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Integrated fermentative process for lipid and β-carotene production from acetogenic syngas fermentation using an engineered oleaginous *Yarrowia lipolytica* yeast

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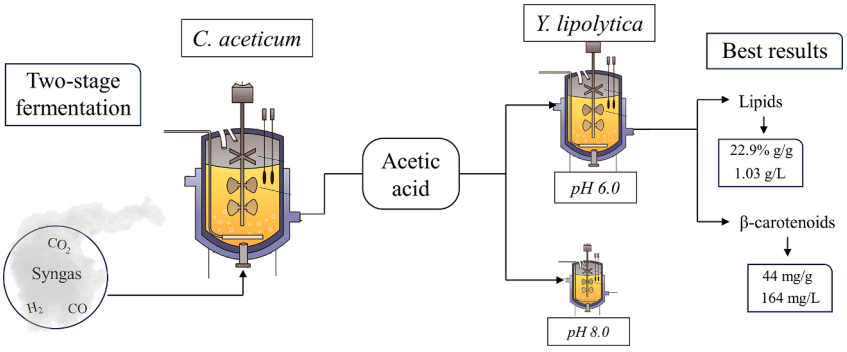
HIGHLIGHTS

- Two-stage fermentation process proposed for efficient bioconversion.
- Engineered *Y. lipolytica* could convert syngas into β-carotene and lipids.
- Tolerance up to 20 g/L acetic acid seen in the yeast strain.
- Optimal lipid and β-carotene production were found at pH 6.0 in bioreactors.
- The dominant lipid identified was C18:1, which is suited for biodiesel production.

GRAPHICAL ABSTRACT



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ABSTRACT

An engineered *Yarrowia lipolytica* strain was successfully employed to produce β-carotene and lipids from acetic acid, a product of syngas fermentation by *Clostridium aceticum*. The strain showed acetic acid tolerance up to concentrations of 20 g/L. Flask experiments yielded a peak lipid content of 33.7 % and β-carotene concentration of 13.6 mg/g under specific nutrient conditions. The study also investigated pH effects on production in bioreactors, revealing optimal lipid and β-carotene contents at pH 6.0, reaching 22.9 % and 44 mg/g, respectively. Lipid profiles were consistent across experiments, with C18:1 being the dominant compound at approximately 50 %. This research underscores a green revolution in bioprocessing, showing how biocatalysts can convert syngas, a potentially polluting byproduct, into valuable β-carotene and lipids with a *Y. lipolytica* strain.

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1. Introduction

According to the recent Paris Agreement and United Nations Framework Conventions on Climate Change (UNFCCC), governments are committed to using renewable resources for the production of chemicals, food, and fuels (Kumar et al., 2017). In this sense, recent research has demonstrated that it is feasible to produce multiple valuable products from sustainable feedstocks and even from pollutants using optimized unit operations or (bio)reactors, thus reducing waste and improving process economics. The simultaneous synthesis of different co-products, as proposed in this article with lipids and β-carotene, might reduce the cost of lipids for fuel production down to a level comparable to that of fossil fuels, converting biorefineries into a viable alternative to the (bio)fuel manufacturing industry (Singh and Singh, 2021).

Recently, interest in lipid-derived biodiesel and jet fuels as sustainable alternative fuels has increased due to their high energy density and biodegradability (Ma et al., 2018; Robles-Iglesias et al., 2023a). Similarly, β-carotene is an orange pigment that is currently produced chemically or biotechnologically using naturally occurring producer microorganisms (Nanou and Roukas, 2016). However, heterologous production based on metabolic engineering is considered a viable strategy to improve β-carotene synthesis and meet the increasing market demand. Therefore, several strategies such as overexpression/elimination of some genes have recently been applied to produce this compound (Larroude et al., 2018).

In terms of environmental pollution, solid wastes, wastewater effluents, and gas emissions are the three major sources of pollution, and their treatment but also valorization is a challenging research area (Kennes, 2023). Regarding the present study, CO_2 and CO are single carbon gases (C_1-gases) that can occur in industrial emissions and are found in synthesis gas (syngas) obtained from the gasification of biomass or solid waste, among others (Bae et al., 2022). These gases can be bioconverted, in the presence of hydrogen, by acetogenic bacteria via the Wood-Ljungdahl pathway (WLP) into products of commercial interest such as short-chain fatty acids including acetic, butyric or hexanoic acids, or even alcohols, e.g., ethanol, butanol or hexanol (Arslan et al., 2021; Fernández-Naveira et al., 2017). Native acetogens cannot produce energy-dense materials such as lipids or carotenoids from C_1-gases due to bioenergetic constraints during autotrophic metabolism and a lack of specialized enzymes (Bae et al., 2022). Therefore, if the goal is to broaden the range of products derived from anaerobic C_1-gas fermentation, it is necessary to either genetically modify bacteria or combine different reactors or microorganisms.

Acetic acid/acetate and other volatile fatty acids (VFAs) have drawn attention as suitable substrates for their bioconversion into high-value compounds. Acetic acid is known to be metabolized by some species of aerobic oleaginous yeasts, demonstrating a remarkable ability to metabolically utilize this carbon source (Park et al., 2018). It would be challenging to cultivate oleaginous yeasts and acetogens together in a single reactor because acetogens are anaerobic bacteria while yeasts require oxygen for growth. Therefore, two separate bioreactors, each with an optimal set of parameters, are required to carry out such an integrated process, i.e., a two-stage fermentation, as proposed in this research. The first stage focused on the bioconversion of C_1-gases to acetic acid by the anaerobic bacterium *Clostridium aceticum*, and a second acetic-converting stage was catalyzed by the engineered oleaginous yeast *Yarrowia lipolytica* to efficiently produce lipids and β-carotene. The tolerance of this yeast to acetic acid was also investigated.

2. Material and methods

2.1. Strains and media

The type strain *Clostridium aceticum* (DSM 1496) was purchased from "DSMZ" (Germany) and stored under cryopreservation conditions following the protocols specified by the supplier.

The *Y. lipolytica* strain JMY6862 (ob-CHCTEFCTEF) was genetically engineered as described in the study carried out by Larroude and colleagues (Larroude et al., 2018). Briefly, strain JMY6862 was derived from the "Obese" strain JMY3501 (Lazar et al., 2014) into which 3 copies of β-carotenoid expression cassettes (Celińska et al., 2017) were sequentially introduced. It was stored at −80 °C until use.

