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Research article

Sequential bioconversion of C_1-gases (CO, CO_2, syngas) into lipids, through the carboxylic acid platform, with *Clostridium aceticum* and *Rhodosporidium toruloides*

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ABSTRACT

Syngas (CO, CO_2, H_2) was effectively bioconverted into lipids in a two-stage process. In the first stage, C_1-gases were bioconverted into acetic acid by the acetogenic species *Clostridium aceticum* through the Wood-Ljungdahl metabolic pathway in a stirred tank bioreactor, reaching a maximum acetic acid concentration of 11.5 g/L, with a production rate of 0.05 g/L·h. Throughout this experiment, samples were extracted at different periods, i.e., different concentrations, to be used in the second stage, aiming at the production of lipids from acetic acid. The yeast *Rhodosporidium toruloides*, inoculated in the acetogenic medium, was able to efficiently accumulate lipids from acetic acid generated in the first stage. The best results, in terms of lipid content, dry biomass, biomass yield ($Y_{(X/S)}$) and lipid yield ($Y_{(L/S)}$) were 39.5% g/g dry cell weight, 3 g/L, 0.35 and 0.107, respectively. In terms of abundance, the lipid profile followed the order: C18:1 > C16:0 > C18:2 > C18:0 > Others. Experiments were also performed to determine the toxicity exerted by high concentrations of acetic acid on *R. toruloides,* resulting in inhibition at initial acid concentrations around 18 g/L leading to a higher lag phase and being lethal to the yeast at initial acetic acid concentrations around 22 g/L and above. This research paves the way for a novel method of growing oleaginous yeasts to produce sustainable biofuels from syngas or C_1-pollutant gases.

1. Introduction

There has never been a greater need to minimise the dependence on fossil fuels and greenhouse emissions than nowadays, given the prospect of fossil resource depletion and global warming (Masson-Delmotte et al., 2021). Alternative prime matters and energy supplies must be urgently found to progress toward greener and sustainable fuel manufacturing and chemical processes. Carbon dioxide (CO_2) and carbon monoxide (CO) are single-carbon gases (C_1-gases) that may be derived from syngas generated from the gasification of municipal wastes, industrial waste gases, or even atmospheric carbon dioxide, which are among the suitable feedstocks and substrates to meet these needs (Bae et al., 2022). Chemical catalysts (Jahangiri et al., 2014) and biocatalysts (Teixeira et al., 2018) have been proposed as two alternatives for converting C_1-gases into value-added compounds. Operational temperatures and pressures for biocatalysts are much lower than in most (thermo)-chemical processes, i.e., around 35 °C and atmospheric pressure *vs* 150–350 °C and 30 bar for chemical catalysts. At the same time, the yield can be very similar (Molitor et al., 2016). Therefore, interest in exploiting microorganisms capable of utilizing C_1-gases has grown considerably.

Acetogenic bacteria, also known as acetogens, are recognized as being among the most effective biocatalysts for the transformation of C_1-gases into chemicals of added value (Fernández-Naveira et al., 2017a). The Wood-Ljungdahl metabolic pathway (WLP) is the most energy-efficient metabolic pathway in terms of CO_2 fixation. Through this metabolic pathway, acetogenic bacteria can use these C_1-gases (CO_2, CO) and H_2 as sole energy sources and carbon sources (Fernández-Naveira et al., 2017b). These microorganisms are known to bioconvert CO_2 into organic acids, alcohols and some other chemicals under anaerobic conditions in an efficient way (Arslan et al., 2021). Their natural range of metabolites is often limited to acetate and,

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occasionally, some longer chain acids (e.g., butyrate), ethanol or other alcohols such as 2,3-butanediol, and a few other products. Due to bioenergetic restrictions during autotrophic metabolism and the lack of specific enzymes, acetogens are unable to synthesize energy-rich substances like lipids or biopolymers from C_1-gases (Bae et al., 2022). Therefore, there is a need to either engineer bacteria or, otherwise, combine different stages and/or microorganisms if the aim is to expand the spectrum of products obtained from anaerobic C_1-gas fermentation.

Considering the advancements achieved in the bioconversion of acetic acid into other compounds, this monocarboxylic acid, or even other volatile fatty acids (VFAs), have gained interest as viable substrates (Kiefer et al., 2021). Different species of aerobic oleaginous yeasts are known to metabolise acetic acid as a carbon source for lipid accumulation, showing an exceptional ability to metabolically exploit these unconventional carbon sources (Llamas et al., 2020; Park et al., 2018; Patel et al., 2021).

Recent interest in lipid-derived biofuels as sustainable alternative fuels has increased because of their high energy density, biodegradability, and environmental friendliness (Robles-Iglesias et al., 2023). Currently, both plants and animal fats are mainly used to manufacture lipid oils at industrial scale. Conversely, research on oleaginous yeasts that can accumulate lipids has become more popular in recent years (Robles-Iglesias et al., 2023). Lipids produced by yeasts are chemically similar to those in mammals or plants. Also, one of their inherent advantages is that they are unaffected by seasonality or climate, with the major benefit being a reduction in the amount of land area needed (Ochsenreither et al., 2016).

