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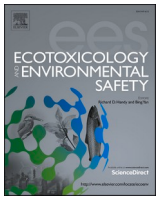

Unlocking the potential of one-carbon gases (CO_2, CO) for concomitant bioproduction of β-carotene and lipids

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ABSTRACT

This study investigates the use of a *Yarrowia lipolytica* strain for the bioconversion of syngas-derived acetic acid into β-carotene and lipids. A two-stage process was employed, starting with the acetogenic fermentation of syngas by *Clostridium aceticum*, metabolising CO, CO_2, H_2, to produce acetic acid, which is then utilized by *Y. lipolytica* for simultaneous lipid and β-carotene synthesis. The research demonstrates that acetic acid concentration plays a pivotal role in modulating lipid profiles and enhancing β-carotene production, with increased acetic acid consumption leading to higher yields of these compounds. This approach showcases the potential of using one-carbon gases as substrates in bioprocesses for generating valuable bioproducts, providing a sustainable and cost-effective alternative to more conventional feedstocks and substrates, such as sugars.

1. Introduction

The biological or catalytic conversion of one-carbon gases (e.g., CO_2, CO), typically present in some industrial emissions, into valuable bioproducts is a growing area of interest (Fernández-Naveira et al., 2017a, 2017b). These same gases can also be found in synthesis gas (syngas), a more complex mixture predominantly comprising carbon monoxide, hydrogen, and carbon dioxide, along with other minor elements. Syngas serves as a versatile substrate for various microorganisms capable of producing biochemicals such as acetic acid, ethanol, and longer carbon-chain compounds (He et al., 2022; Arslan et al., 2022). Among these, acetic acid stands out due to its suitability as a valuable chemical intermediate and for its potential to serve as a sustainable feedstock for other bioproducts (Robles-Iglesias et al., 2023a, 2021).

Various methods are available for synthesizing acetic acid from syngas. Chemical processes like the Monsanto (Knox et al., 1973) and Cativa (Aubigne et al., 1995) methods involve catalytic reactions under high pressures and temperatures. These methods have been traditionally favored for their efficiency in large-scale production. However, biological methods, based on microbial bioconversion, are emerging as viable alternatives. These biological approaches leverage the natural capabilities of acetogenic bacteria, offering advantages such as lower energy requirements, milder operational conditions, and reduced environmental impact compared to their chemical counterparts (Ahmad et al., 2023). The present study focuses on the biological synthesis of acetic acid using *Clostridium aceticum*, a process that aligns with sustainable production goals and demonstrates high efficiency and selectivity for acetic acid production from syngas components.

The use of acetic acid as a feedstock offers a multifaceted avenue for biotechnological advancements. Acetic acid, originating from syngas through microbial pathways involving acetogenic bacteria like *C. aceticum*, stands out as a promising substrate for the biosynthesis of microbial lipids (Robles-Iglesias et al., 2023a). These lipids, serving as crucial biomolecules with applications ranging from the production of biofuels to oleochemicals, can be accumulated by oleaginous microorganisms capable of storing up to 20% lipids in their dry biomass (Robles-Iglesias et al., 2023a; Ma et al., 2018). Moreover, the utilization of acetic acid can circumvent limitations affecting the commercial viability of microbial lipids, primarily arising from the high costs and environmental concerns associated with more conventional feedstocks like glucose (Ochsenreither et al., 2016).

Building upon this premise, a particularly innovative aspect of this

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study lies in the extension of acetic acid utilization to the biosynthesis of carotenoids, specifically β-carotene, using also oleaginous yeasts. Beyond its role in enhancing the spectrum of high-value compounds derived from syngas bioconversion, β-carotene holds substantial commercial significance. As a powerful antioxidant, it finds extensive application in food, cosmetics, and pharmaceutical industries for its health benefits and as a natural colorant (Mata-Gómez et al., 2014). The global market demand for β-carotene underscores the potential of this biotechnological route (Beta Carotene Market, 2022). While the carotenoid-producing capabilities of various oleaginous yeasts such as *Rhodotorula* spp., *Rhodosporidium* spp., *Sporobolomyces* spp., and *Xanthophylomyces* spp. have been previously documented (Mannazzu et al., 2015; Igreja et al., 2021), this study marks a pioneering effort in synthesizing β-carotene from acetic acid derived from one-carbon gases (CO_2, CO) such as also found in some industrial gas emissions as well as syngas.

However, the utilization of acetic acid in microbial lipid production presents significant challenges. A primary concern is the toxicity of acetic acid to oleaginous yeasts such as *Y. lipolytica*, particularly at higher concentrations (Naveira-Pazos et al., 2023). In a recent study, it was reported that the same engineered *Y. lipolytica* strain could tolerate acetic acid concentrations up to 20 g/L (Robles-Iglesias et al., 2023b). Beyond this threshold, a significant drop in growth and lipid production was observed. For instance, at 25 g/L, a marked decline in cell viability and lipid accumulation was evident, underscoring the importance of optimizing acetic acid concentrations for effective commercial application of this biotechnological process.

To sum up, the central hypothesis of this study posits that a two-stage bioconversion system involving *C. aceticum* and *Y. lipolytica* can effectively convert syngas into value-added products, including acetic acid, and subsequently, lipids, and uniquely, β-carotene. This represents a pioneering effort to utilize syngas and, more specifically, one-carbon gases such as CO_2 and CO, for β-carotene production, thereby adding a new dimension to this field of gas bioconversion. The study aims to first optimize an anaerobic process where *C aceticum* converts syngas into acetic acid. This acetic acid then serves as the substrate for an aerobic reactor inoculated with *Y. lipolytica*, with the objective of producing both microbial oils and β-carotene. Through this integrated approach, the research not only aims to offer a more sustainable and cost-effective alternative to conventional feedstocks but also seeks to be sustainable and to significantly enhance the commercial viability of microbial lipids and β-carotene production. This work aims to catalyze a shift in the perception and industrial applicability of microbial bioconversion technologies, particularly in the production of high-value compounds such as β-carotene.

2. Material and methods

2.1. Acetic acid production stage

2.1.1. Microbial strain

C. aceticum DSM 1496 was obtained in freeze-dried pellet form from DSMZ (Germany). Following rehydration, incubations were conducted at 150 rpm and 33 °C.