The medium used for the cultivation of *C. aceticum* was composed (per liter distilled water) of yeast extract (YE), 3.00 g; NH_4Cl, 0.20 g; K_2HPO_4, 8.45 g; KH_2PO_4, 1.75 g; $NaHCO_3$, 10.00 g; $MgSO_4 \cdot 7\ H_2O$, 0.35 g; cysteine-HCl, 0.30 g; $Na_2S \cdot 9\ H_2O$, 0.90 g; Na-resazurin (0.1 % w/v), 1.00 ml; trace metal solution, 2.00 ml; vitamin solution, 2.50 ml. The methodology for the trace metal and vitamin dilution implemented in this study is based on work conducted in our group and reported by Arslan and coworkers (Arslan et al., 2021).

Y. lipolytica was tested in various media. Initially, it was grown on a medium consisting of 10.0 g YE, 20.0 g peptone per liter distilled water, and variable acetic acid concentrations to test toxicity. For the second batch experiment, it was cultured in the *C. aceticum* medium, excluding vitamins but adding the following combinations: 5, 10 and 15 g/L acetic acid (ACE); 5, 10 and 15 g/L glucose (GLU); 10 g/L acetic acid + 25 % YE; 10 g/L acetic acid + 25 % NH_4Cl; 10 g/L acetic acid + 25 % YE + 25 % NH_4Cl; 10 g/L GLU + 10 g/L YE + 20 g/L peptone as control. The percentages of YE and NH_4Cl refer to the % of each reagent added with respect to the optimal amount for *C. aceticum*, e.g., if the optimal medium has a YE concentration of 3 g/L, then "+ 25 % YE" corresponds to 0.75 g/L. In the stirred tank reactor (STR) experiments, the fermented *C. aceticum* medium containing acetic acid (6.6 g/L) was used, with either pH 6.0 or 8.0 as the test variable.

2.2. Feeding strategies

2.2.1. Batch cultivation of Y. lipolytica with acetic acid

To assess the tolerance of *Y. lipolytica* to acetic acid, batch experiments were conducted using five 500 ml flasks, each containing 50 ml medium, and acetic acid levels between 2.5 and 40.0 g/L, and pH was adjusted to 5.5. The rationale for the concentration gradient was based on earlier studies on the toxic limits of acetic acid for yeast cells. Post-sterilization, flasks were inoculated with *Y. lipolytica*, previously grown in YPD and washed, equivalent to an Optical Density OD_{600nm} of 0.5 per milliliter medium. The inoculated flasks were then incubated at 32 °C and shaken at 150 rpm. Throughout the experiment, yeast growth was periodically measured.

2.2.2. Batch cultivation of Y. lipolytica with synthetic medium of C. aceticum

To assess *Y. lipolytica*'s growth in the *C. aceticum* medium, another experiment was performed with 500 ml flasks containing 50 ml of culture medium and pH adjusted to 5.5. Post-sterilization, flasks were inoculated with an amount equivalent to 0.5 OD_{600nm} of *Y. lipolytica*, previously washed to prevent nutrient interference. The inoculated flasks were then incubated at 32 °C on an orbital shaker at 150 rpm. Throughout the experiment, measurements were taken for biomass growth, substrate consumption, and total lipid and β-carotene production.

2.2.3. Bioreactor studies in STR (stirred tank reactor)

Three experiments were conducted in a 2L BIOFLO 120 model bioreactor (Eppendorf, Germany). The first experiment utilized *C. aceticum* under anaerobic conditions in a sterilized environment with pH maintained at 7.5, continuous gas flow at 10 ml/min ($CO:CO_2:H_2:N_2$ / 30:10:20:40), and a constant temperature of 30 °C and agitation at 250 rpm. The chosen syngas composition predominantly included CO, CO_2, and H_2, as these are common components of syngas and some are greenhouse gases amenable to bioconversion via the WLP. Furthermore, N_2 was included in the gas mixture as common gas in some gasification

processes and because of its presence in some industrial gas emissions. It offers a cost-effective approach that eliminates the need for further gas separation. The chosen ratio was based on industrial interest and collaborations and prior studies demonstrating favorable outcomes with *C. aceticum* (Robles-Iglesias et al., 2023b).

The remaining two experiments used an acetic acid-rich culture medium derived from syngas by *C. aceticum*. They varied in their initial pH levels (6.0 and 8.0), maintained throughout fermentation. Post-sterilization, an inoculum with an optical density of 0.5/ml was added. Both experiments were continuously fed synthetic air supplied at a rate of 0.1 vvm, maintained at 32 °C, and with stirring speed of 250 rpm.

2.3. Analytical methods

2.3.1. Assessment of cellular proliferation and biomass density

In both the *C. aceticum* and *Y. lipolytica* experiments, microbial growth was gauged via a Hitachi U-200 spectrophotometer at a 600 nm wavelength, generating a growth curve. Dilutions were applied as needed to maintain spectrophotometer accuracy. For biomass concentration, a culture sample was centrifuged, washed, lyophilized for 48 h, and then weighed, providing biomass measurements.

2.3.2. Quantification of acetic acid and C/N ratio

Acetic acid production and consumption were analyzed using an Agilent HP1100 High-Performance Liquid Chromatograph (HPLC) with a refractive index detector set at 50 °C. Samples of 20 µl were introduced into the Hi-Plex H column, with a mobile phase of 0.005 M H_2SO_4 flowing at 0.80 ml/min. For the determination of the C/N ratio, ammonia concentrations were measured spectrophotometrically at a wavelength of 635 nm, as described in (Calero et al., 2018). Subsequently, the corresponding C/N ratio was calculated.

Within the context of this study, emphasis was placed on quantifying acetic acid and total nitrogen concentrations to assess bioconversion efficiency in the fermentation broth. While recognizing the broth's inherent complexity and the potential influence of unidentified nutrients, a comprehensive compositional analysis was beyond the scope of the current research.

2.3.3. Determination of lipids and fatty acids methyl esters (FAMEs)

Lyophilized samples used for biomass concentration determination were also analyzed for FAMEs. Lipids were extracted using a methanol solution with sulfuric acid (Robles-Iglesias et al., 2021), then identified and quantified with a Thermo Fisher gas chromatograph using a flame ionization detector and an Agilent vf-23 ms column. The carrier gas was helium, with a 2.2 ml/min flow rate. FAMEs were identified by comparison with a known dilution and quantified using an in-house standard technique with commercial C12:0 converted to FAME.

2.3.4. Determination of β-carotene

β-Carotene was extracted from the culture medium using a solution of hexane, ethyl acetate, and butyl hydroxyl toluene. After vortexing and centrifugation, the supernatant was collected until discolored. Its absorbance was then measured at 448 nm using a spectrophotometer, with β-carotene concentration determined from a calibration curve.

