5	20	6.9	3.5	3.4	20	5.1	
							7.6	
							10.1	
RD 1	2.6	1.2	6.3	2.4	2.6	15	1	0.03
RD 2	3.5	2.5	6.9	3.5	3.4	20	5.1	
							7.6	
							10.1	

* Isobutyric, isovaleric and isohexanoic concentrations were negligible.

oleaginous yeasts (Carsanba et al., 2020). Nevertheless, glucose can account for up to 80 % of the entire process production cost (Uthandi et al., 2022). In this sense, the use of low-cost carbon sources obtained from organic waste could boost the economic viability of the microbial lipids production process. SCFAs are organic acids that can be generated by means of anaerobic fermentation (AF) of organic waste. Although it has been proven that some yeasts can use these SCFAs (Llamas et al., 2020a; Žganjar et al., 2024), the available information about oleaginous yeasts' metabolism involved in SCFAs assimilation is still scarce.

Phosphate (PO_4^{3-}), which can be found in significant amounts in digestates depending on the feedstock used during the AF of organic waste, is a crucial element for the synthesis of RNA, DNA, phosphorylated proteins and ubiquitous cofactors (Wang et al., 2018). Nevertheless, it has been observed that high concentrations of this anion can inhibit yeast growth (Shepherd et al., 2021) and are detrimental for microbial lipid production (Huang et al., 2018; Wang et al., 2018; Wu et al., 2010). In the same line, specific concentrations of calcium (Ca^{2+}), that is also present in media derived from waste, have been reported to improve yeast tolerance to some inhibitors such as ethanol, promoting yeast growth (Nabais et al., 1988). Recently, some studies have pointed to PO_4^{3-} as a Ca^{2+} chelating agent, reducing Ca^{2+} availability even when the PO_4^{3-} concentration was as low as 1 g/L (Scudeller et al., 2021). This Ca^{2+} chelation by PO_4^{3-} could restrict the potential positive effect of Ca^{2+} to overcome some inhibitory effects in challenging media such as SCFAs-rich digestates.

When using SCFAs as a carbon source, there is no information on how the chelating capacity of PO_4^{3-} can reduce Ca^{2+} availability, and thereby affecting yeast growth and lipid content from SCFAs. This investigation was designed to cover this gap of knowledge and to elucidate the combined effect of PO_4^{3-} and Ca^{2+} present in SCFAs-rich media on yeast growth and lipid production. For such a purpose, lipid accumulation in *Y. lipolytica* ACA DC 50109 was tested using SCFAs and different PO_4^{3-}:Ca^{2+} ratios on real and synthetic SCFAs-rich media.

2. Material and methods

2.1. Yeast strain and preinoculum conditions

Y. lipolytica ACA DC 50109, obtained from the culture collection of the Agricultural University of Athens, was used in this study. The yeast was maintained at −80 °C in 30 % v/v glycerol. The strain was maintained on yeast extract-peptone-dextrose (YPD) agar plates (containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) at 4 ± 1 °C. Prior to the experiments, a single colony was inoculated into YPD liquid medium (same composition as above mentioned, excluding agar) and incubated overnight at 27 ºC and 150 rpm in a rotary shaker until the late exponential growth phase.

2.2. Fermentation media and conditions

Table 1 provides the SCFAs content in synthetic media (SM) and real digestates (RD) used in this study. Both RD 1 and 2 were obtained as described in Greses et al. (2020) and were centrifuged at 5000 rpm for 30 min using a fixed-angled rotor (Heraeus, Megafuge 16, Thermo Scientific, FiberliteTM F15–6 x 100 y) before use. After centrifugation, digestates were filtered and sterilized by using 0.22 μm filters. The composition of this media in terms of micronutrients can be found in Morales-Palomo et al. (2022a).

SM 1 and SM 2 rich in SCFAs were prepared to replicate the same SCFAs concentration and profiles exhibited by RD 1 and RD 2. In SM 1 and SM 2, 15 g/L and 20 g/L SCFAs were added to Delft medium (7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4$-$_7H_2O$ and 2 mL of trace metals solution). $CaCl_2$ at 0.09 g/L (corresponding to 0.03 g/L Ca^{2+}) was also supplemented. Four concentrations of PO_4^{3-} were attained by adding different amounts of KH_2PO_4 (0 g/L, 5.1 g/L, 7.6 g/L and 10.1 g/L). Thus, PO_4^{3-}:Ca^{2+} ratios in studied media were 0, 170, 253.3 and 336.7, respectively. The selected $CaCl_2$ concentration was based on the results obtained by Nabais et al. (1988), while the PO_4^{3-} concentration was calculated based on previous reports from Wang et al. (2018) and Wu et al., (2011)).