The composition and preparation of the medium used for the inoculum is detailed as follows (per liter of distilled water): 0.20 g NH_4Cl, 3 g yeast extract, 1.76 g KH_2PO_4, 8.44 g K_2HPO_4, 0.33 g $MgSO_4 \cdot 7\ H_2O$, 10 g $NaHCO_3$, 1 mL resazurin (0.1% w/v), 2 mL trace metals solution, 0.30 g cysteine-HCl and 0.92 g $Na_2S \cdot 9\ H_2O$. The composition of the trace metal solution was (per liter distilled water): nitriloacetic acid, 3.50 g; $MgSO_4 \cdot 7\ H_2O$, 6.00 g; $MnSO_4 \cdot H_2O$, 1.50 g; $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$, 0.90 g; $CoCl_2 \cdot 6\ H_2O$, 0.40 g; $ZnSO_4 \cdot 7\ H_2O$, 0.38 g; $CuCl_2 \cdot 2\ H_2O$, 0.04; $NiCl_2 \cdot 6\ H_2O$, 0.05 g; Na_2SeO_4, 0.3 g; $Na_2MoO_4 \cdot 2\ H_2O$, 0.03 g; $FeSO_4 \cdot 7\ H_2O$, 0.2; $CoSO_4 \cdot 7\ H_2O$, 0.36; $CuSO_4 \cdot 5\ H_2O$, 0.02; $KAl(SO_4)_2 \cdot 12\ H_2O$, 0.04; H_3BO_3, 0.02 g. The composition of the vitamin solution was (per liter distilled water): 25 mg D-biotin, 25 mg folic acid, 50 mg pyridoxine-HCl, 50 mg thiamine-HCl, 50 mg riboflavin, 50 mg nicotinic acid, 50 mg D-Ca-pantothenate, 25 mg vitamin B_{12}, 50 mg p-aminobenzoic acid and 25 mg lipoic acid. This medium is recommended by the commercial provider (DSMZ, 2024).

The chemicals were dissolved in 40 mL of distilled water in serum bottles. The medium was flushed with nitrogen, followed by CO, then its pH was adjusted to 8, and it was sterilized at 121 ºC for 20 min.

2.1.2. Continuous syngas-fed bioreactor

Using an Eppendorf BIOFLO 120 bioreactor with a working volume of 1.5 L, the experiment was conducted at a constant temperature of 33 ºC with 250 rpm stirring. Each reactor was filled with the culture medium, which had the following composition per liter distilled water: yeast extract, 2.30 g; NH_4Cl, 1.37 g; KCl, 0.125 g; KH_2PO_4, 1.175 g; K_2HPO_4, 5.07 g; $NaHCO_3$, 6.00 g; $MgSO_4 \cdot 7\ H_2O$, 0.46 g; $CaCl_2 \cdot 2\ H_2O$, 0.05 g; NaCl, 1 g; cysteine-HCl, 0.48 g; $Na_2S \cdot 9\ H_2O$, 0.54 g; Na-resazurin (0.1% w/v), 0.75 mL; trace elements solution, 5 mL; vitamins solution, 4 mL. After autoclaving, cysteine-HCl and $Na_2S \cdot 9\ H_2O$ were added under anaerobic and aseptic conditions. Anaerobicity was ensured by flushing the medium with pure N_2 for two hours, then substituting with a CO, CO_2, H_2, N_2 gas mixture (30:10:20:40). Gas flow was kept at 10 mL/min. The broth's redox potential was tracked with an Ag/AgCl electrode and a transmitter, while the pH was maintained at 8.0 using 1 M HCl or NaOH.

2.2. Lipids and carotenoids production stage

2.2.1. Engineered strain and growth medium

The *Y. lipolytica* strain JMY6862 (ob-CHCTEFCTEF) was utilized in this study for β-carotene and lipid production. This strain is a derivative of the lipid-overproducing 'Obese' JMY3501 strain, as referred to in the work of Lazar Z et al (Lazar et al., 2014). The genetic engineering of JMY6862 was performed through a multi-step synthetic biology approach, as detailed in the research conducted by Larroude et al (Larroude et al., 2018). Initially, *Escherichia coli* DH5α was used for cloning and plasmid propagation under specific growth conditions and antibiotic selection. The *Y. lipolytica* strains were derived from Po1d (wt), which itself is derived from the wild-type *Y. lipolytica* W29 (ATCC20460) strain. The strains were cultivated in specific media, the details of which have been described previously (Larroude et al., 2018). A carotenoid expression cassette, termed 'car-cassette' (Larroude et al., 2018), was designed and assembled using the Golden Gate DNA assembly technique, involving specific restriction enzymes and thermal profiles. This assembly was then transformed into *E. coli* and subsequently into *Y. lipolytica*. Finally, a promoter shuffling strategy was employed to optimize gene expression, and the resulting strains were screened based on color intensity. This process involved the successive introduction of three copies of β-carotenoid expression cassettes, thereby enhancing the β-carotene production capabilities of the engineered strain (Celińska et al., 2017).

This strain was maintained at − 80 ºC, transferred to a Petri plate composed of Yeast Extract-Peptone-Dextrose agar before the start of the experiments, and cultured for 2–3 days. Before inoculating the yeast into the bioreactors, the next step was to culture it in liquid medium from the Petri plate, with a medium composed of Yeast Extract-Peptone-Dextrose (YPD) broth for 24–36 h.

2.2.2. Continuous air-fed bioreactor

Two *Y. lipolytica* bioreactor studies were conducted in an acetic acid-rich medium. The first involved cultivating the yeast with 11 g/L commercial acetic acid, while periodically adding approximately 10 g/L more. The second used a medium from *C. aceticum*'s anaerobic fermentation, enriched with 10 g/L acid, with similar acid additions. Both experiments maintained a pH of 6.0, initial volume of 1 L, constant temperature of 30 ºC, 40% dissolved oxygen content, and variable agitation (150–300 rpm), and airflow rate (0.5–2.0 vvm). Both reactors

were inoculated with an $OD_{600\ nm}$ of 1 of *Y. lipolytica*, pre-washed with 0.9% NaCl.

2.3. Analytical methods

2.3.1. Determination of biomass

Biomass in the acetogenic bacteria experiment was quantified via optical density from a spectrophotometer using a 1 mL sample from the bioreactor measured at 600 nm. Conversely, in the *Y. lipolytica* experiments, biomass determination involved direct measurement of the dry weight of a freeze-dried sample from the reactor.

2.3.2. Acetic acid analyses

Acetic acid quantities in the growth medium were measured using high-performance liquid chromatography (HPLC), with a diode array and refractive index detector at 50 ℃. The mobile phase was a 0.005 M sulfuric acid solution at a flow rate of 0.80 mL/min. 1 mL samples were taken during the bioreactor trials for monitoring acetic acid synthesis and consumption. Samples, centrifuged at 7000 rpm for 5 min and filtered through a 0.22 μm PTFE filter, were injected onto the Agilent Hi-Plex H column at 45 ℃.

2.3.3. Determination of lipid content

Samples were centrifuged at 4000 rpm for 10 min for lipid and β-carotene analysis, and then stored in the freezer with 1 mL of distilled water. Total lipids were extracted from 10–30 mg of freeze-dried biomass using a methanol and sulfuric acid solution (40:1). The resulting fatty acid methyl esters (FAMEs) were measured with a Thermo Fisher gas chromatograph equipped with an Agilent vf-23 column and a flame ionization detector, using helium as a carrier gas. Oven temperatures were regulated for the analysis, and FAMEs were identified by comparison to standards. Lipids were quantified using an internal approach with commercial C12:0 converted to FAME.