2.3.5. Statistical analysis

Linear regression analyses were conducted to evaluate the influence of substrate concentration on β-carotene production under two pH conditions, i.e., pH 6 and pH 8. The analyses were performed using R statistical software, employing the *lm()* function to fit linear models. Key metrics such as the intercept, coefficient for substrate concentration, p-values, R-squared, and F-statistics were extracted to assess the models' fit and significance. The significance level for all statistical tests was set at $\alpha = 0.05$.

3. Results and discussion

3.1. Toxicity of acetic acid to Y. lipolytica strain

The primary aim of this section was to examine the susceptibility and resilience of the *Y. lipolytica* strain against various acetic acid concentrations by monitoring OD_{600nm}.

Fig. 1 shows *Y. lipolytica*'s growth at varying initial acetic acid concentrations. No inhibitory effects were observed at acetic acid concentrations of 2.5 and 5 g/L, as indicated by high OD_{600nm} values (OD_{600nm} at 118 h: 20.6 and 18.8, respectively). At 10 g/L, growth was initially delayed but eventually reached similar OD_{600nm} values (OD_{600nm} at 118 h: 23.9). Concentrations of 20 g/L and above severely inhibited growth, with OD_{600nm} values approaching zero after 118 h. These results concur with existing literature, which suggests that acetic acid toxicity is influenced by the medium's acidity and increases with rising acetic acid concentration (Abbott et al., 2008; Verduyn et al., 1992).

Gao et al. (2020) and Liu et al. (2017) emphasized that yeast strains generally exhibit higher tolerance to acetic acid when subjected to alkaline conditions. This is attributed to a reduction in the dissociated form of the acid, thereby mitigating its cytosolic acidifying effects. Considering these findings, it could be inferred that the alkaline pH in future experiments might facilitate better tolerance and consequently higher lipid and β-carotene yields.

Strain variability is another aspect that cannot be overlooked. Literature reports differences in acetic acid tolerance even among strains of the same genus (Fontanille et al., 2012; Gao et al., 2017; Y. K. Park et al., 2021a). For example, Fontanille et al. (2012) observed that *Y. lipolytica* strain MUCL 28849 could tolerate acetic acid concentrations up to 4 g/L without observable inhibitory effects. Conversely, our engineered *Y. lipolytica* strain showed marked resilience up to 10 g/L, underscoring the importance of strain-specific assessments for industrial applications. Moreover, the delayed growth observed at an initial concentration of 10 g/L may suggest that the *Y. lipolytica* strain undergoes an adaptation phase to acclimate to the toxic environment. Gao et al. reported a similar phenomenon, with longer lag phases observed at acetic acid concentrations above 10 g/L (Gao et al., 2017). This implies that, while high concentrations may initially hinder growth, a period of adaptation might enable the yeast to resume metabolic activity.

3.2. Culture of Y. lipolytica on C. aceticum synthetic medium

3.2.1. Growth and toxicity

The experiment tested the toxicity tolerance of *Y. lipolytica* when grown on the optimal medium for *C. aceticum* and with the addition of different concentrations of acetic acid, glucose, YE and NH_4Cl. The concentrations tested are described in section 2.1.

As per Fig. 2A, *Y. lipolytica* exhibited good growth except at the highest acetic acid concentration (15 g/L). No inhibition was observed at 5 g/L acetic acid, while a longer lag phase was seen at 10 g/L. Table 1

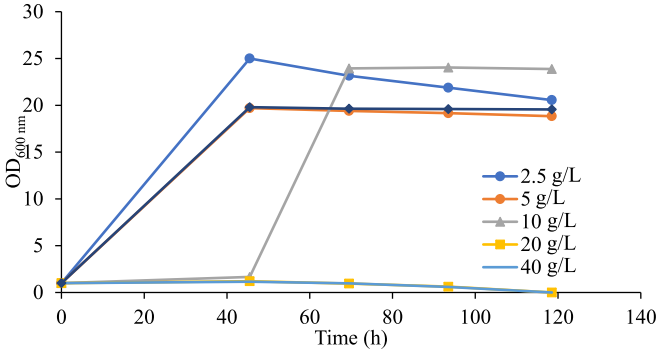

Fig. 1. Growth of *Y. lipolytica* at different initial acetic acid concentrations.

