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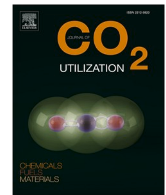

Carbon dioxide bioconversion into single cell oils (lipids) in two reactors inoculated with *Acetobacterium woodii* and *Rhodosporidium toruloides*

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ABSTRACT

Acetobacterium woodii was able to produce high concentrations of acetic acid, i.e., > 20 g/L, from CO_2, in the presence of H_2 as an energy source, which was favoured by maintaining a near-optimal pH of 7.8 in an automated bioreactor. This allows the mitigation of CO_2 emissions for their conversion to acetic acid, which was then further used to produce lipids (single cell oils) by *Rhodosporidium toruloides* in the next assay. The yeast, grown on acetic acid, efficiently accumulated lipids in *A. woodii*'s medium, and further improved bioconversion would result in a highly promising process. Acetic acid inhibitory studies performed with *R. toruloides*, at different initial concentrations of the acid, using the fermented broth of *A. woodii* grown on CO_2, showed that the yeast maintained a constant growth rate and substrate consumption rate up to acid concentrations of 15 g/L. Both rates remained roughly constant at higher initial acetic acid concentrations; except for a more extended lag phase observed in batch assays before the yeast entered in its exponential growth phase.

1. Introduction

Emissions resulting from the use of fossil fuels, mainly CO_2, have globally increased at a rate of 0.9 % per year during the 1990s and increased to 3.0 % during the 2000s, although they decreased then again to a growth rate of 0.9 % per year since 2010 [1]. During the period ranging from 2014 to 2016, there was a globally slight slowdown in emissions of CO_2 as greenhouse gases [2]. Then, in the subsequent three years, this value did significantly rise again, increasing by 2.1 % in 2018 compared to the previous year, with a total of 36.6 ± 1.8 Gt emission per year [3]. According to the International Association for Impact Assessment (IAIA), 41 % of all anthropogenically emitted CO_2 comes from the energy sector, which is the most critical source. According to the IAIA, the three primary sources of such emissions within the energy sector are coal, oil, and gas, with the first two representing approximately 73 % of the total.

On the other side, worldwide energy consumption increased in the last decades due to the increasing world population and the high energy demand [4]. Therefore, much research has recently been focusing on studying new energy sources to become less dependent on oil and coal, and thus significantly reduce CO_2 emissions, targeting a total reduction of 40 % since 1990 according to the 2030 agenda approved by the United Nations (U.N.). Biofuels seem to have some excellent characteristics to gradually replace conventional fuels.

Biofuels are liquid, gaseous, or solid fuels that are derived primarily from biomass or other similar or renewable sources. Indeed, biomass may be converted into a wide range of fuels, which includes, but is not limited to, methanol, ethanol, butanol, biodiesel, Fischer-Tropsch diesel, hydrogen, and methane [5]. Biodiesel is a sustainable biofuel obtained from the transesterification of natural triglycerides, mainly derived from plant oils or animal fats, with alcohol, mostly methanol, to create fatty acid alkyl (methyl) esters [6]. Biodiesel are classified into three different categories according to the oily raw material used, as first generation, second generation, and third-generation biodiesel.

First generation biodiesel is manufactured from vegetable oils extracted from crops such as soybean or rapeseed. This type of biodiesel production results in a series of issues, such as the conflict related to land use since, as mentioned above, first generation biofuels require raw materials and land that can be used for other human needs [5].

Second-generation biodiesel is produced using oils from non-edible plants, which can solve some problems derived from first-generation production. However, it would be necessary to convert large soil areas for the specific production of these raw materials. CO_2 emissions generated from the use of all these lands for biodiesel production would

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hardly reduce the greenhouse impact, which could even significantly increase. So, this option to supply global demand for fuel was recommended to be ruled out [7]. Some studies do even indicate that if all the accessible growing areas would be used to plant second-generation biodiesel raw material, it might not be enough to supply the total energy demand in the world on a global scale [8].