The oleaginous yeast *Rhodosporidium toruloides* was used for the experiment described in this study due to its history of good results in both lipid production and acetic acid assimilation (Huang et al., 2016). However, since this oleaginous yeast requires oxygen for lipid production, it is difficult to culture them alongside acetogens in a single reactor. As a result, two-stage fermentation must be carried out in two independent bioreactors, each with its own optimized set of parameters. In this two-stage co-culture set-up, the first stage is an anaerobic bioreactor containing acetogenic bacteria that transform C_1-gases into acetic acid. Then, the generated acid is supplied to the acetic-converting aerobic system in the second step.

This research evaluated the acetogenic bacterium *Clostridium aceticum* for the bioconversion of C_1-gases into acetic acid and the oleaginous yeast *Rhodosporidium toruloides* for the subsequent bioconversion of acetic acid into lipids.

2. Material and methods

2.1. Anaerobic fermentation stage

2.1.1. Strain, media, and culture conditions

Clostridium aceticum (DSM 1496) was obtained from "*DSMZ*" (Braunschweig, Germany) and maintained in the medium described hereafter. This medium had the following composition (per litre distilled water): yeast extract (YE), 3.00 g; NH_4Cl, 8.45 g; K_2HPO_4, 10.00 g; KH_2PO_4, 0.20 g; $MgSO_4$ x 7 H_2O, 1.75 g; $NaHCO_3$, 0.35 g; cysteine-HCl, 0.30 g; Na-resazurin (0.10% w/v), 1.00 mL; Na_2S x 9 H_2O, 0.90 g; vitamin solution, 2.50 mL; trace metals solution, 2.00 mL. The composition of trace metal dilution used is described in the publication of Robles-Iglesias and co-workers (Robles-Iglesias et al., 2021). The vitamin solution had the following composition (per litre distilled water): biotin, 2.00 mg; pyridoxine-HCl, 10.00 mg; folic acid, 2.00 mg; thiamine-HCl, 5.00 mg; nicotinic acid, 5.00 mg; Ca-D-pantothenate, 5.00 mg; p-aminobenzoic acid, 5.00 mg; vitamin B_{12}, 0.10 mg; (±)-α-lipoic acid, 5.00 mg; riboflavin, 5.00 mg. The temperature was kept at 30 °C, and the pH was adjusted to 7.5. The strain was grown with constant shaking, at 150 rpm, on an orbital shaker for three days to activate the organism before inoculation. The anaerobic conditions required for growth have been ensured by purging the culture media in the bottles with pure nitrogen. The gaseous substrate was a mixture of $CO_2/CO/H_2/N_2$ (10/30/20/40). The strain was maintained through subculturing in serum bottles with a working capacity of 40 mL each.

2.1.2. Batch culture

All of the media mentioned in Section 2.1.1 were dissolved in a total of 40 mL of medium before being introduced in 100 mL glass serum bottles, with the exception of the vitamin solution. Each bottle was flushed with 100% N_2 gas to generate anaerobic conditions and then flushed with the gaseous substrate composed of $CO_2/CO/H_2/N_2$, described above. The pH of each medium in the bottles was adjusted to 7.5 using 2M NaOH and HCl solutions. The bottles were then sealed firmly with aluminium crimps and rubber septa. They were sterilized by autoclaving at 120 °C for 20 min. Before inoculating the medium with 4 mL microbial seed culture (10% of the total working volume), the vitamin solution was added from a sterile stock solution. A thermostat was used to keep the incubation chamber at 30 °C, and at 150 rpm on orbital shaker. A total of eight inoculated bottles were cultured for 120 h.

2.1.3. Bioreactor CSTR (continuously stirred tank reactors)

The bioreactor studies were conducted in a 2 L stirred tank BIOFLO 120 reactor (Eppendorf AG, Hamburg, Germany), containing 1.2 L of the growing media that was discussed before. To ensure the required sterile conditions, the whole system was sterilized using an autoclave for 20 min at 120 °C. After the autoclaving process, Na_2S and cysteine-HCl were added in order to prevent any potential reactions at higher temperatures. After being placed in an autoclave, the medium in the reactor was purged with pure nitrogen gas for a period of 2 h in order to eliminate all traces of oxygen. It was then continuously fed the syngas mixture containing $CO_2/CO/H_2/N_2$ (10/30/20/40). A mass flow controller was used to continually adjust and maintain the gas flow rate at 10 mL/min (Aalborg GFC 17, Germany). Once the temperature was set at 30 °C, with 250 rpm agitation and pH 7.5, the bioreactor was inoculated with 10% (v/v) of *Clostridium aceticum* active culture, which was grown for 72h under the conditions detailed in Section 2.1.2. Peristaltic pumps delivered either a 1 M HCl or 1 M NaOH solution to maintain a consistent pH in the culture broth, which was monitored online.

2.2. Aerobic fermentation stage

2.2.1. Strain, media and culture conditions

The oleaginous yeast *Rhodosporidium toruloides* (DSM 10134) strain was obtained from "*DSMZ*" (Braunschweig, Germany). The strain was maintained at −80 °C on glycerol at 80% and activated at 30 °C on potato dextrose agar (PDA) plates 48h before the inoculation in liquid media. This 50 mL liquid medium was composed of potato dextrose broth (PDB), introduced in a 250 mL flask, previously autoclaved, and then incubated on an orbital shaker at 150 rpm for 36–48 h at 30 °C.