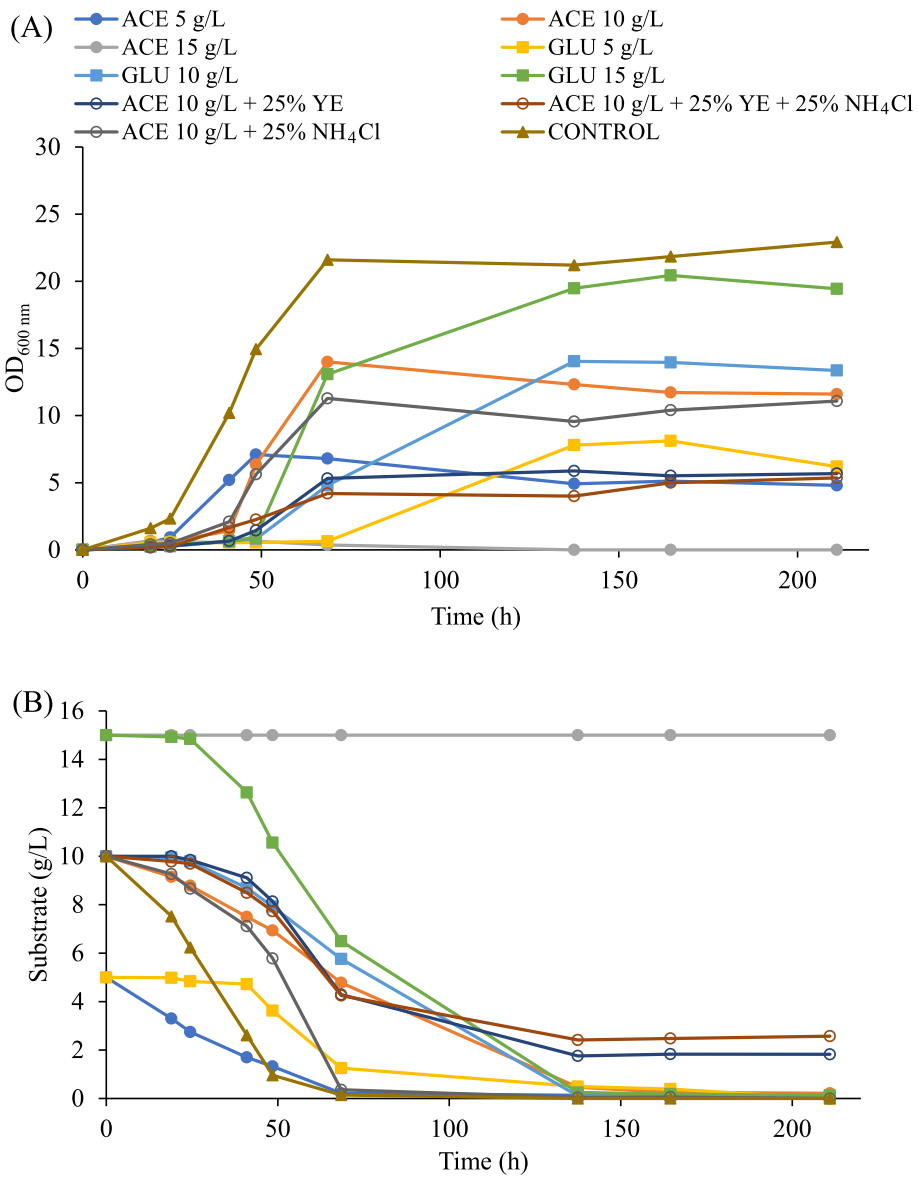

Fig. 2. (A) Growth as a function of optical density (OD_{600nm}) of *Y. lipolytica* with different substrates and at different concentrations. (B) Substrate consumption of *Y. lipolytica* at different concentrations.

shows that the lag phase for 10 g/L acetic acid is 66 % higher than for 5 g/L. The increased glucose concentration improved cell growth, as observed. These results were expected since glucose tends to show some inhibition only at high concentrations for some microorganisms, above 40 g/L (Park et al., 2017). Regarding the flasks with different % YE, NH_4Cl and 10 g/L acetic acid, the one with 25 % NH_4Cl and standard YE concentration showed the best growth. It even outperformed the flask with 5 g/L glucose in terms of biomass. The other two flasks, where YE and NH_4Cl were varied, reached lower biomass concentrations.

Fig. 2B displays substrate consumptions, indicating metabolization in all cases except at 15 g/L acetic acid. Complete substrate consumption was achieved in all flasks except those with varied percentages of YE and NH_4Cl (25 % YE and 25 % YE + 25 % NH_4Cl). This is presumably due to the nutrients and amino acids in YE supporting better yeast growth and acetic acid assimilation. Table 1 shows the maximum substrate consumption rates, with the lowest values in the 5 and 10 g/L acetic acid flasks. However, flasks with 10 g/L acetic acid and varied YE and NH_4Cl demonstrated high maximum consumption rates compared to flasks with unchanged initial reagent percentages.

Compared to other *Y. lipolytica* studies using acids as substrates, the consumption values in this experiment are high. For instance, a study by Llamas et al. reported a maximum acetic acid consumption rate of 0.035 g/L-h, while our minimum value was 0.066 g/L-h, even though the initial acetic acid concentration in the referred study did not exceed 2 g/L (Llamas et al., 2020). The faster and better acetic acid assimilation in our experiment likely results from the enhanced efficiency of the engineered yeast.

3.2.2. *Lipid production*

The results obtained regarding lipids are shown in Table 2, while the composition is shown in Fig. 3. The initial carbon to nitrogen (C/N) ratio, a crucial factor for lipid production, was also calculated for each assay. Adequate C/N ratio allows for biomass construction and carbon reservation for lipid synthesis (Karamerou et al., 2017). High C/N ratios induce stress in the microorganism due to nitrogen deficiency, activating the *de novo* synthesis metabolic pathway, leading to efficient lipid or single cell oils (SCOs) production (Mathiazhakan et al., 2016). However, a balanced C/N ratio is needed to ensure adequate biomass production (Sarantou et al., 2021). In our study, the C/N ratios ranged from 2 to 50, and it was found that lower C/N ratios resulted in higher biomass

Table 1
Different experimental variables in the experiment carried out in *Y. lipolytica* flasks, with different substrates and initial concentrations.

Substrate	Max. growth rate (h^{-1})	Lag phase (h)	Max. consumption rate (g/L-h)	Time to consume all substrate (h)
ACE 5 g/L	0.088	~ 24	0.066	~ 70
ACE 10 g/L	0.074	~ 40	0.087	~ 120
ACE 15 g/L	No consumption	No consumption	No consumption	No consumption
GLU 5 g/L	Not enough data	70–135	0.124	~ 80
GLU 10 g/L	Not enough data	50–70	0.107	~ 120
GLU 15 g/L	0.13	~ 50	0.22	~ 100
ACE 10 g/L + 25 % YE	0.065	~ 50	0.179	Not totally consumed
ACE 10 g/L + 25 % YE + 25 % NH_4Cl	0.033	~ 40	0.159	Not totally consumed
ACE 10 g/L + 25 % NH_4Cl	0.055	~ 40	0.22	~ 70
Control	0.079	<20	0.222	~ 50

production. For instance, the control experiment with C/N = 3 produced the highest biomass concentration of 6.18 g/L.

The experiment with 10 g/L acetic acid + 25 % YE reached the highest lipid content of 33.67 % (g/g dry biomass), corresponding to an initial C/N ratio of 40. Another flask with a high C/N ratio (C/N = 50) resulted in a lipid content of 24.96 % g/g. This decrease in lipid content despite a higher C/N could be attributed to nutrient deficiency as it corresponds to the assay with 25 % YE and standard NH_4Cl. The lowest lipid content of 16.59 % g/g was observed in the flask with 5 g/L glucose. In terms of lipid concentration (g/L), the control flask had the highest value of 1.79 g/L, despite not being the assay with the highest lipid concentration. This can be explained by the fact that the control flask produced almost three times more biomass than the flask with 10 g/L acetic acid + 25 % YE.

Table 2 reveals the yields in terms of biomass and lipids produced relative to the substrate consumed, denoted as $Y_{(X/S)}$ and $Y_{(L/S)}$, respectively. Biomass yield is higher when using glucose as a substrate compared to acetic acid. However, the lipid yield is similar between the substrates, except for the control flask which had the highest lipid yield of $Y_{(L/S)} = 0.179$.

Fig. 3 outlines the lipid compositions from each experiment. While the compositions remained mostly consistent across tests, oleic acid (C18:1) was the dominant compound, followed by palmitic acid (C16:0) and linoleic acid (C18:2). Palmitoleic acid (C16:1) and stearic acid (C18:0) were found at lower percentages. Differences in the lipid profile appeared when *Y. lipolytica* was grown on acetic acid versus glucose,

Table 2
Different experimental variables are summarized for *Y. lipolytica* flasks experiments, with different substrates and initial concentrations.