2.3.4. Determination of β-carotene content

The accumulation of β-carotene within the cells necessitates an extraction procedure. 250 μL of culture media were mixed with 500 μm of glass beads with a diameter of 1 mm, 1.2 mL of a 50:50 mixture of hexane and ethyl acetate, and 0.01% butyl hydroxyl toluene. The orange-colored supernatant was then extracted from the mixture after it had been vortexed, centrifuged, and incubated on ice. This procedure was repeated until the supernatant was no longer stained. The OD of the supernatant was then determined using a Hitachi Model U-200 spectrophotometer at a wavelength of 448 nm. Next, a calibration curve that was previously drawn was used to calculate the β-carotene concentration.

3. Results and discussion

3.1. Acetic acid production by C. aceticum from syngas in bioreactor

Acetic acid was produced by a pure culture of *C. aceticum* in a bioreactor, intended for subsequent use as a substrate for lipid production. Based on existing literature, a pH of 8.0 was chosen to strike a balance between bacterial growth and acetogenesis, while avoiding solventogenesis (Arslan et al., 2019). Likewise, certain trace metals are known to significantly influence the end products of microbial metabolism in acetogenic bacteria. Expanding on the rationale for the exclusion of tungsten (W) from the trace metal solution, prior research strongly indicated that the absence of tungsten may streamline the Wood-Ljungdahl metabolic pathway towards the synthesis of fatty acids over alcohols (Fernández-Naveira et al., 2019; Chakraborty et al., 2020).

After the reactor was inoculated with 10% *C. aceticum* pre-culture, a well-coordinated bacterial growth dynamic was observed (Fig. 1). A minimal lag phase was observed after 16 h, resulting in an $OD_{600\ nm}$ of 0.64 and acetic acid production of 744 mg/L. These values increased

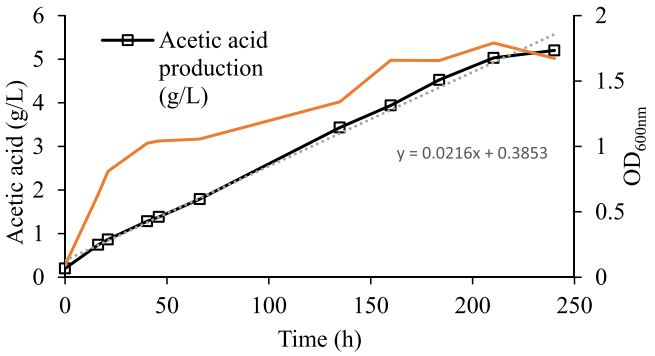

Fig. 1. Acetic acid production and $OD_{600\ nm}$ in *C. aceticum* syngas bioreactor.

until peaking at 208 h with an $OD_{600\ nm}$ of 1.8 and a final acetic acid concentration of 5.2 g/L after 240 h. This suggests an interrelationship between bacterial growth and acetic acid production, which may be governed by complex cellular mechanisms (Fernández-Blanco et al., 2023). Redox potential varied between − 91 mV and − 370 mV, ensuring reducing conditions for fermentation throughout the experiment.

When comparing these results to existing literature, it is observed that both acetic acid production and $OD_{600\ nm}$ were not exceptionally high. However, this discrepancy could be multifactorial, including differences in media composition, reactor configuration, and trace elements. Mayer et al. report different acetic acid production rates under varying CO partial pressures for *C. aceticum* (Mayer et al., 2018). For example, at an initial CO partial pressure of 100 mbar, they observed an acetic acid production rate of 0.4 g/L-h. Conversely, at 200 mbar, the acetic acid production rate dropped to 0.17 g/L-h, highlighting the impact of CO concentration (pressure) on bacterial product formation. The rate of acetic acid production in the present study, with no over-pressure, was 0.02 g/L-h, which is lower than in the study mentioned above. Though the absence of tungsten (W) in our study was designed to streamline metabolic pathways towards the synthesis of acetic acid rather than alcohols, the exact influence of this factor in the context of our experimental setup was not investigated. A study by Arslan and co-workers achieved up to 9.4 g/L acetic acid and 5.6 g/L ethanol using *C. aceticum* grown on syngas (Arslan et al., 2019). In the former study, the utilization of a medium composition different from the one used in the current research, which included W in the trace metal solution, and lowering the pH value somewhat below 7, stimulated ethanol production, thus reducing the accumulation of acetic acid. Instead, when maintaining a higher pH value (e.g., 7.5–8.0) and omitting W, acetic acid becomes the sole end product, as in the present study. Nevertheless, the adaptability of this strain to new environments is evident, as *C. aceticum* was able to grow rapidly, with no lag phase at all. This rapid growth could imply a level of metabolic versatility or resilience in *C. aceticum*, potentially enabling it to adapt to a variety of fermentation conditions.

In the present research, ethanol production was successfully circumvented, a result likely mediated by both the choice of an optimal working pH and by the deliberate exclusion of tungsten from the trace metal solution. As explained above and according to literature in order to observe a shift in the microbial metabolism towards solventogenesis, a lower pH is generally required; specifically, one or two units below the optimum growth pH for acetogens (Ganigué et al., 2016). The absence of W, guided by prior studies, may also have contributed to the preferential metabolic routing towards acetic acid synthesis over ethanol, although this needs further investigation for conclusive evidence.

3.2. Engineered *Y. lipolytica* bioreactor grown on acetic acid-rich media used for the growth of *C. aceticum*

3.2.1. Growth and acetic acid consumption

The same medium used for the growth of *C. aceticum* in the bioreactor, enriched with 10 g/L acetic acid, was used to cultivate *Y. lipolytica* in a separate bioreactor. Fig. 2 shows three acetic acid additions done upon detecting its depletion. The yeast metabolized a total of 42.15 g/L acetic acid in 230 h, with nearly linear consumption illustrated by the square-line. The circular solid line represents acetic acid amounts at each bioreactor sampling, while the optical density at different times, measured at 600 nm, is displayed by the solid line.

Based on the results acquired, it can be inferred that *Y. lipolytica* can grow effectively in a medium that is optimized for the cultivation of an anaerobic bacterium. Despite the yeast exhibiting a lag phase of approximately 75 h before starting growth, once acetic acid consumption began, there were no indications of toxicity or inhibition. The average rate of acetic acid consumption by the yeast was found to be 0.27 g/L-h. The observed 75-hour lag phase in the growth of *Y. lipolytica*, as illustrated in Fig. 2, can be attributed to the yeast's adaptation to the acetic acid-rich medium used. Acetic acid, at certain concentrations, can exert a stress response in yeast cells, necessitating a period of metabolic adjustment. This adaptation phase is crucial for the yeast to effectively acclimatize and commence robust growth under the given conditions.