Third generation biodiesel is based on the use of specific microorganisms, such as fungi, microalgae, bacteria, or yeasts. They represent probably one of the best options compared to the above-described ones, due to various factors, such as avoiding dependence on a large area of farmland. It also manages to reduce the dependence on weather conditions, labour, and high energy consumption. Furthermore, microorganisms have a short life cycle compared to plants. Hence, microorganisms harvest time is shorter, and they grow on a wide variety of substrates, some of which are cheap sources of carbon or even agro-industrial waste [9,10].

The potential of microorganisms in biorefineries has been described for several decades, both from a biotechnological as well as from a biochemical point of view. Numerous researchers have attempted to manufacture biodiesel using microbial lipids produced from oleaginous microorganisms such as bacteria, yeasts, fungi, and algae [9]. Oleaginous microorganisms have been identified as capable of accumulating a high amount of lipids, typically more than 20 % of their biomass weight [10]. Some studies even suggest that they can accumulate or store more than 70 % of their dry weight when grown under limited nitrogen conditions [11].

Of all the different types of known oleaginous microorganisms, yeasts were chosen in this study due to their advantages compared to other microorganisms. The lipid accumulation in microalgae can often be high since it has been shown that they can reach up to 80 % of the total lipid content of their dry weight, but compared to bacteria, fungi, and yeasts, they grow much more slowly, they require more space and exhibit a higher risk of contamination [12]. Although some bacteria and fungi can accumulate lipids, they have been quite less considered than algae for the generation of biodiesel. A large part of all published research has been focusing on their ability to produce specific lipids such as docosahexaenoic acid (DHA), omega-6 fatty acids (GLA), eicosapentaenoic acid (EPA), or arachidonic acid (ARA), which are not used for biodiesel production [13]. Regarding the use of oleaginous yeasts for biodiesel production, it should be noted that they can accumulate large amounts of lipids, often around 40–60 % of their dry weight. Also, lipids are recovered more efficiently from lyophilised or thawed yeast cells, which is a more feasible extraction alternative when compared with methods based on disintegration or drying at moderate temperatures [14].

In this type of technology, the cost of the substrate is a determining factor since, in some studies, it may represent 40–50 % of the total cost of the process [15]. For instance, a techno-economic analysis of lipid synthesis utilising glucose as a substrate determined that the unit cost of producing biodiesel is around 5.9 $/kg. In contrast, the retail price of biodiesel is around 1.2 $/L [16]. In that study, glucose contributed 35 % of the total production cost. Therefore, the substrate chosen to feed yeasts should be as cheap as possible. In that sense, substrates that involve industrial wastes or pollutants have recently gained attention. Some recent studies reported the efficient use of acetic acid and other volatile fatty acids as raw materials for yeasts, reaching lipid yields of 50–65 % with yeasts belonging to the *Cryptococcus* and *Rhodosporidium* genera [17,18]. Volatile fatty acids, and especially acetic acid, are cheaper than glucose and can also be obtained through the bioconversion of gases as an innovative alternative.

There is only limited research focusing on the production of biofuels or bioproducts from acetic acid and other types of volatile fatty acids derived from pollutants, agro-industrial wastes or directly from industrial waste gases containing CO or CO_2 [19,20]. Obtaining fatty acids from greenhouse gases, through acetogenesis, converts those gases into a raw material rather than being considered as simple pollutants to get rid of [21]. Several studies yielded favourable and scalable results related to the use of such acids as raw material for yeasts. The main objective of this research was to combine the anaerobic acetogenic bioconversion of gases such as CO_2 into volatile fatty acids in a first stage, with a second stage based on the subsequent conversion of those acids, by oleaginous yeasts, accumulating lipids, for their potential use as biofuel (i.e., biodiesel).

2. Material and methods

2.1. Gas fermentation stage

2.1.1. Strain and media used in the gas fermentation stage

The type strain *Acetobacterium woodii* (DSM 1030) was obtained from "*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*" (Braunschweig, Germany). It was kept in a medium described by the company from which the strain was purchased.