Substrate	C/N	Dry biomass (g/L)	Lipid content (% g/g)	Lipid concentration (g/L)	$Y_{(X/S)}$	$Y_{(L/S)}$	Relative fatty acid content (%)					
							C16:0	C16:1	C18:0	C18:1	C18:2	Others
ACE 5 g/L	13	2.12	19.05	0.404	0.424	0.081	16.2	15.6	2.6	46.7	18.8	0
ACE 10 g/L	18	3.62	27.25	0.986	0.362	0.099	19.9	9.9	3.9	46.2	18.4	1.7
ACE 15 g/L	23	–	–	–	–	–	–	–	–	–	–	–
GLU 5 g/L	14	2.64	16.59	0.438	0.528	0.088	17.1	11.7	3.1	45.5	19.8	2.9
GLU 10 g/L	19	4.12	24.48	1.008	0.412	0.101	16.8	12.6	2.3	50.1	15.4	2.9
GLU 15 g/L	24	4.98	28.35	1.412	0.332	0.094	19.2	12.2	3.1	51.1	12.1	2.3
ACE 10 g/L + 25 % YE	40	2.38	33.67	0.801	0.29	0.098	25.4	8.6	5.7	42.9	16.5	0.9
ACE 10 g/L + 25 % YE + 25 % NH_4Cl	50	2.34	24.96	0.584	0.312	0.078	25.7	9.3	5.2	43	15.7	1.1
ACE 10 g/L + 25 % NH_4Cl	20	2.82	25.11	0.708	0.282	0.071	21.8	9.7	4	45.1	18	1.4
Control	3	6.18	28.98	1.791	0.618	0.179	24.3	11.6	4.1	44.3	12.5	3.2

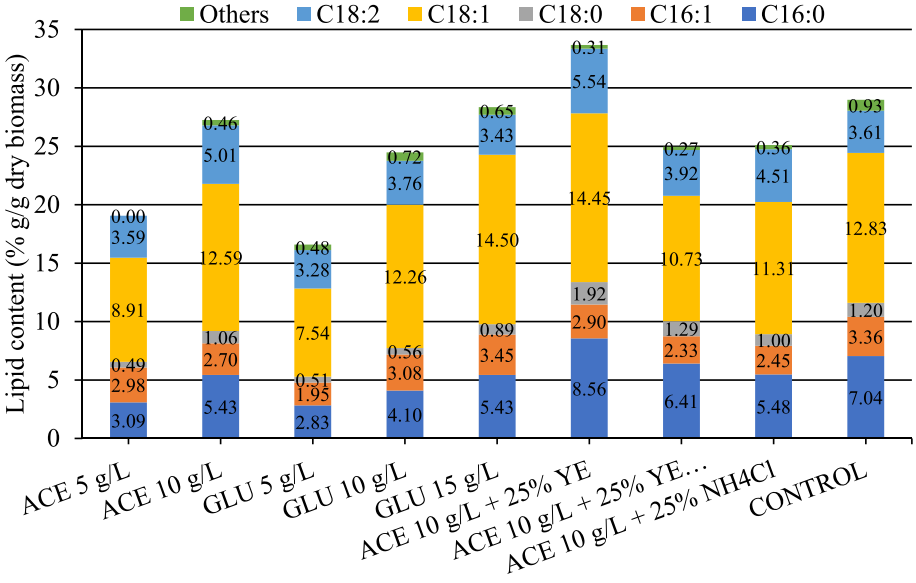

Fig. 3. Composition of lipids obtained in *Y. lipolytica* flasks experiments.

with a higher proportion of C18:2 compound produced when grown on acetic acid.

The lipid profile of yeasts is closely related to experimental conditions and the yeast strain (Hansson and Dostálek, 1986), typically with C18:1 acid as the most dominant, comprising up to 66 % of total lipids (Manzoor, 2017). For example, Carsanba and colleagues showed that variations can exist even between different yeasts of the same species (Carsanba et al., 2020). For our experiment, the maximum C18:1 was 51.1 % from 15 g/L glucose (Table 2). Comparing this lipid profile with similar studies, our findings are largely consistent. For instance, two types of *Y. lipolytica* strains grown on glucose and later given VFAs also yielded similar C18:1 and C18:2 percentages, but with minor differences in C16:0, C16:1, and C18:0 percentages (Pereira et al., 2021). Another study growing *Y. lipolytica* with glycerol yielded similar results, with the exception of a higher C18:2 value in our study (Magdouli et al., 2017).

3.2.3. *β-carotene production*

The experiment measured β-carotene produced by *Y. lipolytica* over time. Fig. 4A shows the results of all flasks in mg/L, while Fig. 4B shows the results as a function of mg/g of biomass. The control flask produced the highest β-carotene concentration (45 mg/L), followed by assays with 10 g/L acetic acid, 15 g/L glucose, and 10 g/L acetic acid + 25 % NH_4Cl. Notably, the 10 g/L acetic acid flask outperformed the 15 g/L glucose flask, indicating a higher product-substrate yield. Assays with 25 % NH_4Cl also performed well, exceeding average values. However, looking at Fig. 4B, the control experiment did not exhibit the highest β-carotene concentration despite its overall high biomass production. The best results in terms of β-carotene per dry weight were found in the 10 g/L acetic acid and 15 g/L glucose experiments.

The optimal C/N ratio for β-carotene production is between the values that promote lipid and biomass production. Higher C/N ratios enhance lipid production, whereas lower ones are conducive for biomass growth. Ma et al. determined that the best β-carotene yield occurs at C/N = 9, whereas the lowest was observed at C/N = 43, where it produced almost half the amount of carotenoids (Ma et al., 2022). It is worth noting that Ma's experiments maintained a constant initial glucose concentration of 50 g/L, only varying the nitrogen content sourced from YE and peptone.