3.2.2. Lipid production

Throughout the bioreactor experiment, samples were taken at different intervals to assess *Y. lipolytica*'s lipid production as it consumed varying amounts of acetic acid. It is of paramount importance to underscore the relationship between lipid production patterns and acetic acid availability. Fig. 3A shows that the lipid content remained stable at 17–20% within 12–30 g/L acetic acid consumption, then increased to 25.8% at 42 g/L. Conversely, biomass increased from 4 to 8 g/L as acetic acid consumption rose from 12 to 30 g/L, then stabilized at 8 g/L even at 42 g/L. Meanwhile, the lipid concentration progressively increased from 1.1 g/L to 2.1 g/L with increasing acetic acid consumption.

Y. lipolytica's metabolic pathways can explain its behavior: with excess acetic acid, it produces and accumulates lipids, causing an increase in lipid content. Once acetic acid becomes limited, it prioritizes biomass production, leading to continued biomass growth even as lipid content plateaus. This metabolic toggling is likely steered by shifts in the C/N ratio, a critical determinant in lipid and biomass production dynamics. Starting with a C/N ratio of 10, additional acetic acid supply without supplementary nitrogen sources progressively increases this ratio. Literature corroborates that lower C/N ratios predominantly facilitate biomass generation, whereas elevated C/N ratios, usually around 100, are conducive for lipid accumulation (Awad et al., 2019). In summary, this argues for a tightly regulated carbon flux which, based on the C/N ratio, allocates resources between biomass and lipid synthesis

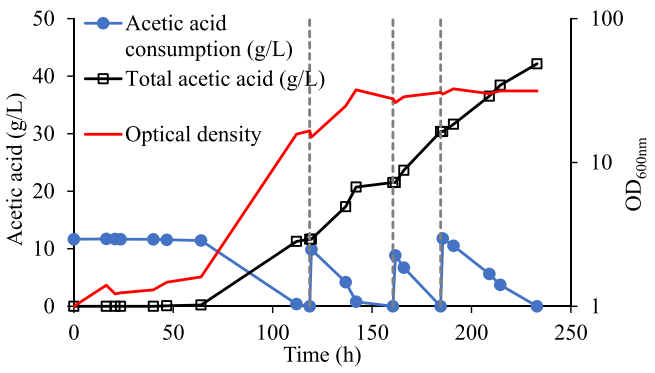

Fig. 2. Growth of *Y. lipolytica* as a function of optical density, and acetic acid consumption over time, in commercial acetic acid.

(Karamerou et al., 2017). Additionally, it is worth noting that the initial increase in biomass concentration can also be attributed to the fact that the microorganism is adapting to its environment and establishing its growth rate. Overall, these observations provide insight into the metabolic behavior of *Y. lipolytica* in the presence of varying concentrations of acetic acid consumed.

Fig. 3B presents the lipid profile obtained in each sample extracted at different concentrations of acetic acid consumed. It shows the percentage of individual lipid types, including C16:0, C16:1, C18:0, C18:1, C18:2, and others. As acetic acid consumption increases, there is an upward trend in C16:0, C18:0 accumulation, while the amount of C16:1, C18:1, and C18:2 compounds decreases. This predilection towards saturated fatty acids at elevated acetic acid levels suggests metabolic adaptation; it might imply a mechanism to harness energy more efficiently. Conversely, the decline in unsaturated fatty acids possibly indicates a scarcity of longer-chain precursors needed for their biosynthesis.

For a more visual interpretation, Fig. 3C deploys a heatmap to elucidate the normalized lipid profile values. This figure complements the data of Fig. 3B by offering a more immediate visual representation of how lipid profiles shift. The color gradient, ranging from white to red, represents the normalized values of individual lipid types. As acetic acid consumption increases, there is a discernible upward trend in the normalized values for C16:0 and C18:0, while those for C16:1, C18:1, and C18:2 generally decline. The increase in normalized values for saturated fatty acids like C16:0 and C18:0 can be attributed to *Y. lipolytica*'s metabolic adaptation to utilize acetic acid more efficiently. Conversely, the decline in normalized values for unsaturated fatty acids, like C16:1 and C18:1, could be indicative of a reduced availability of longer-chain precursors for their biosynthesis.

Fig. 3D employs a heatmap to represent the Z-scored lipid profile values in *Y. lipolytica* across different acetic acid concentrations. The Z-score normalization technique allows for a more nuanced understanding of how each lipid type deviates from the mean, providing a standardized metric that is independent of the unit of measurement. In this figure, the color gradient from blue to red represents the Z-score, with blue indicating values below the mean and red indicating values above the mean.

Upon examination of Fig. 3D, it is evident that the lipid types C16:0 and C18:0 generally exhibit positive Z-scores as acetic acid consumption increases, particularly at concentrations of 37 and 42 g/L. This suggests that these saturated fatty acids are produced in higher quantities relative to the mean at these acetic acid concentrations. Conversely, the unsaturated fatty acids, notably C16:1 and C18:1, predominantly show negative Z-scores, especially at higher acetic acid levels. This indicates that the production of these unsaturated fatty acids is less than the mean at these concentrations. The Z-score heatmap thus corroborates the observations made in the normalized heatmap (Fig. 3C) but adds an additional layer of analytical depth. It quantitatively underscores *Y. lipolytica*'s metabolic flexibility in lipid production, adapting to varying acetic acid concentrations by modulating its lipid profile. To sum up, Fig. 3D provides a statistical measure of the significance of these changes or trends, thereby enriching the overall interpretation of *Y. lipolytica*'s lipid metabolism in response to acetic acid consumption.

3.2.3. β-carotene production

The modified *Y. lipolytica* strain was also able to accumulate β-carotene in the presence of the medium high in acetic acid. This presents an intriguing scenario where a single carbon source—acetic acid—serves to fuel multiple biosynthetic pathways. The graphical representation depicted in Fig. 4 illustrates the relationship between the quantities of acetic acid consumed and the resulting production of β-carotene content and concentration.