A. woodii was grown in bottles containing 40 mL medium with the following composition (per liter distilled water): yeast extract (YE), 2 g; NH_4Cl 1 g; KH_2PO_4, 0.33 g; K_2HPO_4, 0.45 g; $NaHCO_3$, 10 g; $MgSO_4$ x 7 H_2O, 0.10 g; cysteine−HCl, 0.5 g; Na_2S x 9 H_2O, 0.5 g; D-fructose, 10 g; Na-resazurin (0.1 % w/v), 0.5 mL; trace element solution, 2 mL. The composition of the trace metals solution was (per liter distilled water): Nitrilotriacetic acid, 15 g; $MgSO_4$ x 7 H_2O, 30 g; $MnSO_4$ x H_2O, 5 g; NaCl, 10 g; $FeSO_4$ x 7 H_2O, 1 g; $CoSO_4$ x 7 H_2O, 1.8 g; $CaCl_2$ x 2 H_2O, 1 g; $ZnSO_4$ x 7 H_2O, 1.8 g; $CuSO_4$ x 5 H_2O, 0.1 g; $KAl(SO_4)_2$ x 12 H_2O, 0.2 g; H_3BO_3, 0.1 g; Na_2MoO_4 x 2 H_2O, 0.1 g; $NiCl_2$ x 6 H_2O, 0.25 g; Na_2SeO_3 x 5 H_2O, 3 mg; Na_2WO_4 x 2 H_2O, 4 mg. The pH was maintained at 7.8 and the temperature at 30 °C. The culture medium in the bottles was flushed with pure N_2 in order to ensure anaerobic conditions. The gaseous substrate was composed of $CO_2/H_2/N_2$ (25/35/40), and the bottles were agitated on an orbital shaker at 150 rpm.

2.1.2. Bioreactor studies in CSTR (continuously stirred tank reactor) with a continuous gas feed

The bioreactor experiments were carried out in a 2 L stirred tank BIOFLO 110 system (New Brunswick Scientific, Edison, NJ, USA), introducing 1.2 L of the culture medium described above. The bioreactor with the medium was autoclaved at 120 °C for 20 min in order to avoid any possible contamination. In this experiment, cysteine−HCl, Na_2S and fructose were added after autoclaving to avoid possible reactions at high temperatures. The medium, in the reactor, was then flushed for two hours with pure N_2 to remove all oxygen. The bioreactor inoculated with the *A. woodii* was fed continuously a gas mixture containing $CO_2/H_2/N_2$ (25/35/40) as substrates. The gas flow rate was continuously regulated and maintained constant, at 10 mL/min by means of a mass flow controller (Aalborg GFC 17, Germany). The bioreactor was maintained at a constant temperature of 30 °C, and the fermentation broth was agitated at a constant speed of 250 rpm throughout the experiment. 10 % of an actively growing bacterial culture, which was grown for 72 h on the above-mentioned gas mixture, was used as inoculum and was aseptically seeded into the bioreactor's medium. The pH of the culture broth was measured online, and it was regulated at a constant value of 7.8 through the addition of either a 1 M NaOH or a 1 M HCl solution, fed with a peristaltic pump.

2.2. Lipids production stage

2.2.1. Strain and media used in the lipids production stage

The strain *Rhodosporidium toruloides* (DSM 10,134) was used for the lipids production stage. It was obtained from "*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*" (Braunschweig, Germany), and was maintained on potato dextrose agar (PDA) medium.

2.2.2. Batch reactor cultivation experiments

Samples of 40 mL medium were obtained from the bioreactor

experiments performed with *A. woodii* as described in previous sections and containing acetic acid concentrations ranging from 4 to 21 g/L, depending on the samples. These samples were then used as a culture medium for experiments performed with the species *R. toruloides*.

First, the *R. toruloides* strain was cultivated on PDA for 2–3 days. Afterwards, it was transferred to a potato dextrose broth (PDB) and grown in a liquid medium for 24 h. Then, it was transferred to the fermented *A. woodii* medium to start the lipids production experiments. In order to avoid carrying over nutrients from the PDB medium, the seed cultures were collected via centrifugation (3600 g, 5 min), washed with standard saline medium, and resuspended in the bottles containing the culture medium at an optical density (OD_{600}) of 0.5 for 1 mL of culture. The experiments were carried out in 40 mL of previously fermented *A. woodii* medium, using 250-mL flasks in each experiment. The bottles were continuously shaken at 200 rpm and maintained in a thermostated room at 30 °C. During the experiments, biomass growth, pH, acetic acid consumption and lipids production were monitored regularly. In these batch assays, the initial pH of the medium was 6.0, and it was then allowed to vary freely throughout the experiment.