RD 1 was supplemented with the same amounts of KH_2PO_4 mentioned above (5.1 g/L, 7.6 g/L and 10.1 g/L), 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). 0.09 g/L $CaCl_2$ was also added to mimic the media composition of SM. In this case, the achieved PO_4^{3-}:Ca^{2+} ratios were 336.7, 253.3, 170 and 33.3, respectively. Additionally, RD 2 was obtained from RD 1. For that, SCFAs were added until reaching a final concentration of 20 g/L

Three replicates of each experiment were carried out in baffled 250-mL Erlenmeyer flasks with 100 mL of fermentation media at pH

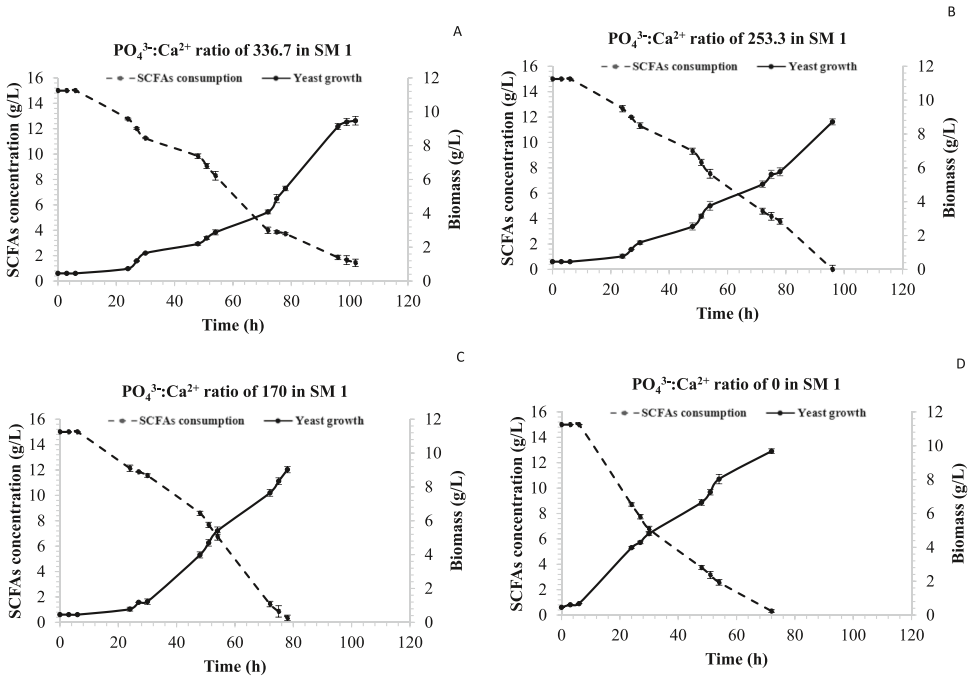

Fig. 1. *Y. lipolytica* growth (**—**) and substrate consumption (–) under PO_4^{3-}:Ca^{2+} ratios of 336.7 (A), 253.3 (B), 170 (C) and 0 (D) in SM 1 with 15 g/L SCFAs.

6 (no control pH was carried out). An initial optical density (OD) of 1 at 600 nm (OD_{600}) (equivalent to 0.45 g dry weight cells/L) was set as inoculum size. Fermentations were incubated at 170 rpm and 27 ºC until 95–100 % of SCFAs were consumed. Regular sampling was conducted throughout the fermentation process to assess yeast growth and SCFAs consumption. At the end of the fermentation, lipids analysis was performed.

2.3. Analytical methods

2.3.1. SCFAs, PO_4^{3-} and Ca^{2+} determination

SCFAs were quantified using high pressure liquid chromatography with an Agilent 1260 HPLC-RID system (Agilent, Santa Clara, CA, USA), equipped with a Cation H Refill Cartridge Microguard column (Biorad, Hercules, CA, USA) and an Aminex HPX-87 H ion exclusion column (300 × 7.8 mm I.D.) (Biorad). The analysis was performed using an isocratic elution mode with a mobile phase consisting of 5 mM H_2SO_4 and a flowing rate of 0.6 mL min^{-1}. A sample volume of 20 µL was injected into the system. The detector and oven temperatures were maintained at 35 °C and 50 °C, respectively.

PO_4^{3-} concentration was measured by using the method of Vanadate-Molybdate (Tandon et al., 1968). Ion chromatography (ICS 3000, Dionex) equipped with pre-columns and separation columns CG 16 and CS 16 (3 mm ø) for cations was used to measure Ca^{2+}. The mobile phase was 1 M HNO_3 + 0.1 M oxalic acid with a flow rate of 0.8 mL min^{-1}. The injected sample volume was 10 µl.

2.3.2. Yeast growth and lipid determination

Cell growth was measured at OD_{600} with Spectroquant® Pharo 100 spectrophotometer. For dry weight determination, 5 mL of culture was filtered through a pre-weighted 0.45 µm glass fiber membrane (Millipore, MA, USA) and dried at 105 ºC until constant weight (Lipps et al., 2024).

The specific growth rates, µ (h^{-1}) were determined as the amount of the biomass produced (g/L) versus the initial biomass concentration (g/L) and the time taken (h) during the fermentation.