The data in Fig. 4 illustrate a direct correlation between the level of acetic acid consumption and β-carotene production in *Y. lipolytica*. This indicates that acetic acid acts as a primary driver for the biosynthesis of secondary metabolites rather than merely serving as an auxiliary

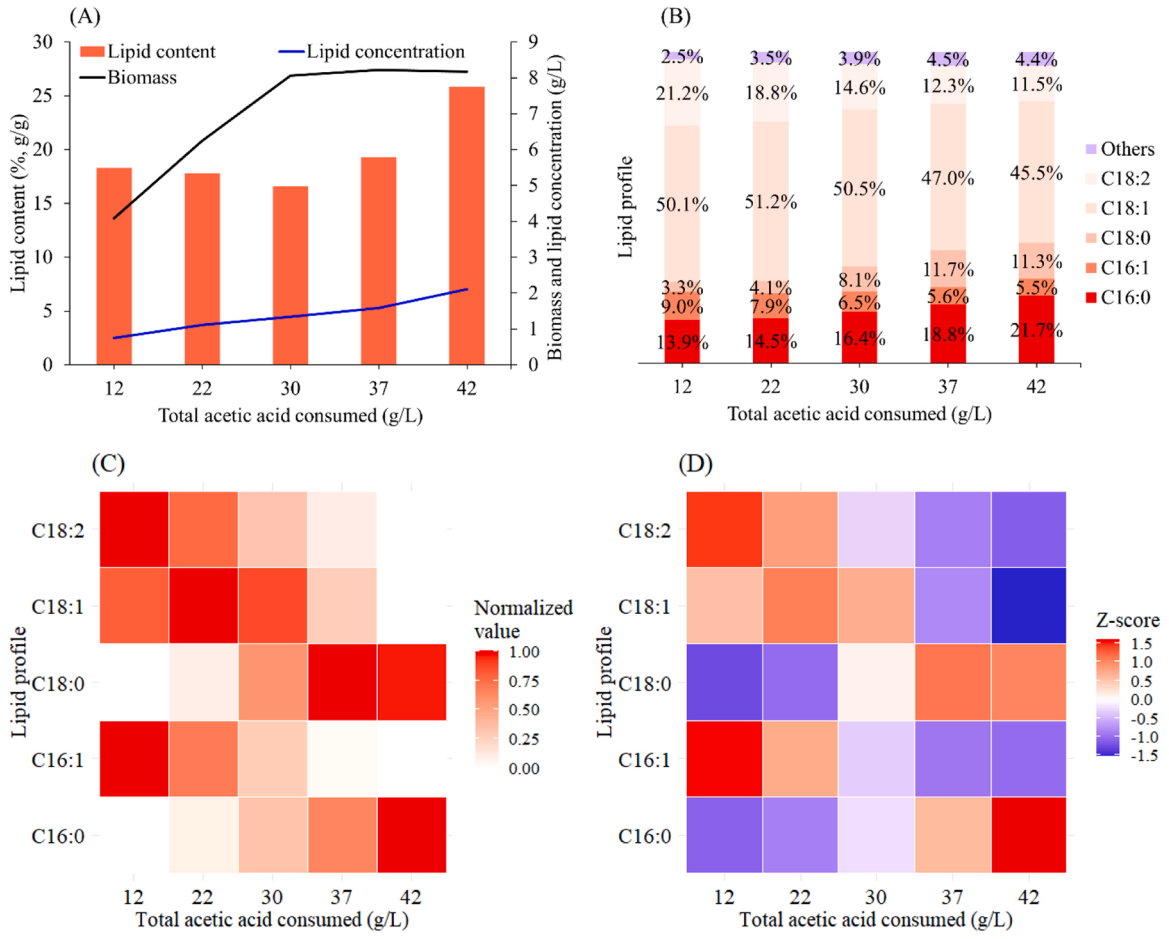

Fig. 3. (A) Lipid content (% g/g), lipid concentration (g/L) and biomass (g/L) as a function of acetic acid consumed. (B) Evolution of the lipid profile of *Y. lipolytica* as a function of the acetic acid consumed. (C) Heatmap of normalized lipid profile values in *Y. lipolytica* at varying levels of acetic acid consumption. (D) Heatmap of Z-scored lipid profile values in *Y. lipolytica* across different acetic acid concentrations. Experiment with acetic acid.

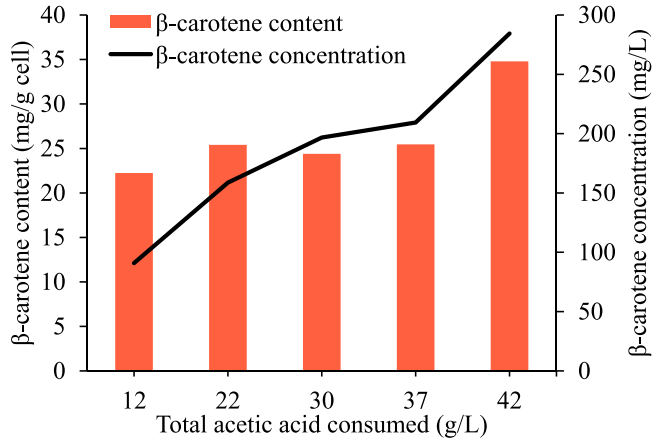

Fig. 4. Evolution of β-carotene content and concentration as a function of the amount acetic acid consumed in experiment with commercial acetic acid.

substrate. A dosage-dependent response is suggested, where higher acetic acid levels may enhance β-carotene biosynthesis pathways. At 12 g/L acetic acid, β-carotene accumulation was 22.2 mg/g cell and 90.9 mg/L, increasing to 34.8 mg/g cell and 284.4 mg/L at 42 g/L, supporting a 'flux overflow' model of metabolism. Such data highlight the organism's metabolic flexibility and suggest that excess acetic acid prompts a regulatory shift towards carotenoids accumulation.

3.3. Engineered Y. lipolytica bioreactor with fermentation-derived acetic acid-rich medium obtained from C. aceticum bioreactor

3.3.1. Growth and acetic acid consumption

This assay used *C. aceticum*'s anaerobically fermentation-derived acetic acid medium, enriched to an initial acetic acid concentration of about 10 g/L, as *Y. lipolytica*'s substrate. Fig. 5 illustrates acetic acid consumption over time, *Y. lipolytica*'s growth in optical density terms, and three subsequent acetic acid additions after complete substrate

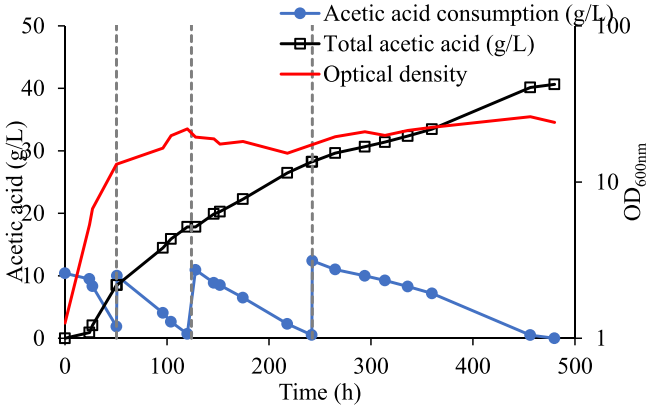

Fig. 5. Temporal dynamics of *Y. lipolytica*'s growth and acetic acid consumption in a fermentation-derived acetic acid-rich medium.

depletion, represented by dashed vertical lines.