2.3. Analytical methods

2.3.1. Determination of biomass concentration

Growth of the microorganisms (bacteria and yeast) were determined spectrophotometrically (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) in each experiment, measuring the optical density of 1 mL samples at a wavelength of 600 nm (OD_{600}), which allowed to plot the corresponding growth curves.

At the end of each culture, the samples were centrifuged at 3500 rpm for10 minutes, and the pellet was washed with distilled water and frozen in order to lyophilise the samples after 48 h. The accumulation of biomass was determined by measuring the weight of a known volume of the lyophilised samples.

2.3.2. Determination of acetic acid production and consumption

In order to measure the amount of acetic acid (HAc) present in the fermented medium, a high-performance liquid chromatograph (HPLC) (HP1100, Agilent Co., USA) was used. It was equipped with a diode array detector and a refractive index detector maintained at 50 °C. For analysing the composition and concentration on the HPLC, 1 mL sample was withdrawn regularly from the corresponding batch or bioreactor experiments. They were centrifuged at 7000 rpm for 5 min, and, subsequently, the supernatant was filtered through a 0.22 μm PTFE filter before HPLC analyses. A 0.005 M sulphuric acid solution with a flow rate of 0.80 mL/min was used as the mobile phase. Samples of 20 μL were taken and injected into the Agilent Hi-Plex H Column (300 × 7.7 mm) at 45 °C.

2.3.3. Determination of lipids content

Total lipids accumulated in the cell were extracted from 10−30 mg lyophilised biomass and converted to their methyl ester equivalents, according to the method described by Larroude and co-workers previously using a solution of methanol and sulphuric acid (40:1) [22]. A Thermo Fisher gas chromatograph (GC) was used for their quantification. The GC was equipped with an FID and an Agilent vf-23 ms column, whose specifications are 60 m, 0.25 mm, and 0.25 μm. Helium was used as a carrier gas, and the initial temperature of the oven was 190 °C, increasing by 4 °C min^{-1} up to 235 °C, and holding these conditions for 2 min. The fatty acids were identified by comparing with standard fatty acid methyl ester (FAME) solutions prepared in the laboratory. For the quantification of lipids, an internal standard method was used with the addition of 50 μg of commercial C12:0 converted to FAME (Sigma). Washed and freeze-dried cells of a known volume were used to measure the dry cell weight (DCW).

3. Results and discussion

3.1. Cell growth and acetic acid production from carbon dioxide

3.1.1. Acetobacterium woodii bioreactor

The *A. woodii* strain was grown in the bioreactor under the conditions described in Materials and Methods, continuously feeding the gaseous mixture composed of $CO_2/H_2/N_2$. Acetogenic bacteria are efficient at using carbon dioxide as a carbon source, while they need an electron donor such as hydrogen, or other possible electron donors, and as an energy source for the anaerobic bioconversion of CO_2. This experiment focused on optimal acetic acid production by the acetogenic strain for the subsequent yeast bioconversion of acids into lipids (microbial oils). pH is known to be one of the parameters that most affects the products obtained by acetogenic bacteria [23–25], and it was therefore decided to work at constant pH 7.8, which is near-optimal for growth and acetic acid production in this organism.

Fig. 1 shows the optical density measured at 600 nm (OD_{600nm}) and acetic acid production throughout the experiment. As can be observed in the figure, the acetogenic *A. woodii* strain grew rather fast on carbon dioxide in the presence of hydrogen and efficiently produced acetic acid at high concentrations, exceeding 21 g/L. The growth curve shows its fastest exponential biomass increase during the first 48 h after inoculation, to later slow down during the subsequent growth phase, from t = 48–140 h, and reach its maximum cell concentration after 209 h, corresponding to a maximum OD_{600nm} = 1.91. Later on, after maintaining a stable biomass concentration for several hours, from t = 140–230 h, bacterial decay was finally observed when the acetic acid concentration reached about 17 g/L (Fig. 1).