The quantification of lipid content (% w/w) was conducted through fluorimetric analysis, following the optimized procedure outlined in Morales-Palomo et al. (2022b). Briefly, cells were stained with a final concentration of Nile Red of 1 µg/mL and fluorescence intensity was determined at λex/em = 488/570 nm, ex/em slit = 10 nm (PerkinElmer® LS 55 Fluorescence Spectrometer). Total areas were calculated with Origin (Pro), version 8.5 (OriginLab Corporation, Northampton, MA, USA) and used to obtain the quantum yield values of each sample**.** Lipid yields were calculated as the grams of lipid produced / grams of consumed SCFAs (w/w).

2.3.3. Data analysis

Cell growth (OD_{600}) and yeast lipid content (% w/w) were evaluated using a parametric one-way ANOVA with a confidence interval of 95 %. Statistical significance was determined at a p-value < 0.05.

Table 2
Y. lipolytica growth, lipid content and yield in SM 1 (15 g/L SCFAs) and SM 2 (20 g/L SCFAs) at different PO_4^{3-}:Ca^{2+} ratios.

SCFAs concentration (g/L)	PO_4^{3-}:Ca^{2+} ratio	Fermentation time (h)	Growth rate$_{72\ h}$ (h^{-1})	Biomass production (g/L)	Lipid content (% w/w)	Lipid yield (w/w)
15	336.7	102	0.12	9.5 ± 0.3	20.2 ± 0.6	0.14 ± 0.01
15	253.3	96	0.15	8.7 ± 0.1	22.4 ± 0.4	0.14 ± 0.01
15	170	78	0.23	9.0 ± 0.1	23.7 ± 0.3	0.15 ± 0.01
15	0	72	0.29	9.7 ± 0.1	40.9 ± 0.4	0.27 ± 0.01
20	336.7	144	0.04	10.3 ± 0.1	22.7 ± 0.5	0.14 ± 0.01
20	253.3	72	0.41	13.9 ± 0.2	24.4 ± 0.8	0.15 ± 0.01
20	170	72	0.41	13.5 ± 0.2	28.6 ± 0.6	0.17 ± 0.01
20	0	48	0.49*	10.8 ± 0.4	52.7 ± 0.9	0.31 ± 0.01

* Growth rate at 48 h.

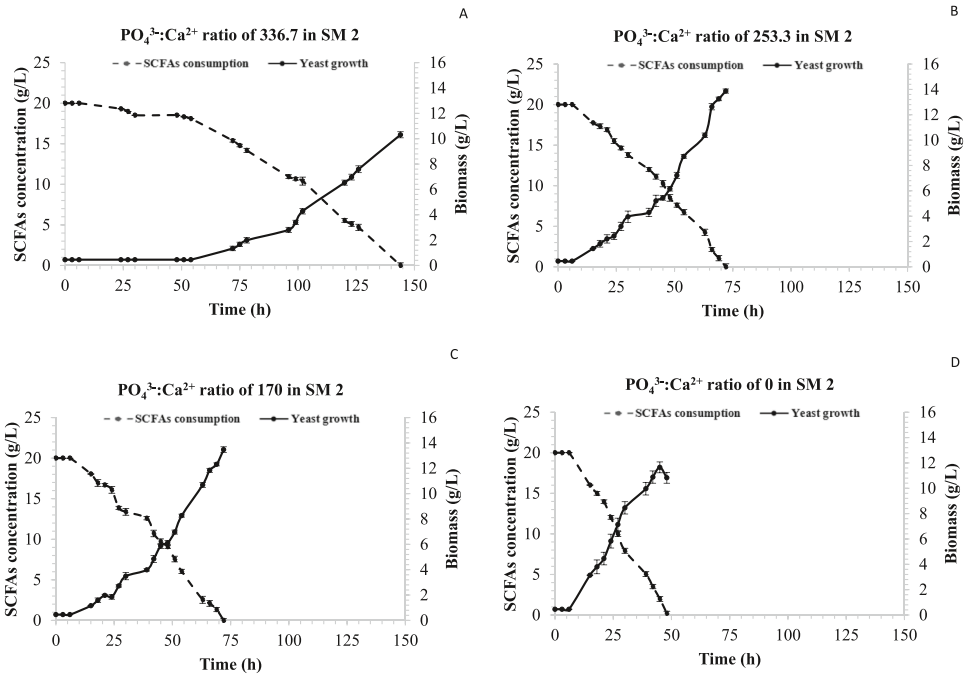

Fig. 2. *Y. lipolytica* growth (—) and substrate consumption (–) under PO_4^{3-}:Ca^{2+} ratios of 336.7 (A), 253.3 (B), 170 (C) and 0 (D) in SM 2 with 20 g/L of SCFAs.