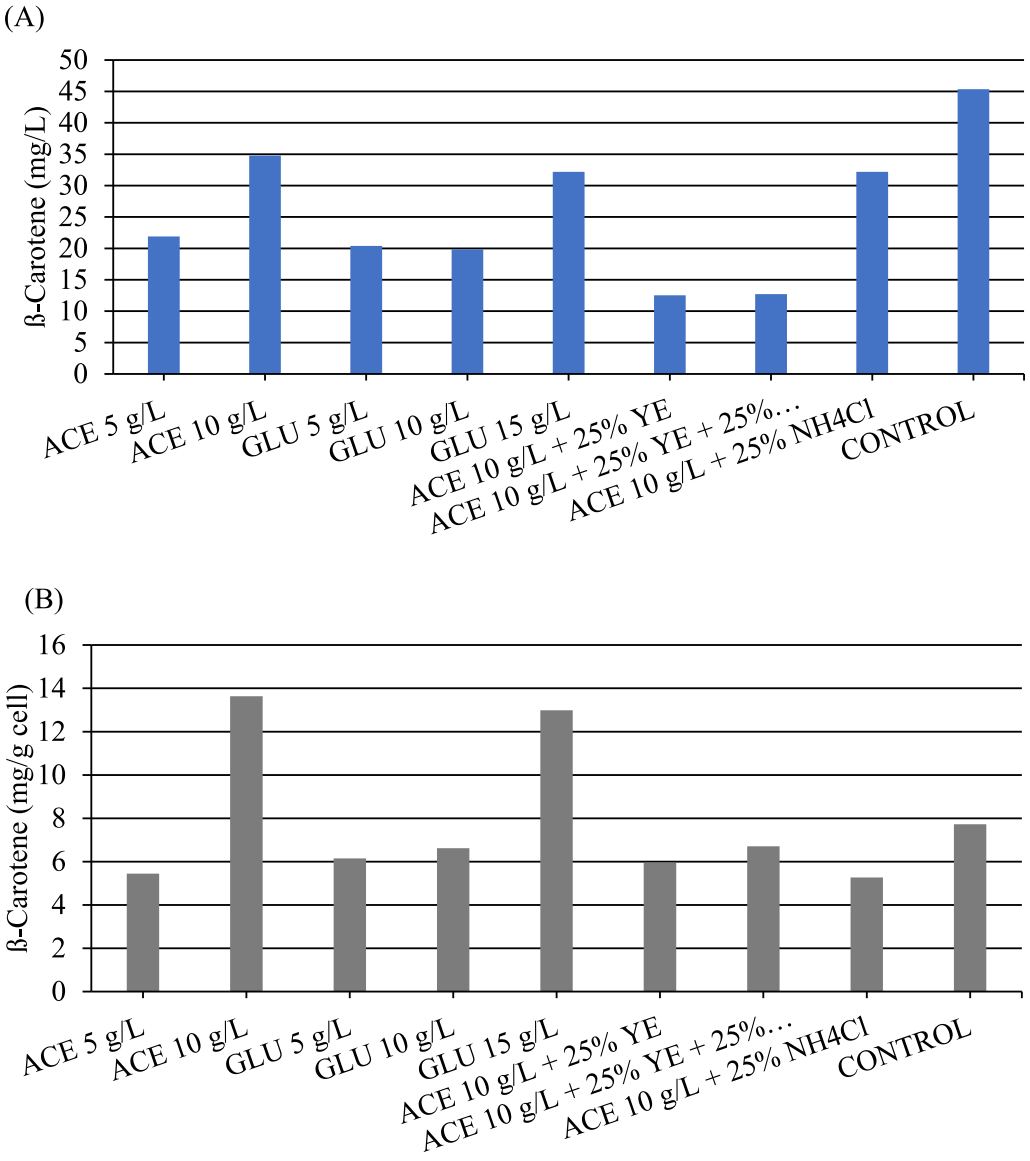

Fig. 4. Maximum (A) β-carotene concentration and (B) β-carotene content obtained in each flask, with different substrates and initial concentrations.

3.3. *Y. lipolytica* cultivation on *C. aceticum* fermented medium from syngas

3.3.1. Acetic acid production by *C. aceticum*

C. aceticum bacteria were grown on syngas in an STR. Although this bacterium can produce other types of products (Arslan et al., 2019), the focus was on acetic acid production to supply second-stage *Y. lipolytica* reactors. To stimulate acid production and prevent alcohol formation, the pH was set and maintained constant at around 7.5 or higher, known as the optimal pH for these conditions (Fernández-Blanco et al., 2022).

C. aceticum's growth and acetic acid accumulation were monitored over time, with the bacteria producing up to 7 g/L acetic acid 230 h post-inoculation. The maximum growth rate was $\mu_{max} = 0.025\ h^{-1}$, which is somewhat lower than on pure CO (Arslan et al., 2021), while the acetic acid production rate remained nearly constant at 0.05 g/L-h , decreasing only slightly until the end of the experiment.

An experiment with *C. aceticum* fed syngas at pH 8.0 produced 9.4 g/L acetic acid (Arslan et al., 2019), while another experiment under identical conditions as in the present study yielded 11.5 g/L acetic acid 387 h after inoculation (Robles-Iglesias et al., 2023b). The time this reactor was kept active is much longer than the time of the present experiment, perhaps that is why a higher concentration was reached. Nevertheless, it was decided to stop our experiment at 230 h because the production rate of acetic acid decreased considerably. Comparatively, our production rate of acetic acid is substantially lower than other *C. aceticum* experiments; for instance, using pure CO as substrate yielded a maximum production rate of 0.26 g/L-h, compared to 0.05 g/L-h in our experiment (Arslan et al., 2021). This suggests that pure CO is a more effective substrate than a syngas mixture for *C. aceticum*. Under optimal conditions, this organism can even produce acetic acid concentrations nearing 20 g/L (Arslan et al., 2021). The medium from the *C. aceticum* experiment was stored, then divided into two STRs, sterilized, and inoculated with *Y. lipolytica* under various experimental conditions as described in the "Materials and Methods" section.

3.3.2. Effect of pH on lipid production in a bioreactor with *Y. lipolytica* from the acetic acid-rich *C. aceticum* fermented medium

The medium from the previous *C. aceticum* experiment was divided into two 0.9 L reactors. Both reactors had identical operating conditions, except for pH, maintained at 6.0 in one and 8.0 in the other. Optical density data and acetic acid (HAc) concentrations can be found in Fig. 5A and 5B, respectively. Due to the rapid consumption of acetic acid, extra feeds were added at 160 and 220 h, bringing the total amount consumed to around 20 g/L. The initial acetic acid concentration decreased slightly from 7 g/L to 6.6 g/L due to pH adjustment.

Y. lipolytica grew effectively in both reactors at different pHs, as shown in Fig. 5A, with a similar lag phase of approximately 38 h (Table 3), consistent with previous flask experiments. The growth kinetics in the reactor experiments surpassed those in the flask experiments, likely due to constant pH and air-flow control. Maximum biomass concentrations were 5.05 g/L and 3.07 g/L in the pH 6.0 and pH 8.0 reactors, respectively. When comparing these results to those obtained in the previous experiment (Table 2), high biomass is typically obtained in reactor experiments using acetic acid as a substrate, while biomass amounts produced with glucose in flasks are generally similar. On the other hand, Fig. 5B shows data of concentrations of acetic acid in the reactor, and thus the substrate consumption by *Y. lipolytica*. The maximum acetic acid consumption rate was slightly higher in the experiment performed at pH 6.0 (0.268 g/L-h) than at pH 8.0 (0.223 g/L-h). It is notable that the consumption rate in the pH 8.0 reactor decreased following the first acetic acid addition, compared to the pH 6.0 reactor.