2.2.2. Toxicity batch assays

Due to the low acetic acid concentration (0.8 g/L) obtained in the 8 bottles of the *C. aceticum* batch experiments, different concentrations of acetic acid were added to this medium in order to test a range of acetic acid concentrations from 5 to 40 g/L. These media were divided into 8 bottles with 40 mL aqueous phase introduced in 500-mL baffled flasks, each with a different acid concentration, and *R. toruloides* was then inoculated into each bottle. Before transferring the *R. toruloides* strain to the acetic acid-rich media, seed cultures were collected by centrifuging at 3600 rpm for 5 min and washing this seed culture with NaCl 0.9% to remove the nutrients present in the PDB medium. The optical density (OD_{600nm}) at inoculation was 1. The experiments were carried out at 30 °C, pH 6.0 and with orbital shaking at 150 rpm. Yeast growth and acetic acid consumption were followed throughout the whole experiment.

2.2.3. Lipid accumulation by the yeast

The CSTR investigations on *C. aceticum*, which were discussed in previous sections, yielded a variety of samples, each of around 40 mL, with acetic acid concentrations between 8 and 12 g/L. The *R. toruloides* strain was then used to grow cultures in these samples for different experiments. The seed cultures were washed before transferring the *R. toruloides* strain to acetic acid-rich media. Then, they were resuspended in batch culture medium bottles at an OD_{600nm} of 1. The experiments were carried out in 500-mL baffled flasks, shaken at 150 rpm, maintained at 30 °C, and at initial pH of 6.0. The growth, consumption of acetic acid, and lipid production were measured during the cultivation.

2.3. Analytical methods

2.3.1. Biomass concentration and growth

Microbial growth was assessed spectrophotometrically in both of the studies, one with yeasts and one with bacteria, by checking the optical density (OD) at 600 nm. This allowed the generation of growth curves. Occasionally, to determine the correct growth-related optical density, the samples had to be diluted.

Medium samples were centrifuged at the end of each experiment at 4000 rpm for 10 min. The pellet was then washed with distilled water and refrigerated for a period of 48 h in order to lyophilise the samples. The weight of lyophilised sample was used to determine the biomass accumulation.

2.3.2. Production and consumption of acetic acid

Utilizing a high-performance liquid chromatography (HPLC) (HP1100, Agilent Co., USA), the acetic acid contained in the medium as a result of its generation or consumption was quantified. The HPLC was fitted with a 50 °C-maintained refractive index detector (RID) and diode array detector (DAD). Each batch or bioreactor experiment was sampled for HPLC measurement of chemical content and concentration. Before undergoing HPLC analysis, the samples were first subjected to a 5-min centrifugation at 7000 rpm, after which the supernatant was passed through a 0.22-μm PTFE filter. The mobile phase was a solution of H_2SO_4 with a concentration of 0.005 M, and the flow rate was 0.80 mL/min. At a temperature of 45 °C, 20 μl of each sample was injected into an HPLC system that was fitted with an Agilent Hi-Plex H Column measuring 300 mm × 7.7 mm.

2.3.3. Fatty acids methyl ester (FAME)

After collecting microbial cells, total lipid accumulation was calculated using 15–30 mg of lyophilised biomass and then converted into methyl ester equivalents using the extraction protocol and method described elsewhere (Larroude et al., 2018). Samples for these measurements were taken at the end of the *R. toruloides* batch experiment, i.e., after 100 h. A gas chromatograph was used (Thermo Fisher) for the quantification of lipids. The GC conditions, column and flow of carrier gas are described by Robles-Iglesias et al. (2021). It was determined which FAMEs were present by comparing them to standard FAME solution that had been prepared. An internal technique of adding a known amount of lauric acid (C12:0) to the extraction solution was used as a method of quantification. *R. toruloides* does not naturally produce C12:0, so lipids were quantified by area differences in the chromatograms. The dry cell weight (DCW) was measured using freeze-dried and washed cells of a specified volume.

3. Results and discussion

3.1. Anaerobic fermentation: production of acetic acid from C_1-gases by *C. aceticum* in bioreactor (CSTR)

The *Clostridium aceticum* strain was grown on the gaseous mixture and in the bioreactor described in Materials and Methods. Several *Clostridium* spp. are known to use carbon monoxide as carbon and energy source or CO_2 as a carbon source in the presence of hydrogen, or another possible electron donor, following the Wood-Ljungdahl metabolic pathway (Fernández-Naveira et al., 2017a). *C. aceticum* is known to produce other compounds than acids from gaseous substrates, e.g., ethanol (Fernández-Blanco et al., 2022; Arslan et al., 2019). However, this will only occur under stress conditions, among others, at pH below optimal, usually below pH 6.9 for that species, which has a somewhat alkaline optimal growth pH, contrary to most other acetogens generally preferring slightly acidic conditions (Fernández-Naveira et al., 2019). Considering that the aim of this experiment is the single production of acetic acid, it was decided to maintain a pH level of 7.5 throughout the operation to favour acidogenesis and the accumulation of this acid as the sole end metabolite.

Fig. 1 represents the acetic acid production and growth pattern of *C. aceticum* over the 400 h experimental run. The dashed vertical lines also depict the exact points when the 40 mL samples were extracted for the subsequent yeast experiments at different acid concentrations.