3. Results

3.1. Effect of phosphate chelation over calcium on lipid production in synthetic media

As observed in Fig. 1, lag phases of around 20 h were observed in SM 1 containing 5.1 g/L, 7.6 g/L and 10.1 g/L of PO_4^{3-}. In similar SM, Morales-Palomo et al. (2023) reported lag phases as long as 51 h when PO_4^{3-} concentration was higher than 5.1 g/L and 0.003 g/L Ca^{2+} was used (corresponding to the trace metal concentration in Delft media). Considering the chelating effect of PO_4^{3-} over Ca^{2+}, the higher Ca^{2+} concentration employed in the current study resulted in higher concentration of Ca^{2+} available for the yeast (0.03 g/L), which boosted cell growth and reduced lag phase (Fig. 1). The higher Ca^{2+} availability may help the yeast to adapt to harsh media. In fact, when using SM 1 without PO_4^{3-} (0 g/L) and, therefore, with the highest availability of Ca^{2+}, the yeast reduced its lag phase to only 6 h (Fig. 1 D).

As observed in Fig. 1, the fermentation time required to consume all SCFAs was reduced from 102 h in SM 1 with PO_4^{3-}:Ca^{2+} ratio of 336.7–72 h in SM 1 with PO_4^{3-}:Ca^{2+} ratio of 0. Thus, the lower the PO_4^{3-} concentration (lower PO_4^{3-}:Ca^{2+}), the higher the carbon source consumption and the growth rates (h^{-1}) (Table 2). It is worth mentioning that, regardless of the PO_4^{3-}:Ca^{2+} ratio used, no major differences in biomass production were observed using SM 1 (Table 2). Taking this into account, it can be inferred that while Ca^{2+} supplementation may assist the yeast in adapting to the medium or overcoming adverse conditions, it does not influence yeast metabolism in a manner that affects biomass production.

Concerning lipid content, the yeast accumulated 40.9 ± 0.4 % lipids w/w in SM 1 when no PO_4^{3-} was added (with PO_4^{3-}:Ca^{2+} ratio of 0), resulting in a lipid content 2.02-fold higher than that observed in SM 1 with PO_4^{3-}:Ca^{2+} ratio of 336.7. These results are in good

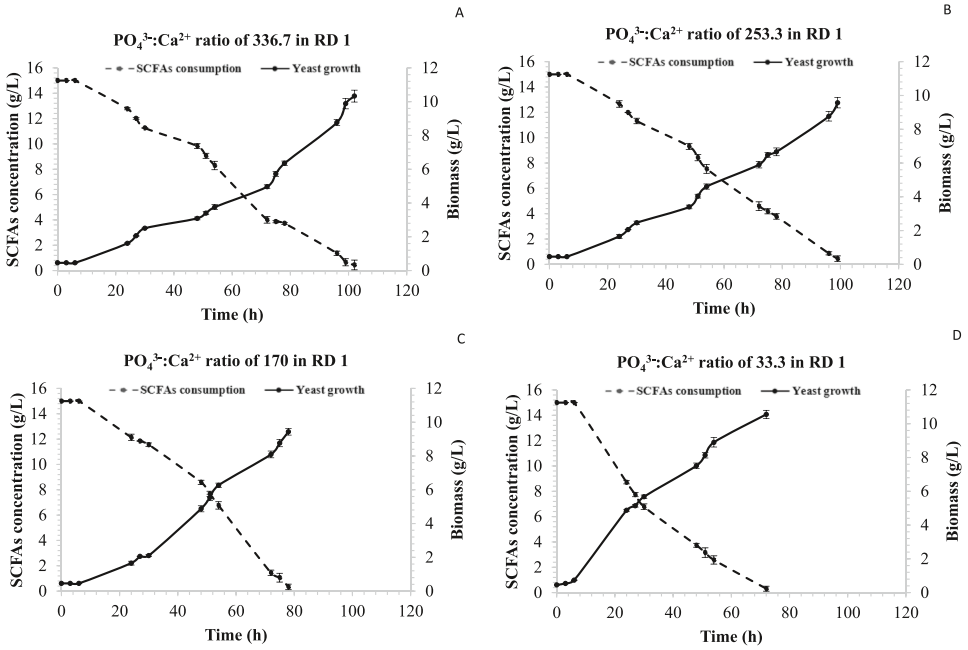

Fig. 3. *Y. lipolytica* growth (—) and substrate consumption (–) under PO_4^{3-}:Ca^{2+} ratios of 336.7 (A), 253.3 (B), 170 (C) and 33.3 (D) in RD 1 with 15 g/L SCFAs.

agreement with previous studies that demonstrated that PO_4^{3-} limitation promoted lipid production in yeast (Huang et al., 2018; Wierzchowska et al., 2021). Remarkably, Morales-Palomo et al. (2023) described a 1.92-fold increase when comparing the lipid content attained in SM with 15 g/L of SCFAs and 0 g/L and 10.1 g/L of PO_4^{3-} (44.4 ± 0.9 % w/w and 23.1 ± 0.5 % w/w, respectively). This increase in lipid content between SM with 0 g/L and 10.1 g/L of PO_4^{3-} was similar to that obtained in this investigation despite the fact that Morales-Palomo et al. (2023) did not supplement the medium with Ca^{2+} (0.003 g/L of Ca^{2+}). Moreover, no significant differences were observed in the lipid content achieved in this investigation with 5.1 g/L and 7.6 g/L of PO_4^{3-} (22.4 ± 0.4 % w/w and 23.7 ± 0.3 % w/w, respectively) compared to those reported by Morales-Palomo et al. (2023) with 15 g/L of SCFAs and these same PO_4^{3-} concentrations without Ca^{2+} addition (23.0 ± 0.7 % w/w and 22.1 ± 0.5 % w/w, respectively). These results indicated that Ca^{2+} availability did not have a significant effect on lipid content, but it did influence the yeast growth rate (Table 2).