Based on the findings presented in Fig. 5, it can be inferred that *Y. lipolytica* exhibited a minimal lag phase, lasting less than 24 h. This short lag phase suggests that *Y. lipolytica* readily acclimated to the fermentation-derived medium. Furthermore, a noteworthy observation is the diminishing rate of acetic acid consumption as the experiment progressed. This phenomenon can be quantitatively captured; for instance, during the initial 50 h, the rate of acetic acid consumption peaked at 0.27 g/L-h. As the experiment progressed, after around 300 h, with acetic acid concentrations surpassing 30 g/L, a marked decrease in consumption rate down to 0.04 g/L-h was evident.

When comparing Figs. 2 and 5, it appears that Fig. 2 shows a steady average acetic acid consumption rate of 0.27 g/L-h, whereas Fig. 5 mirrors this rate only initially. The variance in consumption rates can potentially be attributed to the differential distribution of nutrients between the two distinct growth media. The medium in the first experiment was supplemented with nutrients, including ammonium and yeast extract, thereby facilitating a more robust growth for *Y. lipolytica*. In contrast, the second experiment leveraged a medium that had undergone fermentation by *C. aceticum*, during which essential nutrients and nitrogen sources were expectedly partly metabolized, thus becoming less available for *Y. lipolytica*'s growth. The nitrogen-limited condition in the fermentation-derived medium likely invoked metabolic shifts that curtailed the yeast's growth rate and consequently decelerated acetic acid consumption.

It should be noted that follow-up studies have been initiated to substantiate the nitrogen-limitation hypothesis (non published data). These offer an exploration into how nutrient availability modulates acetic acid consumption rates. Preliminary results suggest a tenfold increase in substrate utilization rates during periods where nitrogen was not a limiting factor.

By comparing both experiments, it becomes evident that nutrient sufficiency plays a crucial role in expediting acetic acid consumption. This could elucidate why the first experiment was completed in a considerably shorter period of 230 h, while it took up to 480 h for acetic acid to be consumed in the fermentation-derived medium.

3.3.2. Lipids production

During the bioreactor experiment, samples were taken at various intervals to evaluate *Y. lipolytica*'s lipid synthesis as it consumed differing acetic acid levels. The results, along with corresponding biomass measurements, are presented in Fig. 6A.

The data presented in Fig. 6A reveal a relatively stable lipid content, around 26%, within the acetic acid consumption boundaries of 22 to 41 g/L. This observation suggests a consistent flux distribution towards lipid biosynthesis pathways within this range of acetic acid concentrations. Accordingly, there is a corresponding 30% increase in biomass concentration when acetic acid consumption increases from 9 to 22 g/L. This correlation may signify an optimal substrate-to-biomass conversion efficiency in this specific range. Beyond the 22 g/L value, an initial decline in biomass is noted, which subsequently rebounds to peak at 6.14 g/L upon consuming 41 g/L acetic acid. The lipid concentration pattern generally reflects the biomass trajectory, culminating in a maximum observed lipid concentration of 1.6 g/L after 41 g/L acetic

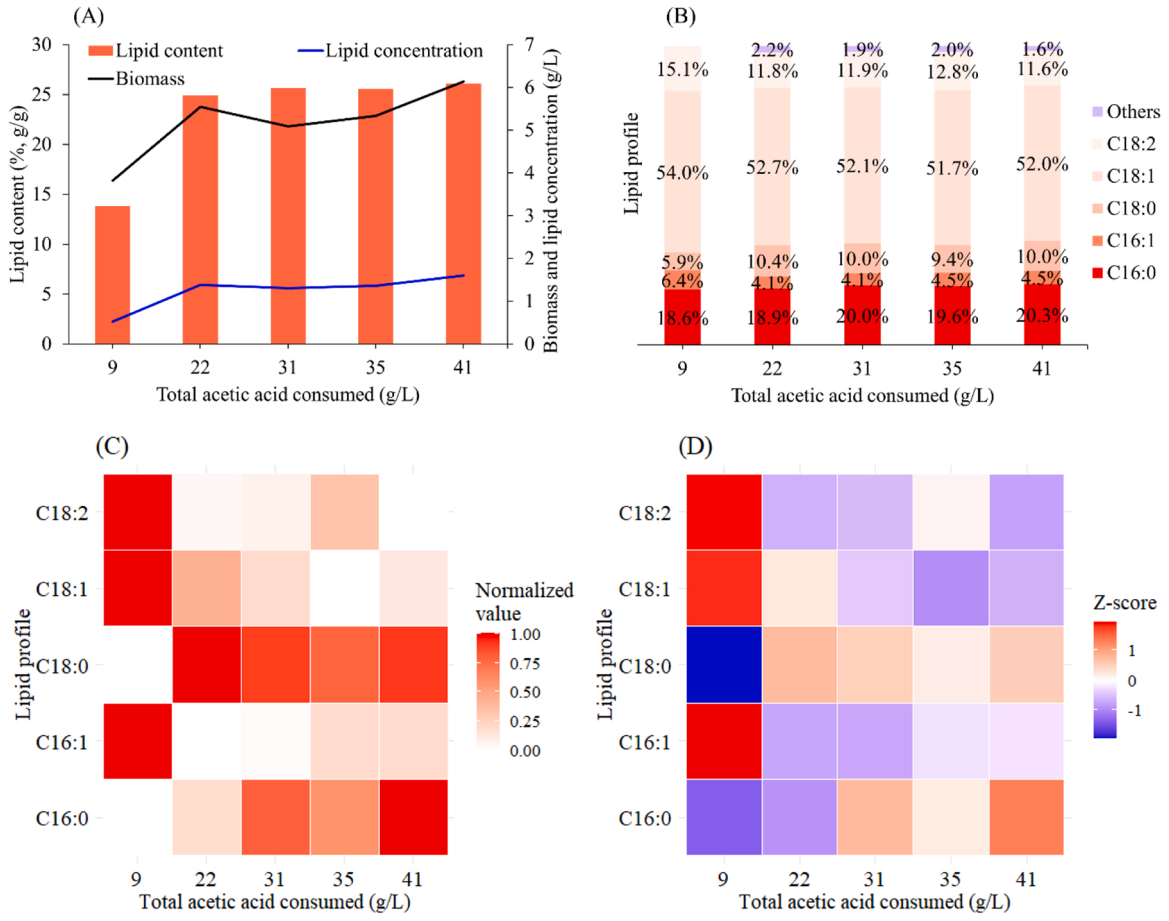

Fig. 6. (A) Lipid content (% g/g), lipid concentration (g/L) and biomass (g/L) as a function of acetic acid consumed. (B) Evolution of the lipid profile of *Y. lipolytica* as a function of the acetic acid consumed. (C) Heatmap of normalized lipid profile values in *Y. lipolytica* at varying levels of acetic acid consumption. (D) Heatmap of Z-scored lipid profile values in *Y. lipolytica* across different acetic acid concentrations. Experiment with fermentation-derived acetic acid coming from syngas fermentation.

acid consumption.