The acetogenic *C. aceticum* strain grew efficiently on syngas and generated acetic acid, reaching 11.50 g/L after 387 h, as seen in Fig. 1. During the first hours of the experiment, the growth curve shows that the optical density increased exponentially after inoculation, reaching a maximum growth rate (μ_{max}) of 0.057 h^{-1}. Once the stationary phase was reached, the growth curve levelled off and tended to remain constant, achieving the highest absorbance after 123 h, corresponding to $OD_{600nm} = 1.65$. This point was reached when the concentration of acetic acid produced was about 2.50 g/L. This suggests that the rate of growth of the biomass was originally rapid, but it subsequently slowed down and eventually ceased, while acetic acid production kept going on and further increased up to 11.50 g/L. Metabolic uncoupling causes this behaviour; the cell number no longer expands, but the bacteria remain metabolically active. The strain continued producing acetic acid at comparable high rates until its early decline phase, indicating that it remained highly metabolically active.

The highest concentration of acetic acid, 11.50 g/L, is close to values reported in earlier experiments carried out with the same strain, though Fig. 1 suggests that higher values could still have been reached. In another research, when feeding a reactor of the same characteristics with $CO_2/CO/H_2/N_2$ (5/30/15/50) at constant pH 8.0, 9.40 g/L acetic acid was obtained (Arslan et al., 2019). Still, when feeding a reactor of the same characteristics with pure carbon monoxide at constant pH 8.0, 18.0 g/L acetic acid was produced by *C. aceticum* through acidogenesis (Arslan et al., 2021). This substantiates the findings that were derived from those research and leads to the conclusion that, in comparison to $CO_2 + H_2$ or syngas mixes (for example, $CO_2 + CO + H_2$), pure CO would be an excellent carbon and electron source (Arslan et al., 2021). It happens because pure CO allows for more efficient utilisation of energy and carbon by acetogenic bacteria through the Wood-Ljungdahl pathway. Syngas requires additional energy expenditure by the

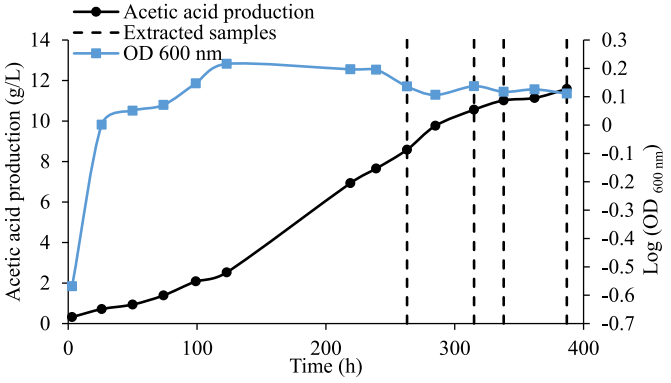

Fig. 1. Acetic acid production and growth of *C. aceticum* in CSTR at pH 7.5.

bacteria to convert first CO_2 to CO, which is then used in the pathway. This additional step results in energy loss and reduces the efficiency of the process. In contrast, pure CO can be directly used by the bacteria in the Wood-Ljungdahl pathway, leading to more efficient carbon and energy utilisation. The study with CO presented a maximum acetic acid production rate of 0.26 g/L·h, which is approximately five times higher than in the present research, i.e., 0.05 g/L·h. The maximum growth rate is similar, reaching $\mu_{max} = 0.057\ h^{-1}$ in this study and $\mu_{max} = 0.052\ h^{-1}$ in work reported by Arslan and co-workers (Arslan et al., 2021). The greater ATP output per electron pair generated from CO and/or the necessity to lower CO_2 in the initial stage might explain the higher efficiency of acetic acid production from CO. The maximum growth rates of acetogenic bacteria growing on C_1-gases are typically of the same order of magnitude. For example, *C. carboxidivorans*, grown on pure CO, exhibited an average $\mu_{max} = 0.081\ h^{-1}$ (Fernández-Naveira et al., 2016), while this value was $0.072\ h^{-1}$ on a syngas mixture containing CO_2, CO, H_2, N_2 (Fernández-Naveira et al., 2017c). Interestingly the maximum growth rate in such species is similar and hardly slightly higher on soluble substrates such as glucose ($\mu_{max} = 0.087\ h^{-1}$) (Fernández-Naveira et al., 2017d).

Following the procedures outlined in the section titled "Materials and Methods," samples totalling approximately 40 mL were extracted in order to carry out the aerobic portion of the experiments that were to follow. This portion of the research was centred on the production of microbial oils and lipids by yeasts, using acetic acid that was generated from an assay of bioconversion of syngas.

3.2. Aerobic fermentation: lipids production from acetic acid by R. toruloides

3.2.1. Toxic effects of acetic acid on R. toruloides

To determine the potential toxicity exerted by different acetic acid concentrations on *R. toruloides* grown in the fermented medium of *C. aceticum*, left over from the gas fermentation stage, first a batch experiment was performed, as described in section 2.1.2, in which eight 40 mL bottles were used to grow the anaerobic species *C. aceticum*. This experiment with the anaerobic bacterium lasted four days, and this period was sufficient to grow *C. aceticum* and produce approximately 1 g/L acetic acid in each bottle. This result is considered relatively low but expected due to the small volume of medium used in the experiment and the non-continuous feeding of gases. This experiment aimed to determine the potential toxicity of acetic acid on the oleaginous yeast in the bacterial medium. The starting concentration of 1 g/L of acetic acid is expected to be tolerable for *R. toruloides* (Huang et al., 2016). Pure commercial acetic acid was then added to reach a concentration range between 5 and 35 g/L, further divided into eight baffled flasks. *R. toruloides* was cultivated in each one of these eight flasks, adjusting the pH to 6.0.