In this sense, low amounts of PO_4^{3-} (≤ 5.1 g/L) would not only result in higher availability of Ca^{2+} but also will promote lipid production by diverting carbon metabolisms towards lipid accumulation instead of growth.

To further evaluate the positive effect that Ca^{2+} has on aiding yeast to overcome certain inhibitory barriers, such is the case of SCFAs concentrations higher than 15 g/L (Žganjar et al., 2024), SM 2 containing 20 g/L of SCFAs was used (Section 2.2).

As observed in Fig. 2, the yeast presented a lag phase as low as 6 h when SM 2 with a PO_4^{3-}:Ca^{2+} ratio of 253.3 and 170 were used in media containing 20 g/L SCFAs. Nonetheless, a lag phase of 54 h was observed when SM 2 with PO_4^{3-}:Ca^{2+} ratio of 336.7 was used. These results are consistent with what was previously observed with SM 1, suggesting that higher concentrations of PO_4^{3-} can reduce the availability of Ca^{2+} leading to longer lag phases. This effect can also be observed in the growth rate achieved in SM 2. With PO_4^{3-}:Ca^{2+} ratio of 253.3 and 170, the yeast attained a growth rate 10.3-fold higher (0.41 h^{-1} in both cases in the first 72 h) when compared with PO_4^{3-}:Ca^{2+} ratio of 336.7 (0.04 h^{-1} during the first 72 h).

It should be noted that the growth rates obtained in SM 2 with 20 g/L of SCFAs and PO_4^{3-}:Ca^{2+} ratios of 253.3, 170 and 0 were 2.7-, 1.8- and 1.7-fold higher, respectively, than those obtained when using SM 1 with 15 g/L of SCFAs and the same PO_4^{3-}:Ca^{2+} ratios. Higher growth rates are achieved with higher carbon:phosphate (C:PO_4^{3-}) ratio (Morales-Palomo et al., 2023). When the SCFAs concentration was increased to 20 g/L in SM 2, the resulting C:PO_4^{3-} ratio was higher than in SM 1, allowing the yeast to increase its growth rates. Nevertheless, when SM 2 with PO_4^{3-}:Ca^{2+} ratio of 336.7 was used, the growth rate was 3-fold lower than that achieved in SM 1 with PO_4^{3-}:Ca^{2+} ratio of 336.7. In this case, although SM 2 had higher C:PO_4^{3-} ratio than SM 1, the high PO_4^{3-} concentrations (> 7.6 g/L) could prevent the yeast from increasing its growth rate as in the previous cases.

When SM 2 with a PO_4^{3-}:Ca^{2+} ratio of 0 was used, the yeast maintained the same lag phase observed in SM 2 with a PO_4^{3-}:Ca^{2+} ratio of 253.3 and 170 (6 h) but reduced the time required to consume all SCFAs from 72 h to 48 h (Table 2). This higher SCFAs consumption rate might be due to the absence of PO_4^{3-} in SM 2 (PO_4^{3-}:Ca^{2+} ratio of 0), which prevented any chelating effect and left more Ca^{2+} available for yeast growth. Noteworthy, in this case, biomass production (10.8 ± 0.4 g/L) was reduced when compared with SM 2 with a PO_4^{3-}:Ca^{2+} ratio of 253.3 and 170, but lipid content was increased up to 52.7 ± 0.9 % w/w (Table 2).

Regarding biomass production, 13.9 g/L and 13.5 g/L for PO_4^{3-}:Ca^{2+} ratio of 253.3 and 170 were reached, respectively. The fact that biomass increased with PO_4^{3-}:Ca^{2+} ratio of 253.33 and 170 when compared to media with PO_4^{3-}:Ca^{2+} ratio of 336.7 (10.3 ± 0.1 g/L), corroborated the hypothesis about yeast growth difficulties in media with high concentrations of PO_4^{3-} (> 7.6 g/L) and SCFAs (>

Table 3
Y. lipolytica growth, lipid content and yield in RD 1 (15 g/L SCFAs) and RD 2 (20 g/L SCFAs) at different PO_4^{3-}:Ca^{2+} ratios.