It is interesting to compare the growth curve with the data of acetic acid production versus time. This shows that, although the acetic acid production rate remained roughly constant, the biomass growth rate was initially fast to slow down later and even stop once an acid concentration of about 9 g/L had accumulated in the medium. This behaviour occurs due to metabolic uncoupling; the cells no longer grow but remain metabolically active. In any case, the strain remained active during its initial decay phase, as it continued to produce acetic acid at similar high rates. Kantzow and co-workers reported product inhibition in *A. woodii* in a continuous gas fermentation experiment when the acetic acid concentrations exceeded 8−12 g/L, causing a decrease in biomass and acetic acid production rate [26].

Moreover, the maximum product concentration in the medium reached 21.1 g/L, i.e., 0.352 M, after 354 h. This value is relatively high in comparison to studies carried out recently by other researchers. Indeed, Karekar and co-workers reached a maximum acetic acid concentration of 7.83 g/L (0.130 M), using glucose as the initial soluble substrate and feeding an H_2/CO_2 gas mixture in batch culture at a pH value near 7.0, adjusting its value daily [27]. In a more recent study by the same authors, also with an H_2/CO_2 (70/30) mixture, the amount acetic acid produced was 6.4 g/L (0.106 M), which was achieved after

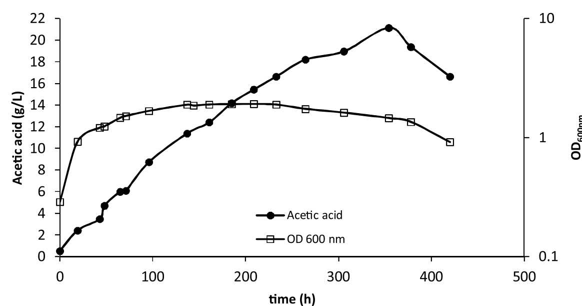

Fig. 1. Growth of *A. woodii* measured as OD_{600nm} and represented on a logarithmic scale, and acetic acid production in the continuous gas-fed bioreactor at constant pH 7.8.

10 days experiment in batch fermentation at pH 6.5 [28] and corresponded to less than one third of the concentration obtained in the present study over the same period of time. Performing a fed-batch experiment and maintaining a constant near-optimal pH certainly helped to reach high acetic acid concentrations in our assays, as that acid has been observed to be more toxic and inhibitory in clostridia at low pH, which is also related to the amount of dissociated and undissociated acid form in the medium [29]. In our experiments, the maximum growth rate in the exponential phase was 0.03 h^{-1} from the beginning of the assay up to 48 h. On the other side, the acetic acid production rate remained roughly constant during the whole experiment, corresponding to approximately 1.92 g/L day^{-1}. This value is significantly lower than in studies published by Demler and Weuster-Botz, reporting a productivity of 7.4 g/L day^{-1} in a stirred tank bioreactor of 2 L, with continuous gas feeding of H_2, CO_2 and CO at 0.25 vvm [30]. The lower acetic acid production rates can result, among others, from the different hydrogen partial pressures (pH_2) of the gas fed to the reactor. As indicated in that study, the initial volumetric productivity increased from 1.2 g/L day^{-1} at pH_2 of 400 mbar to 7.4 when increasing pH_2 to 1700 mbar, clearly showing the importance of that parameter [30].

As explained in Materials and Methods, samples of about 40 mL were taken from time to time to perform the second part of the experiment, described below, on inhibitory effects and the production of lipids and microbial oils from acetic acid coming from this gas (CO_2) bioconversion assay.

3.2. Effect of the acetic acid concentration on the growth of R. toruloides

It has been reported in the literature that, at relatively high levels of volatile fatty acids (VFAs) in the culture medium (including acetic acid), toxicity and inhibitory effects may show up and may affect the growth and activity of oil accumulating yeasts [31]. It has also been suggested that it can have an inhibitory effect on the accumulation of lipids and that depending on the origin of the acetic acid substrate (from where it is obtained), different impacts may be observed at different concentrations [32].