According to the results obtained in the experiment, it can be seen that from an initial concentration of approximately 18 g/L acetic acid, inhibition starts to be observed. It was found that, at this concentration, the lag phase increased considerably, i.e., the lag phase was approximately 30 h. Conversely, at lower concentrations, substrate consumption started already a few hours after inoculation (Fig. 2). No significant lag phase differences were observed below the initial concentration of 14 g/L. It can also be deduced from the data that a concentration above 18 g/L can significantly inhibit yeast growth and may result in a longer lag phase, but some growth may still occur after some longer period of time. In the case of 22 g/L initial acetic acid, this concentration was lethal (Fig. 2). As for the time used by *R. toruloides* to consume all the acetic acid contained in the medium, it should be noted that, despite the inhibition found in the bottle with 18 g/L initial acetic acid, the results obtained show a high rate of consumption, with full substrate consumption completed in less than three days. Nevertheless, at lower acid concentrations, complete substrate metabolization ended faster; for example, for 9 g/L initial acetic acid concentration, the approximate

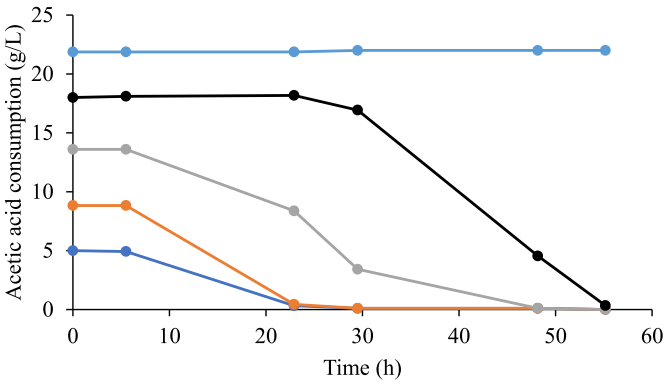

Fig. 2. Consumption of acetic acid by *R. toruloides* varying initial concentrations.

total consumption period was 20 h while it was 55 h at 18 g/L, which is 37% more than for 9 g/L due to partial inhibition (Fig. 2).

Authors such as Huang and collaborators observed similar toxicity results when growing *R. toruloides* in a medium composed mainly of commercial acetic acid, where the maximum tolerance reached without observing any inhibition was at an initial concentration of 10 g/L of the acid. In comparison, with 20 g/L, the yeast still managed to grow but with a prolonged lag phase (Huang et al., 2016). In some other of our own recent studies, where *R. toruloides* was grown with a medium fermented by the acetogenic bacterium *A. woodii*, some inhibition was also observed to translate into a prolonged lag phase for initial concentrations of acetic acid of 16–19 g/L, while from 20 g/L onwards the acid concentration was lethal for the yeast (Robles-Iglesias et al., 2021). Other authors such as Gao and co-workers concluded that the initial pH plays a key role in the tolerance of acetic acid and other VFAs, stating that an alkaline pH favours the assimilation of acids by yeasts, i.e., at pH > 8.0 (Gao et al., 2020). Another study performed by Kolouchová and colleagues concluded that acetic acid is the least toxic VFA for yeasts compared to longer chain fatty acids (Kolouchová et al., 2015).

After testing the toxicity range of acetic acid in *R. toruloides,* it was concluded not to dilute or add further acetic acid to the samples extracted from the *C. aceticum* CSTR bioreactor experiment described in section 3.1.

3.2.2. Growth of R. toruloides and acid consumption from fermented C. aceticum broth

Although the concentration of acetic acid naturally produced by *C. aceticum* in the C_1-gas-fed CSTR was not changed for the yeast cultivation, the pH was adjusted to 6.0 before inoculating *R. toruloides* into the baffled flasks. The four acetic acid samples extracted from the CSTR had the following concentrations: 8.6, 10.6, 11 and 11.5 g/L. Since the last three extracted samples had rather similar acetic acid concentrations, they were used for a triplicate assay at an initial acetic acid concentration of 11 ± 0.5 g/L, allowing at the same time to accurately check and confirm reproducibility. Before inoculating *R. toruloides*, all extracted samples were subjected to a sterilisation process to eliminate any presence of bacteria and possible subsequent contamination, by autoclaving at 120 °C for 20 min.