The amount lipids produced in the reactors at different pH values also varied. The maximum lipid content was 22.9 % g/g at pH 6.0 and 17.6 % g/g at pH 8.0. The lipid concentrations were 1.03 and 0.50 g/L at pH 6.0 and 8.0, respectively. Interestingly, alkaline conditions (pH 8), in the second experiment, did not validate the expectations drawn from the initial toxicity experiment, nor did they coincide with findings from existing literature. For instance, Gao et al. (2020) demonstrated that *Y. lipolytica* produced a significantly higher lipid yield at pH 8.0 compared to pH 6.0 (29.3 % *vs* 16.4 % g/g). This discrepancy between our findings and Gao et al.'s might be attributed to the different initial concentrations of acetic acid used—30 g/L in their study compared to 6.6 g/L in the current work. At a lower initial acetic acid concentration, the strain could find an optimal pH closer to 6.0 for growth and lipid production. However, at higher initial concentrations, such as 30 g/L, the toxicity of the dissociated acids might be effectively reduced at a higher pH, thus enhancing lipid yields.

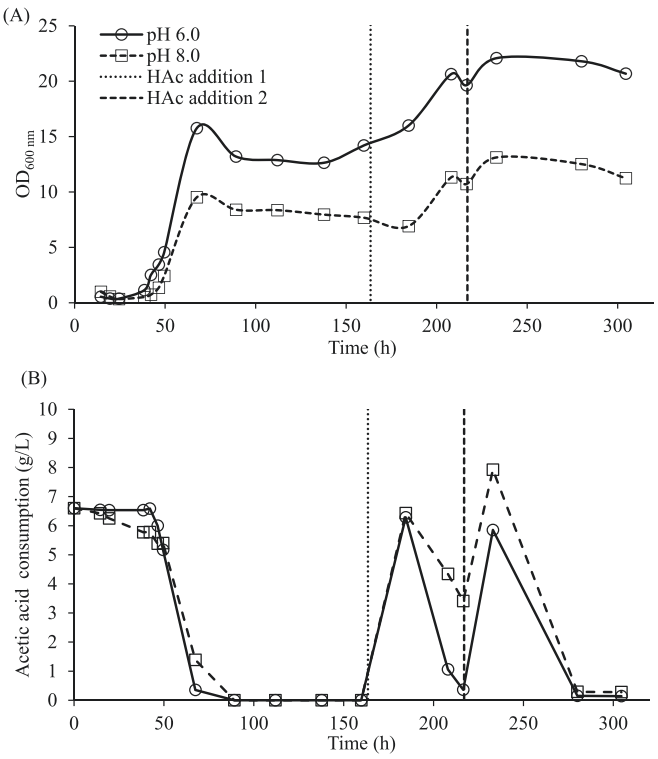

Fig. 5. (A) Growth and (B) acetic acid consumption of *Y. lipolytica* in bioreactor at pH 6.0 and 8.0.

The lipid content and concentration in the bioreactors at both pH are similar to those obtained in the previous flasks experiments with 5 g/L glucose and 5 g/L acetic acid (Table 2). However, the amount lipids is higher in the flasks of the previous experiment. The reason is probably related to the C/N ratio. To determine the C/N ratio of the reactor experiment, acetic acid was assumed to be the only carbon source, while for nitrogen, the amount of ammonium present in the medium was determined. Since the bacteria had previously consumed virtually all of the ammonium available for growth, the ammonium remaining in the medium to grow the yeast was zero and this leads to a C/N ratio reaching infinity. Previous research by Ye et al. on *R. toruloides* and different C/N ratios concluded that the lipid content peaked at a C/N ratio of 200, then decreased, with infinite C/N ratio not yielding the best results (Ye et al., 2021).

Table 3 also displays the relative amounts of extracted FAMEs, showing similar lipid profiles at both pH values and comparable to prior flask experiments. It suggests that the substrate used impacts the lipid profile more significantly than the medium's pH.

3.3.3. Effect of pH on β-carotene production

The experiment monitored β-carotene production by *Y. lipolytica*, with Fig. 6 displaying the results. The reactor at pH 6.0 achieved better results compared to pH 8.0, with the maximum concentration being 164

Table 3
Different experimental data obtained in the *Y. lipolytica* bioreactor experiments at pH 6.0 and pH 8.0.

pH	C/N	Max. growth rate (h^{-1})	Lag phase (h)	Dry biomass (g/L)	Max. consumption rate (g/L-h)	Lipid content (% g/g)	Lipid concentration (g/L)	$Y_{(X/S)}$	$Y_{(L/S)}$	Relative fatty acid content (%)					
										C16:0	C16:1	C18:0	C18:1	C18:2	Others
6	∞	0.12	38	5.04	0.268	22.9	1.03	0.24	0.055	19.3	7.5	4.9	49.3	17.3	1.7
8	∞	0.14	38	3.07	0.223	17.6	0.5	0.15	0.024	18.4	9.8	4.7	45.5	18	3.7