Metabolic pathways of *Y. lipolytica* can explain the observed behavior. The yeast consumes nutrients for macromolecule synthesis, including lipids, during its growth phase. Increased acetic acid consumption results in more carbon for lipid synthesis, raising lipid content and biomass. However, after a certain point, nutrient and growth factor availability declines due to resource depletion, resulting in decreased biomass and lipid production. This is seen after consuming 22 g/L acetic acid. From an economic standpoint, based on these outcomes, one could deduce that the utility of acetic acid fermented as a substrate for lipid production, beyond the consumption level of 22 g/L, does not confer significant economic advantages.

When comparing Figs. 3A and 6A, distinct variations in lipid production are observed depending on whether the medium has been fermented by *C. aceticum* or not. Although the graphs exhibit analogous trends with respect to biomass, lipid content, and concentration as a function of the amount acetic acid consumed, there are notable differences in the thresholds and maximal values. The lipid content in Fig. 3A remains stable within a range of 12 to 30 g/L acetic acid consumption and then increases to 25.8% at 42 g/L. Conversely, Fig. 6A exhibits an initial lipid content of approximately 14% at 9 g/L acetic acid consumed, which increases to around 26% within a 22 to 41 g/L acetic acid consumption range. These differences can be ascribed to the C/N ratio of the respective media. Oleaginous yeasts, like *Y. lipolytica*, reconfigure their metabolic pathways under nitrogen limitation to prioritize lipid accumulation. The medium fermented by *C. aceticum* likely better optimizes the C/N ratio to induce earlier lipid accumulation, thus shifting the threshold from 42 g/L in commercial acetic acid media to 22 g/L.

Additionally, while biomass in Fig. 3A consistently increases, the biomass in Fig. 6A experiences an initial upturn, followed by a slight downturn before culminating at 41 g/L. This suggests that the medium composition in Fig. 3A likely lacks growth-limiting factors, possibly due to excess of a nitrogen source.

Table 1 presents a comparative analysis between the two most successful outcomes achieved in the aforementioned experiments (considering, as the best result, the 22 g/L consumption in the bioreactor with fermentation-derived acetic acid) and other relevant studies from the scientific literature using *Y. lipolytica* as a biocatalyst for lipid production. It is worth noting that a recent study by Robles-Iglesias et al. (2023) also utilized acetic acid generated from syngas fermentation by *C. aceticum*, achieving a lipid content of 39.5% g/g dry cell weight, biomass concentration of 3 g/L, and lipid yield of 0.107 using the yeast *Rhodosporidium toruloides* in the second bioconversion stage in this case (Robles-Iglesias et al., 2023a). Because of the use of *R. toruloides* rather than *Y. lipolytica*, this study is not included in Table 1.

Fig. 6B provides a breakdown of the lipid profile of *Y. lipolytica*, categorized into five fatty acids, i.e., C16:0, C16:1, C18:0, C18:1, C18:2, and Others. The data reveal an influence of acetic acid concentration on the lipid composition. Fig. 6C, a heatmap of normalized values, offers a quantitative perspective on these shifts. Specifically, the heatmap highlights a potential bottleneck in the biosynthesis of unsaturated fatty acids, such as C16:1 and C18:1, possibly due to limitations in precursor availability. In Fig. 6D, Z-score normalization is employed to statistically quantify the significance of these compositional changes. This adds an additional layer of rigor to the analysis, enabling a more robust interpretation of the metabolic shifts observed.

When comparing Figs. 6B, 6C and 6D with Figs. 3B, 3C, and 3D, the influence of the acetic acid source on lipid metabolism becomes even more pronounced. For instance, Figs. 3C and 3D show more significant modulations in lipid profiles at higher acetic acid concentrations (37 and 42 g/L) than what is observed in Figs. 6C and 6D. This divergence suggests that not just the concentration, but also the origin of acetic acid—whether gas fermentation-derived or commercial acetic acid media—plays a crucial role in shaping the lipid profile. Moreover, comparison between Figs. 3C and 6C reveals that the metabolic responses are not uniform across different acetic acid sources. This could be attributed to the presence of other metabolites or trace elements in the fermentation-derived acetic acid, which may interact with specific metabolic pathways in *Y. lipolytica*.

Lastly, it is worth noting that residual microbial biomass or enzymes in the acetic acid fermentation-derived source could introduce additional variables that influence lipid metabolism. This complexity underscores the need for a holistic approach when studying the metabolic effects of acetic acid on *Y. lipolytica*, considering both its concentration and source.

3.3.3. β-carotene production

In a way similar to the previous experiment involving commercial acetic acid media, it was observed that *Y. lipolytica* was capable of producing β-carotene when cultured in the bioreactor.

Similarly to the previous experiment, the results obtained from the experiment, showed in Fig. 7, suggest a correlation between the consumption of fermentation-derived acetic acid by *Y. lipolytica* yeast and the production of β-carotene content and concentration. As the concentration of fermentation-derived acetic acid consumed increased, there was an overall increase in both β-carotene content and concentration. For instance, at 9 g/L of fermentation-derived acetic acid consumed, the β-carotene content was 17.1 mg/g cell, and the β-carotene concentration was 65.6 mg/L. However, when the concentration of fermentation-derived acetic acid consumed increased to 41 g/L, the β-carotene content increased to 62.3 mg/g cell and the β-carotene concentration increased to 382.7 mg/L, exhibiting the most favorable outcomes of the entire experiment. Additionally, the non-linear relationship observed between the concentration of fermentation-derived acetic acid and β-carotene content and concentration implies that an optimal concentration of fermentation-derived acetic acid may exist for maximum β-carotene production.

The results from Fig. 4 (commercial acetic acid) and Fig. 7 (fermentation-derived acetic acid) show that there are notable

Table 1
Some of the most relevant results in terms of lipid production and yields obtained with *Y. lipolytica*.