The acid consumption profiles obtained with the different initial acetic acid concentrations are plotted in Fig. 3. As shown in that figure, it was decided to stop the experiments after 100 h because, for the highest initial concentrations, consumption slowed down when minor substrate concentrations remained leftover towards the end of the studies. It can be observed that, for example, for the initial concentration of 8.6 g/L, the time to consume all the acetic acid is almost 80 h. If we compare this result with those obtained in the toxicity experiment, with a commercial initial acetic acid concentration of 9 g/L (Fig. 2), it turns out that it takes much longer to consume the fermented acetic acid coming entirely from

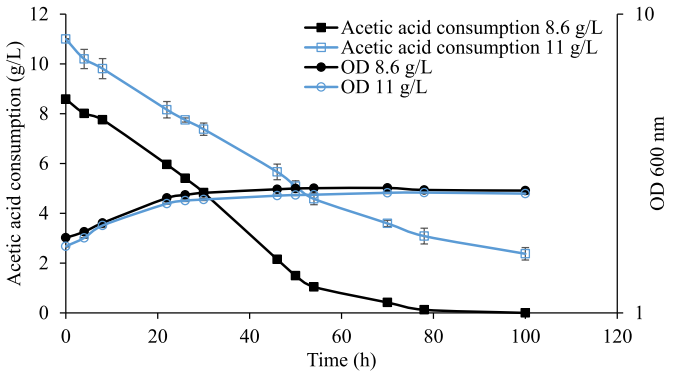

Fig. 3. Growth (OD_{600nm}) and consumption of acetic acid by *R. toruloides* at various starting concentrations of acetic acid generated from *C. aceticum* anaerobic fermentation.

C. aceticum, which may even be up to four times longer (Fig. 3). There could be several reasons for the slower consumption rate of fermented acetic acid compared to commercial acetic acid by *R. toruloides*. One possibility is that the fermented acetic acid may contain impurities or byproducts from the bacterial fermentation that could be inhibitory to the yeast's growth or metabolic activity. Another possibility is that the fermented acetic acid may have a lower nitrogen concentration due to its complete consumption by the bacteria, and therefore, a high C/N ratio that could affect the rate of biomass formation. It is also possible that the yeast is not fully-adapted to the specific composition of the fermented medium with acetic acid, and may require a longer period of acclimation or adaptation to manage to consume the carbon source at optimal rate.

Overall, the growth of *R. toruloides* in acetic acid fermented by *C. aceticum* fed syngas was favourable, as no lag phase was observed. However, the latter was also expected as the experiment was performed at concentrations below potential toxicity. The final amount of biomass obtained, expressed in g/L per dry cell weight, together with the biomass yield obtained and the maximum rate of acid consumption, are shown in Table 1.

According to the results shown in Table 1, the experiment with the lowest initial acetic acid concentration (8.6 g/L) produced a somewhat lower amount of biomass and had a lower biomass yield. In contrast, the rate of acid consumption was almost identical.

If one compares the data of acetic acid consumption by the yeast with other similar studies, the values are pretty low. For example, when *R. toruloides* was cultivated in a media rich in acetic acid generated from the fermentation of CO_2 by the anaerobic bacteria *A. woodii*, the consumption rates of acetic acid were around 0.33 g/L·h (Robles-Iglesias et al., 2021), which is more than twice higher than in this study. On the other hand, in terms of substrate-to-biomass conversion yields, the present experiments, with the fermented medium of *C. aceticum*, yielded better results with values approximately 20–30% higher than in the *A. woodii* fermented medium of our previous study (Robles-Iglesias et al., 2021). Huang and colleagues also studied the effect of different initial concentrations of acetic acid on the growth of this yeast, reaching a biomass-to-substrate yield of $Y_{(X/S)} = 0.84$ for concentrations similar to the present research, more than twice as much as in our experiments (Huang et al., 2016). Conversely, the amount biomass obtained in that study only exceeded the present experiments by 25% (Huang et al., 2016). These differences could be due to the concentration of nitrogen in the culture medium. The higher the concentration of nitrogen relative to carbon (in terms of C/N ratio), the higher the expected amount biomass produced. For example, for yeasts of the same *Rhodosporidium* genus, it was found that the difference in terms the amount of dry biomass obtained between two experiments where the C/N ratio varied from 100 to 2 was up to 4 times higher at C/N = 2 (Méndez Polo et al., 2021). However, the low nitrogen concentration, which could have affected biomass growth in these experiments, are considered to be better for optimal accumulation of lipids, which was indeed the case in this study, in which high lipid contents, around 40%, were reached, as described below.

Table 1
Dry biomass, biomass yield and maximum consumption rate of acetic acid varying the initial acetic acid (HAc) concentrations in *R. toruloides*.

Initial HAc concentration (g/L)	Biomass (g/L)	$Y_{(X/S)}$	Max. rate of acid consumption (g/L·h)
8.6 ± 0.5	2.56 ± 0.10	0.23 ± 0.02	0.123
11 ± 0.5	3.01 ± 0.04	0.35 ± 0.02	0.113

3.2.3. *Effect of the concentration of acetic acid produced by C. aceticum on lipid production*

As described in previous sections, different bottles were inoculated with the yeast *R. toruloides*, using the medium from the *C. aceticum* experiments, at different acetic acid concentrations. As previously stated, the last three extracted samples had similar acetic acid concentrations, and it was decided to mix them to perform the experiment in triplicate at an initial concentration of acids of 11 ± 0.5 g/L. Fig. 4 shows that the initial acetic acid concentration affects the amount of lipid produced, both lipid concentration (g/L) and lipid content (% g/g DCW). The highest total lipid content of 39.5% (g/g DCW) was achieved at an initial acetic acid concentration of 11 ± 0.5 g/L. The highest lipid concentration measured was 1.18 g/L, which also corresponds to the initial concentration of acetic acid of 11 ± 0.5 g/L. Productivity was thus 0.28 g/L·day.