SCFAs concentration (g/L)	PO_4^{3-}:Ca^{2+} ratio	Fermentation time (h)	Growth rate$_{72\ h}$ (h^{-1})	Biomass production (g/L)	Lipid content (% w/w)	Lipid yield (w/w)
15	336.7	102	0.15	10.3 ± 0.3	16.4 ± 0.8	0.14 ± 0.01
15	253.3	99	0.18	9.6 ± 0.1	18.8 ± 0.7	0.14 ± 0.01
15	170	78	0.24	9.4 ± 0.1	18.3 ± 0.5	0.15 ± 0.01
15	33.3	72	0.32	10.6 ± 0.1	36.6 ± 0.5	0.27 ± 0.01
20	336.7	144	0.04	12.7 ± 0.1	20.4 ± 0.5	0.14 ± 0.01
20	253.3	78	0.31	13.7 ± 0.6	20.9 ± 0.5	0.17 ± 0.01
20	170	78	0.34	13.7 ± 0.2	20.2 ± 0.7	0.19 ± 0.01
20	33.3	54	0.61*	13.6 ± 0.2	42.4 ± 0.8	0.29 ± 0.01

* Growth rate at 54 h.

15 g/L).

Although lipid content in SM 1 with 15 g/L of SCFAs was slightly higher than that achieved in SM 1 with 20 g/L of SCFAs and with the same PO_4^{3-} concentration (20.2 ± 0.6 % w/w and 22.7 ± 0.5 % w/w, respectively), the yeast reached a final biomass production similar to that obtained in this media under the same conditions (9.5 ± 0.3 g/L and 10.3 ± 0.1 g/L, respectively) (Table 2). Since all SCFAs were consumed in SM 2, it was expected that the lipid content or biomass production would be significantly higher compared to SM 1. Nonetheless, the yeast did not show a substantial increase in lipid content or biomass production. This suggests that the high concentrations of PO_4^{3-} (10.1 g/L) and SCFAs (20 g/L) were very restrictive, causing the yeast to divert the carbon source for cell maintenance instead of growth.

In addition, the lipid content was 1.3-fold higher (28.6 ± 0.6 % w/w) when SM 2 with PO_4^{3-}:Ca^{2+} ratio of 336.7 and 170 were compared. These results indicated that once the amount of PO_4^{3-} in SM 2 was ≤ 7.6 g/L (PO_4^{3-}:Ca^{2+} ratio ≤ 253.3) media composition would no longer hinder yeast growth. Thus, the yeast was able to use the carbon source to increase its growth and slightly increase lipid content.

In this study, PO_4^{3-} limitation allowed yeast to redirect its metabolism towards lipid production and Ca^{2+} supplementation did not affect lipid metabolism. Thus, without PO_4^{3-} in the medium, higher SCFAs concentration resulted in higher lipid content as the yeast was able to divert more carbon source to produce lipids. The attained lipid yield (0.31 ± 0.1 w/w) was similar to those reported in the literature with glucose and xylose (0.32 w/w and 0.34 w/w, respectively), and in previous studies where nitrogen and PO_4^{3-}-limitation strategies were used to increase lipid content with sugars as carbon source (Hapeta et al., 2020; Wang et al., 2020; Wierzchowska et al., 2021). These results confirmed that Ca^{2+} helps yeast overcome the inhibition caused by high PO_4^{3-} concentration. Nevertheless, there must be enough Ca^{2+} so that it is not entirely chelated by PO_4^{3-}.

3.2. Fermentation in real digestate corroborated the effect of calcium on yeast growth rate and lipid content production

The chelating effect of PO_4^{3-} and Ca^{2+} availability on yeast growth was verified in SCFAs-rich RD. As it can be seen in Fig. 3, the yeast exhibited a lag phase of 6 h in all cases (RD 1 with PO_4^{3-}:Ca^{2+} ratio of 33.3, 170, 253.3 and 336.7). Previous studies identified RD as a nutrient-rich environment resulting in improved yeast growth when compared with SM (Llamas et al., 2020b). In this sense, nutrients present in RD could help the yeast to achieve a lower lag phase compared to those obtained with SM 1 and 2, regardless of the PO_4^{3-}:Ca^{2+}.

In RD 1 with a PO_4^{3-}:Ca^{2+} ratio of 33.3 (1 g/L PO_4^{3-}), the growth rate was 0.32 h^{-1} during the first 72 h of fermentation, achieving a biomass production of 10.6 ± 0.1 g/L. This value was 1.09-fold higher than the biomass reached in SM 1 without PO_4^{3-} (Table 2). This improvement in growth could be due not only to the nutrient-rich environment of RD but also to the difference in PO_4^{3-} concentrations between the two media. When RD 1 with a PO_4^{3-}:Ca^{2+} ratio of 170 was used, a growth rate of 0.24 h^{-1} during the first 72 h of fermentation and a total biomass at the end of the fermentation of 9.4 ± 0.1 g/L were reached. These values were 2.9- and 1.4-fold higher than the growth rate and the biomass concentration attained by Morales-Palomo et al. (2023) under these conditions without Ca^{2+} supplementation (0.003 g/L). These results highlighted the negative effect of high PO_4^{3-} concentrations (≥ 5.1 g/L), combined with low Ca^{2+} concentrations, on both yeast growth rate and biomass production.