Medium composition	Substrate concentration (g/L)	Lipid content (% g/g)	Lipid concentration (g/L)	Biomass (g/L)	Biomass yield ($Y_{X/S}$)	Lipid yield ($Y_{L/S}$)	Ref.
Commercial acetic acid	42	25.8	2.11	8.18	0.194	0.05	This study
Fermentation-derived acetic acid media	22	24.9	1.38	5.55	0.25	0.062	This study
Fermentation-derived acetic acid media	20	22.9	1.03	5.04	0.24	0.055	(Robles-Iglesias et al., 2023b)
Food and vegetable fermented waste-derived VFAs	22	26.3	2.77	10.52	0.474	0.125	(Gao et al., 2020)
Synthetic VFAs	36.9	13	0.3	2.24	0.06	0.008	(Naveira-Pazos et al., 2022)
Synthetic VFAs mimicking food waste fermented	13.9	27	0.83	± 3	0.61	0.16	(Pereira et al., 2023)
Glucose + synthetic VFAs	20 + 18	37	3.5	9.7	0.29	0.1	(Pereira et al., 2022)

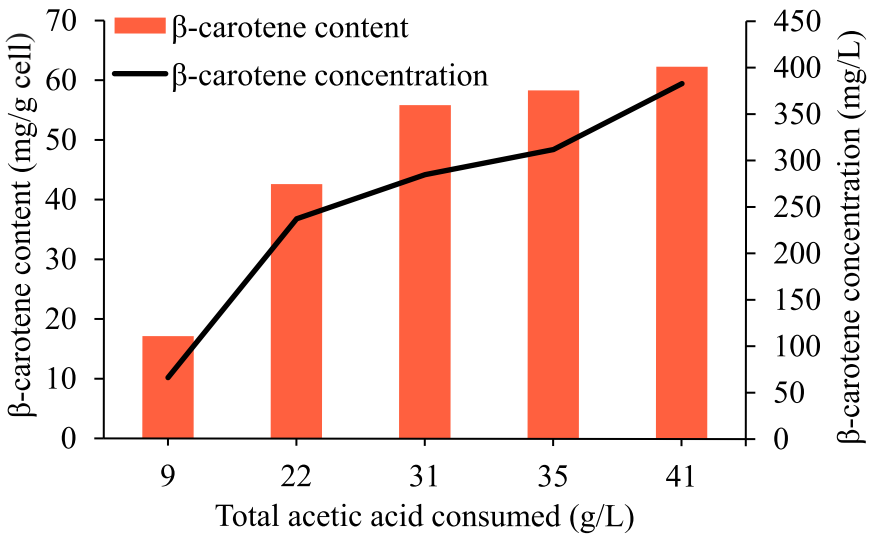

Fig. 7. Evolution of β-carotene content and concentration as a function of the acetic acid consumed. Experiment with fermentation-derived acetic acid coming from syngas fermentation.

differences in the amount of β-carotene produced under each condition. Fig. 7 shows a higher β-carotene content and concentration compared to Fig. 4. For instance, at 22 g/L of acetic acid consumed, the β-carotene content was 25.4 mg/g cell and the β-carotene concentration was 158.7 mg/L in the commercial acetic acid experiment (Fig. 4), while in the fermentation-derived acetic acid experiment (Fig. 7), the β-carotene content was 42.6 mg/g cell and the β-carotene concentration was 236.7 mg/L, representing an increase of 67.7% and 49.2%, respectively.

One possible explanation for the observed elevated β-carotene content and concentration in *Y. lipolytica* cultivated in fermentation-derived acetic acid, relative to commercial acetic acid, centers on the biochemical milieu of the fermentation process. The fermentation-derived acetic acid is likely to be accompanied by secondary metabolites which may act synergistically to modulate *Y. lipolytica* metabolic pathway, thereby catalyzing increased β-carotene synthesis. Comparatively, a recent study by Robles-Iglesias and colleagues also analyzed β-carotene production in *Y. lipolytica* using both commercial (in flasks) and fermentation-derived acetic acid (in bioreactor) under different pH conditions (Robles-Iglesias et al., 2023b). They reported a β-carotene content of 13 mg/g cell and a concentration of 35 mg/L with commercial acetic acid, which significantly increased to 44 mg/g cell and 164 mg/L when using fermentation-derived acetic acid at a controlled pH of 6.0 (Robles-Iglesias et al., 2023b). Another avenue for exploration is the initial C/N ratio of the growth medium. In the commercial acetic acid setup, the initial C/N ratio was 10, a value generally considered suboptimal for secondary metabolites production. This lower C/N ratio possibly facilitated a nitrogen-rich environment conducive for *Y. lipolytica*ś growth yet inimical to β-carotene production due to nitrogen-mediated suppression of secondary metabolism (Paul et al., 2023). The deficiency in nitrogen availability could potentially instigate a stress response in *Y. lipolytica*, leading to the upregulation of β-carotene synthesis. For instance, according to Braunwald and colleagues' findings, the lipid production in *R. glutinis* was not increased by a C/N ratio above 70 to 120 when glucose was used as carbon source; however, it had a favorable impact on the synthesis of carotenoids (Braunwald et al., 2013). This could explain why the β-carotene content and concentration in the commercial acetic acid experiment were lower compared to the fermentation-derived acetic acid experiment.

Larroude et al. employed the same engineered strain in a reactor with glucose as the substrate and an airflow velocity of 2.0 vvm to produce β-carotene (Larroude et al., 2018). The aforementioned investigation yielded a β-carotene concentration of 90 mg/g cell and a total concentration of 6500 mg/L, whereas a lower concentration of 62.3 mg/g cell and 382.7 mg/L was obtained in the present research. This significant difference in β-carotene concentration could be attributed to the utilization of glucose as a substrate, as it facilitates higher biomass production by the microorganism under favorable medium conditions. However, it is important to consider that glucose holds a market value approximately four times greater than that of acetic acid (Robles-Iglesias et al., 2023a), making it more commercially advantageous to employ acid as a substrate. Further optimizing culture conditions, as demonstrated by Lv et al. in their work on optimizing dissolved oxygen parameters, may enhance β-carotene yields (Lv et al., 2020). In summary, the differential β-carotene yields across varying conditions accentuate the multifactorial nature of microbial metabolism and product synthesis. Substrate selection and physico-chemical environment—namely the C/N ratio and potentially dissolved oxygen levels—emerge as significant variables that could be targeted for further research and optimization in industrial applications.

4. Conclusions

This research methodically scrutinized lipid and β-carotene biosynthesis in *Y. lipolytica* utilizing acetic acid derived from syngas fermentation as the substrate. A direct proportional relationship was evident between the concentration of acetic acid consumed and the production of both lipids and β-carotene, under both commercial and fermentation-derived acetic acid media scenarios. Interestingly, the lipid yield remained relatively invariant between the two conditions, thereby ruling out significant metabolic shifts in lipid synthesis pathways. The divergence between the two conditions was manifested in terms of β-carotene production, where fermentation-derived acetic acid exhibited superior efficiency. This elevated yield can be attributed to either the complex metabolic landscape introduced by additional fermentation by-products or altered C/N ratios that favored a heightened secondary metabolic activity. While β-carotene concentrations reached here were lower than those from glucose-based studies, acetic acid presents a cost-effective alternative due to its lower market value, highlighting the potential for industrial, cost-effective, and eco-friendly *Y. lipolytica* use.

CRediT authorship contribution statement

Fernández-Blanco Carla: Data curation, Investigation, Writing – original draft, Writing – review & editing. **Robles-Iglesias Raúl:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Kennes Christian:** Conceptualization, Data curation, Funding

acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Veiga María C.:** Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Nicaud Jean-Marc:** Investigation, Writing – review & editing.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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