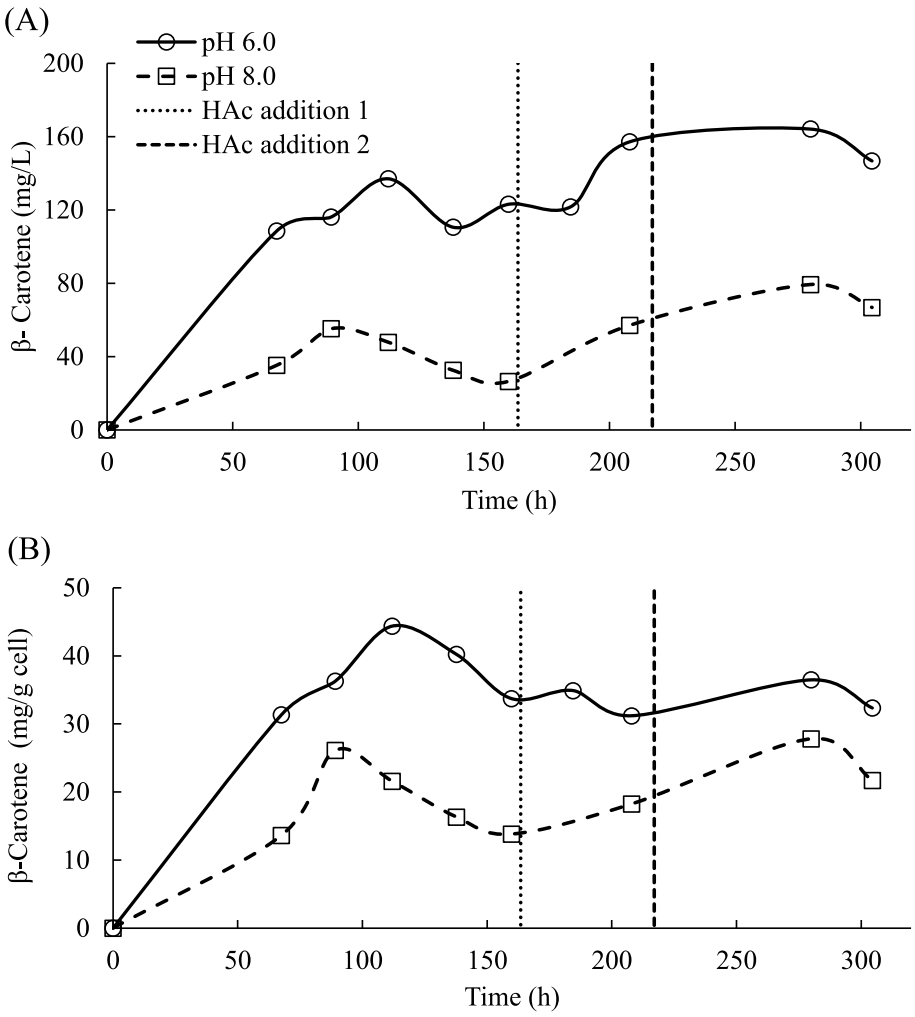

Fig. 6. (A) β-carotene concentration and (B) β-carotene content per gram dry cell produced by *Y. lipolytica* at pH 6.0 and 8.0.

mg/L and 44 mg/g cell at t = 110 h. The best results at pH 8.0 were 79 mg/L and 27.8 mg/g cell. It can be concluded that pH change influenced β-carotene production more than lipid accumulation in this *Y. lipolytica* strain. Shifting from pH 8.0 to 6.0 saw lipid content rise by roughly 30 %, while β-carotene content increased by 58 %.

In a similar reactor study using the same yeast but with glucose as substrate, a β-carotene concentration of 90 mg/g cell was achieved, which is nearly twice our result (Larroude et al., 2018). However, glucose is 4–20 times more costly than acetic acid (Bonatsos et al., 2020; G. W. Park et al., 2021b). Besides, in that previously published study, an optimal medium was used for the yeast rather than an acetogenic fermented bacterial medium, as in the present research. It is therefore interesting to calculate the yield ($Y_{C/S}$) obtained. In the present experiment, the yield in terms of β-carotene obtained is $Y_{C/S}$ = 0.0082 g/g acetic acid; however, in the experiment carried out by Larroude and coworkers, a yield of $Y_{C/S}$ = 0.036 g/g glucose was achieved, which is four times higher. To sum up, compared with acetic acid obtained from fermented syngas, the experiment with glucose had a yield four times higher and a β-carotene content two times higher. Therefore, for these conditions, as long as the price of acetic acid is at least eight times (or more) lower than the price of glucose, acetic acid can be considered a preferred substrate to produce β-carotene.

To complete the analysis, two linear regression models were constructed using software R to investigate the relationship between substrate consumption and β-carotene production in reactors operating at pH 6 and pH 8. In the model at pH 6, the coefficient for substrate concentration is 4.291 with a p-value of 0.0114, whereas in the model at pH 8, the coefficient is 3.736 with a p-value of 0.0201. Both coefficients are statistically significant at a 95 % confidence level, but the magnitude of the coefficient at pH 6 is slightly larger, suggesting a more substantial increase in β-carotene production for each unit increase in substrate concentration at this pH level. Concerning the fit of the models, the R-squared value is 0.7524 at pH 6 and 0.6931 at pH 8. The F-statistics, 15.19 at pH 6 and 11.29 at pH 8, further support the models' goodness of

fit, although the model at pH 6 appears slightly stronger. To sum up, while substrate concentration is a significant factor for β-carotene production in both pH conditions, the model suggests that its impact is more pronounced at pH 6, making it a more robust predictive model compared to its pH 8 counterpart.

3.4. Comparison between the experiments

Firstly, the toxicity experiment shows a decrease in growth and acetic acid utilization with higher substrate concentration, which is indicative of potential substrate inhibition. The second experiment with unfermented acetic acid, however, presents a more complex interplay of nutrients and substrates, where higher growth and lipid production occur under a combination of acetic acid and low yeast extract. The divergence in these results suggests that the inhibitory effects of acetic acid, witnessed in the first experiment, may be alleviated or mitigated by the presence of other nutrients, as in the second experiment.

Secondly, the bioreactor experiment inoculated with fermented acid containing broth coming from *C. aceticum* fermentation introduces an additional variable, i.e., pH control. Here, *Y. lipolytica* exhibits stable growth and acetic acid consumption. This contrasts sharply with the first experiment, where there might have been an uncontrolled pH fluctuation due to high acetic acid concentrations, leading to decreased growth.

Lastly, lipid and β-carotene productions present distinct optimization landscapes across the experiments. Unfermented acetic acid experiment maximizes lipid yield with additional commercially sourced acetic acid and yeast extract, while β-carotene production peaks in bioreactor experiment under controlled pH conditions. Notably, acetic acid in this experiment is provided from fermentation derived from a *C. aceticum* reactor, which introduces the possibility that dead biomass or other by-products may provide some nutrients that might favor one metabolic pathway over another. This divergence in peak production suggests that the metabolic pathways for lipid and β-carotene biosynthesis are influenced differently by environmental and nutritional variables. While pH stability appears to optimize β-carotene yield, a well-balanced nutrient profile is essential for lipid production. Therefore, the bioconversion performance of *Y. lipolytica* is multi-factorial and may require further sophisticated approaches to simultaneously optimize both lipid and β-carotene production.

This additional variable strengthens the imperative for further comprehensive research on understanding metabolic flux distributions and nutrient contributions, especially when relying on a fermentative source like *C. aceticum* for acetic acid.

4. Conclusions

This study effectively revealed the capabilities of *Y. lipolytica*, adapted to an acetic acid-rich medium, to simultaneously produce lipids and β-carotene from syngas-derived carboxylic acids. It established the yeast's acetic acid tolerance, key to its growth and productivity. By optimizing the experimental parameters, the process could be enhanced further. The achieved lipid profile, with nearly 50 % C18:1 compound, is significant for biodiesel production, thus promising an economical and efficient production method from syngas.

CRediT authorship contribution statement

Raúl Robles-Iglesias: Data curation, Investigation, Methodology, Writing – original draft. **Jean-Marc Nicaud:** . **María C. Veiga:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Christian Kennes:** Conceptualization, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christian Kennes reports financial support was provided by Spain Ministry of Science and Innovation. Maria C. Veiga reports financial support was provided by Government of Galicia.

Data availability

Data will be made available on request.

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