Since high VFAs concentrations have an inhibiting impact on yeast development, low concentrations of VFAs are more often used in research. In other experiments carried out in batch, with the same yeast strain and acetic acid coming from anaerobic gas fermentation with *A. woodii*, the results showed an evident inhibition on lipid production from initial acid concentrations of 18 g/L onwards (Robles-Iglesias et al., 2021). The highest lipid content of 17.8% g/g of DCW was obtained in that study, with a 15.4 g/L initial acetic acid concentration (Robles-Iglesias et al., 2021). This result is lower than the one obtained in the present experiment. It may be due to the nutrients consumed/generated in the previous anaerobic fermentation process (Ye et al., 2021), as different acetogenic bacteria were tested and they were grown in different media, expected to be near-optimal for each specific bacterial strain. For instance, in this study, 0.2 g/L NH_4Cl was used to grow the acetogenic *C. aceticum* bacteria. In contrast, in another of our recently reported research, with *A. woodii*, the amount ammonium chloride was

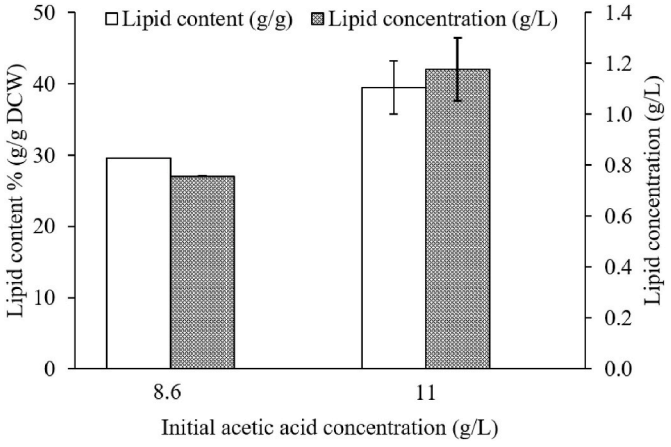

Fig. 4. Influence of the initial concentration of acetic acid on the amount of lipids produced after 100 h incubation.

1 g/L, which affects the initial C/N ratio and may affect yeast growth. Several studies have shown that a high C/N ratio favours the accumulation of lipids inside oleaginous yeasts due to nitrogen deficiency in the culture medium (Sarantou et al., 2021). *R. toruloides* was also cultured by Huang and colleagues with acetic acid serving as the only source of carbon for the synthesis of lipids (Huang et al., 2016). These authors, on the one hand, grew the yeast with initial concentrations of acetic acid between 5 and 40 g/L, obtaining the best results at an initial concentration of 20 g/L, reaching 23.5% g/g lipid content. On the other hand, they tested different initial C/N ratios reaching a maximum lipid content of 48.2% g/g at C/N = 200 (Huang et al., 2016). In terms of lipid content that *R. toruloides* produces, one of the highest results that has been reported in the scientific literature is 73.5% g/g (Liu et al., 2021). This result was obtained in a 6-cycle sequencing-batch reactor by using synthetic sugar-containing wastewater anaerobic fermentation broth with 5 g/L acetic acid.

Table 2 compares the data of lipid content and yield with those of other studies using different strains fed acetic acid at different concentrations as the sole carbon substrate. The highest yield obtained in our study was 0.107 ± 0.015 g/gC from an initial acetic acid concentration of 11 ± 0.5 g/L. Although this research did not reach the highest lipid yield ($Y_{L/S}$) compared to other references cited in Tables 2 , it reached one of the highest lipid contents compared to the different strains. It is also worth observing that the total amount substrate (acetic acid) supplied in this study was lower compared to other references, while supplying more substrate might have increased the production of lipids.

3.2.4. Lipid profile

It is essential to know the composition of the lipids obtained from the yeast fermentation stage, as this may affect their suitability for specific applications, e.g., to produce biodiesel or other fuels. Some studies have focused on the design of desired FAME components to improve biodiesel's cold flow and oxidative stability (Pinzi et al., 2009). According to the literature, an ideal composition would include relatively low levels of polyunsaturated fatty acids (FA), e.g., to prevent oxidative instability, low quantities of saturated (FA), e.g., to decrease cold flow issues, and high ratios of monounsaturated FA. Previous studies showed that oleic acid (18:1) and palmitoleic acid (16:1) offer the optimal mix of oxidative stability and cold flow without producing significant cetane number changes (Pinzi et al., 2009).

Table 3 shows the lipid composition obtained in this research and typical compositions found in *R. toruloides* when using different feedstocks. Our study shows that oleic acid (C18:1) is slightly dominant and closely followed by palmitic acid (C16:0). Stearic and linoleic acids (C18:0 and C18:2, respectively) are also present, though at lower concentrations. On the basis of the results obtained, the concentrations of each one of them were as follows (g/L): 0.29 ± 0.06, 0.12 ± 0.03, 0.33 ± 0.05, 0.13 ± 0.02, 0.08 ± 0.02 of C16:0, C18:0, C18:1, C18:2 and others, respectively.

Apart from the aforementioned fatty acids, the main identified other fatty acids generated by this yeast are myristic acid (C14:0) and linolenic acid (C18:3), both produced in small amounts. Besides, Fig. 5 shows the average FAME composition calculated from the data obtained at different acetic acid concentrations, showing that there is only limited standard deviation and that the acid concentration hardly affects the final composition.