The growth rate in RD 1 with PO_4^{3-}:Ca^{2+} ratios of 253.3 and 336.7 was as low as 0.18 h^{-1} and 0.15 h^{-1}, respectively (Table 3). Biomass production (g/L) was similar to that obtained in RD 1 with PO_4^{3-}:Ca^{2+} ratio of 33.3 and 170. It is worth mentioning that the yeast was able to grow in RD 1 with 7.6 g/L and 10.1 g/L of PO_4^{3-}, contrary to what was previously described by Morales-Palomo et al. (2023), where no yeast growth was observed.

The lipid content obtained in RD 1 with a PO_4^{3-}:Ca^{2+} ratio of 33.3 (36.6 ± 0.5 % w/w) (Table 3) was lower than that achieved in SM 1 with PO_4^{3-}:Ca^{2+} ratio of 0 (40.9 ± 0.4 % w/w) (Table 2). This decrease in lipid content could be due to the difference in PO_4^{3-} concentration in media, which shifted the yeast metabolism towards higher lipid production rather than biomass production in the medium without PO_4^{3-} (SM 1 with PO_4^{3-}:Ca^{2+} ratio of 0). Nonetheless, similar lipid yields were reached in both cases (0.27 ± 0.01 w/w). The lipid content attained with PO_4^{3-}:Ca^{2+} ratios of 253.3 and 336.7 did not differ from what was obtained with a PO_4^{3-}:Ca^{2+} ratio of 170 (Table 3). Remarkably, after using SM with 15 g/L of SCFAs and 7.6 g/L of PO_4^{3-} without Ca^{2+} addition, Morales-Palomo et al. (2023) reported a similar lipid content than that observed in this investigation with RD 1 and 7.6 g/L of PO_4^{3-} (18.8 ± 1.1 % w/w and 18.3 ± 0.5 % w/w, respectively). These results confirmed that Ca^{2+} only affected the yeast growth rate and had no impact on lipid production.

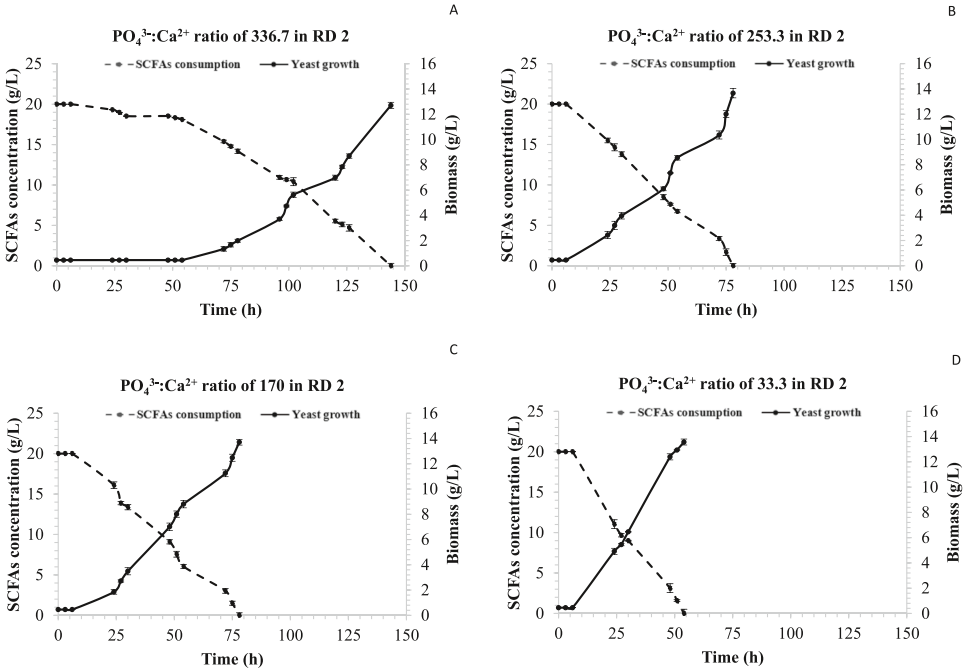

Fig. 4. *Y. lipolytica* growth (—) and substrate consumption (–) under PO_4^{3-}:Ca^{2+} ratios of 336.7 (A), 253.3 (B), 170 (C) and 33.3 (D) in RD 2 with 20 g/L of SCFAs.