Although palmitoleic acid (C16:1) was not detected, the lipids composition, high in oleic acid (C18:1), has a great potential for application in biodiesel production. The lipid profile observed in this experiment resembles the profile published by Huang and co-workers, who grew this same species with commercial acetic acid (Huang et al., 2016). In general, the differences observed were as follows: (1) in the present experiment, the amount of C18:1 was lower, ranging between 34.8 and 36.8%, while, in the afore cited study, with commercial acetic acid, it ranged from 41.3 to 49.5%, at an initial acid concentration of 4 g/L (Huang et al., 2016). This may be due to the initial concentration of acetic acid rather than the origin of acetic acid; as in another study using acetic acid from another acetogenic bacteria, the C18:1 concentration decreased from 50.0 to 34.7%, when the initial concentration of acetic acid increased from 8.7 to 19 g/L (Robles-Iglesias et al., 2021); (2) the concentration of C16:0 in this experiment is higher, with values of around 30%, while in the referenced study, they did not even reach 20%, regardless of the initial concentration of acetic acid (Huang et al., 2016).

Overall, from the aforementioned results, it can be considered that acetic acid produced by *C. aceticum* from C_1-gas valorization can be efficiently converted into lipids whose fatty acid profile matches the typical composition of biodiesel; so, this technique has a high potential for biodiesel manufacture.

4. Conclusions

The present work investigated, on the one hand, the ability of the acetogenic bacterium *C. aceticum* to convert C_1-gases, such as syngas, into an interesting platform chemical, i.e., acetic acid. On the other hand, this research evaluated the ability of the yeast *R. toruloides* to accumulate lipids using acetic acid from the fermented medium of *C. aceticum* originally grown on C_1-gases. In short, syngas could be efficiently converted into acetic acid, with subsequent conversion of that acetic acid into lipids. Some inhibition of the yeast was observed at initial acetic acid concentrations around 18 g/L, and the acid was lethal at concentrations around 22 g/L and above. Highly encouraging results were achieved in terms of lipid content, with a maximum of 39.5 ± 3.7% g/g dry cell weight, when the yeast was grown with 11 ± 0.5 g/L initial acetic acid concentration. Due to the high lipid content obtained and the capacity for improvement in this field, this study opens the door to a new strategy for growing oleaginous yeasts, using syngas or C_1-pollutant gases as the first stage of the overall process.

Credit author statement

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

Table 2
Lipid content (% g/g) and lipid yield production ($Y_{(L/S)}$) using different initial concentrations of acetic acid for different strains.

Yeast strain	Initial concentration of HAc (g/L)	Culture mode	Lipid content % (g/gDCW)	$Y_{(L/S)}$ (g/gC)	Reference
C. curvatus ATCC 20509	20	Batch	41.3	0.110	Lian et al. (2012)
Y. lipolytica CICC 31 596	30	Batch	29.3	0.175	Gao et al. (2020)
R. toruloides 10134	15.4	Batch	17.8	0.047	Robles-Iglesias et al. (2021)
R. toruloides AS 2.1389	5	Batch	18.4	0.277	Huang et al. (2016)
	10	Batch	19.1	0.162	
	20	Batch	23.5	0.187	
	40	Batch	No growth	–	
R. toruloides 10134	8.6	Batch	29.6	0.088	This study
	11 ± 0.5	Batch	39.5 ± 3.7	0.107 ± 0.015	This study

Table 3
FAME composition of *R. toruloides* with different feedstocks and concentrations.

Feedstock	Concentration (g/L)	Relative amount of total FAs (%, w/w)					Ref.
		C16:0	C18:0	C18:1	C18:2	Others	
Acetic acid	8.6	27.6	10.2	36.8	17.6	7.9	This study
Acetic acid	11.0	31.8 ± 0.6	12.6 ± 1.0	34.8 ± 0.7	12.6 ± 1.7	8.2 ± 0.9	This study
Acetic acid	4.0	15.6	8.2	49.5	13.7	13.1	Huang et al. (2016)
Acetic acid	20.0	16.6	16.1	38.3	16.5	12.5	Huang et al. (2016)
Sucrose	40.0	24.4	7.8	31.4	20.2	16.2	Ye et al. (2021)
Wood hydrolysate	50.0 (sugars)	23.8	14.1	47.3	7.9	6.9	Saini et al. (2021)
Crude glycerol	60.0	22.2	12.6	46.0	12.1	7.1	Chmielarz et al. (2021)
Pure glycerol	60.0	22.2	5.7	21.3	39.5	11.3	Gao et al. (2016)

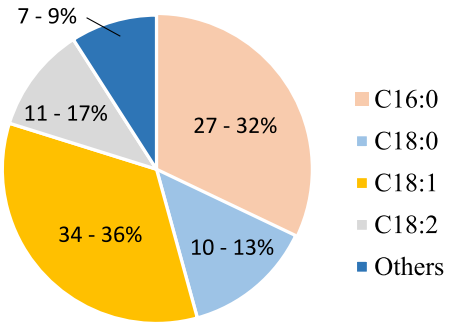

Fig. 5. Average FAME composition obtained from different initial acetic acid concentrations.

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 Ministry of Science Technology and Innovations. Christian Kennes reports a relationship with University of A Coruna that includes: employment.

Data availability

Data will be made available on request.

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