When RD 2 with a PO_4^{3-}:Ca^{2+} ratio of 33.3 was used (Fig. 4 D), the yeast presented a lag phase of 6 h and was able to consume all SCFAs in 54 h. When using RD 2 with a PO_4^{3-}:Ca^{2+} ratio of 170 and 253.3, the yeast exhibited the same lag phase observed in RD 2 with a PO_4^{3-}:Ca^{2+} ratio of 33.3 (6 h) but increased the fermentation time up to 78 h. Growth rates were 0.34 h^{-1} and 0.31 h^{-1} during the first 72 h of fermentation in RD 2 with a PO_4^{3-}:Ca^{2+} ratio of 170 and 253.3, respectively. With RD 2 with PO_4^{3-}:Ca^{2+} ratio of 336, the lag phase was increased up to 54 h and the growth rate during the first 72 h of fermentation was reduced to 0.04 h^{-1}. Similar biomass production was achieved in all media (Table 3). These results demonstrated that despite the longer lag phase and growth rates caused by increased PO_4^{3-} (1 g/L vs 10.1 g/L PO_4^{3-}), which led to greater chelation of Ca^{2+}, the available Ca^{2+} (0.03 g/L) was sufficient for the yeast to grow under these challenging conditions and maintain consistent biomass production. Furthermore, these results supported the initial hypothesis that a lower PO_4^{3-}:Ca^{2+} ratio (meaning more Ca^{2+} is available and not chelated by PO_4^{3-}) makes it easier for the yeast to start growing in the media.

As was previously observed with SM 2, the growth rates obtained in RD 2 with 20 g/L of SCFAs and PO_4^{3-}:Ca^{2+} ratios of 253.3, 170 and 1 were 1.7-, 1.4- and 4.3-fold higher, respectively, than those reached in RD 1 with 15 g/L of SCFAs as the C:PO_4^{3-} ratios of RD 2 were higher. RD 2 with PO_4^{3-}:Ca^{2+} ratio of 336.7 presented lower growth rate (3.8-fold lower) than that observed in RD 1 with PO_4^{3-}:Ca^{2+} ratio of 336.7, which would corroborate that PO_4^{3-} higher than 7.6 g/L would prevent the yeast from improving its growth rate regardless of the C:PO_4^{3-} ratio.

As shown in Table 3, in RD 2 with a PO_4^{3-}:Ca^{2+} ratio of 33.3, the biomass production (13.6 ± 0.2 g/L) was 1.3-fold higher and lipid content (42.4 ± 0.8 % w/w) was 1.2-fold lower when compared with SM 2 with a PO_4^{3-}:Ca^{2+} ratio of 0. Similar results were obtained by Huang et al. (2018), in which case, 1.8-fold higher biomass and 1.7-fold lower lipid content were attained with *Cutaneotrichosporon curvatus* in SM with 10 g/L of SCFAs with 0 g/L and 1 g/L of PO_4^{3-}, respectively. These results indicated that yeast could not divert all the carbon source towards lipid production as long as there is PO_4^{3-} in the medium. Nonetheless, a lipid content increase from 36.6 ± 0.5 % w/w to 42.4 ± 0.8 % w/w was observed when comparing RD 1 and RD 2 with PO_4^{3-}:Ca^{2+} ratio of 33.3 after increasing the amount of total SCFAs (from 15 to 20 g/L, respectively). Likewise, the lipid content attained in RD 2 with PO_4^{3-}:Ca^{2+} ratio of 170, 253.3 and 336.7 was 1.2-fold higher than that achieved in RD 1 with the same PO_4^{3-}:Ca^{2+} ratios. These results are consistent with what was previously mentioned, as the yeast was able to assimilate more carbon sources and shift part of its metabolism to increase lipid content under these conditions in RD 2.

4. Conclusion

This work evidenced the chelating effect of PO_4^{3-} on Ca^{2+} and the importance of Ca^{2+} availability on yeast growth. Although Ca^{2+} supplementation (0.03 g/L) did not directly affect the lipid content in *Y. lipolytica* ACA DC 50109 using SCFAs, it allowed the use of up to 20 g/L SCFAs, which indirectly led to an increase in lipid content (up to 52.7 % w/w). This resulted in the obtention of similar lipid yields (0.31 w/w) to those reported in the literature when using sugars as carbon source. These results demonstrated that the concentration of Ca^{2+} in the media must be sufficient for the yeast, considering that some of this cation will be chelated by PO_4^{3-}. Despite

the improvements herein described, future investigations should focus on elucidating optimal and limiting Ca^{2+} concentrations (and other cations) to enhance yeast lipid production in SCFAs-rich media.

CRediT authorship contribution statement

Sergio Morales-Palomo: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Elia Tomás-Pejó:** Writing – review & editing, Supervision, Data curation. **Cristina Gonzalez:** Writing – review & editing, Supervision, Data curation.

Declaration of generative AI and AI-assisted technologies in the writing process

Not applicable.

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.

Acknowledgements

The project BIOMIO+H2 (PID2020–119403RBC21) funded by MCIN/AEI/10.13039/501100011033, OLEOFERM (EraBoBiotech; PCI2021–121936) funded by MCIN/AEI/10.13039/501100011033 and "European Union NextGenerationEU/PRTR" supported this research. We also acknowledge the grants PRE2018–086477 and RYC2019–027773-I funded by "ESF Investing in your future" and by MCIN/AEI/10.13039/501100011033.

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