In this study, *R. toruloides* was grown on different medium samples withdrawn from the bioreactor of the previous experiment at different acetic acid concentrations. The initial pH of all experiments was 6.0, varying freely during the assay. The experiment was carried out in different batch assays with acetic acid concentrations ranging from 4.7–21.1 g/L. Fig. 2 shows the acetic acid consumption patterns during each experiment at different initial amounts of the acid.

The figure shows that the yeast's lag phase and/or substrate consumption rates may vary depending on the initial acetic acid (HAc) concentration. It can be concluded that at initial acetic acid concentrations exceeding 16 g/L, *R. toruloides* suffers from significant inhibitory effects resulting in different HAc consumption rates and apparently longer lag phases. For example, below 16 g/L initial acetic acid, the lag phase was 10 h, while at higher initial concentrations of 19 g/L, substrate consumption started only about 53 h after inoculation (Fig. 2). Table 1 shows the calculations of the acetic acid consumption rates at different initial concentrations, together with growth yields. The data are calculated for concentrations ranging between 14.2 and 21.1 g/L, as there was no inhibition found below 14.2 g/L. It can also be observed that above 20 g/L, there is no substrate consumption, meaning that acetic acid is lethal for the yeast above this concentration. The maximum acetic acid consumption rate reached in the experiment was 8.04 g/L day^{-1}, achieved in the experiments where no inhibition was observed. The results suggest that the initial acid concentration had only a limited effect on its consumption rate, which remained roughly constant between about 7–8 g/L day^{-1}. Its main effect was on the lag phase. In terms of growth yield, as the initial acetic acid concentration increased, the growth yield slightly decreased, reaching the minimum value of 0.183 g/gC at high acetic acid concentrations (Table 1).

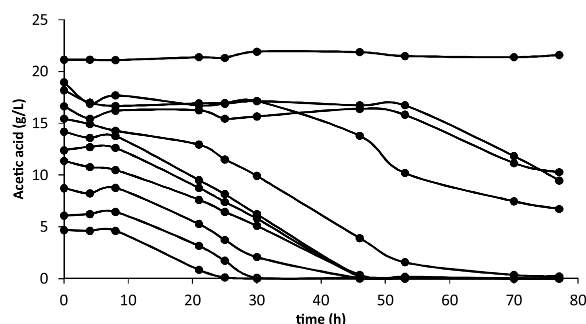

Fig. 2. Acetic acid consumption by *R. toruloides* in batch experiments at different initial concentrations.

Some other toxicity studies were reported with yeasts and different VFA. It was observed that using acetic acid as a substrate was more effective for the production of lipids than with other types of volatile fatty acids such as propionic acid due to the higher threshold inhibitory concentration of acetic acid and its better assimilation [33]. Moreover, recent studies carried out by Gao et al. [34] claimed that a more alkaline pH, above 7.0, is suitable to significantly reduce the inhibitory effects, with some oleaginous yeasts being able to assimilate as much as 30 g/L acetic acid at pH 8.0 in the case of a *Yarrowia lipolytica* species. However, our *R. toruloides* strain could not assimilate the highest concentration of 21.1 g/L acetic acid in the experiments at the initial pH of 6.0. The composition of the medium and other experimental conditions may also play a role in potential inhibitory effects. The pH was monitored throughout the experiments, and it was observed that the final value, for the cases in which acetic acid was consumed, was between 9.0 and 10.0. The pH evolution was directly related to the substrate consumption, as in the experiments with a more extended lag phase, the increase in pH exhibited a similar delay.

The growth curves of the yeast in the media containing different initial acetic acid concentrations are plotted in Fig. 3. This shows a very similar growth behaviour at all substrate concentrations below 16 g/L acetic acid. This threshold value is similar to the substrate consumption pattern previously shown in Fig. 2, where it was observed that the total consumption of acetic acid was possible in all cases below this acid concentration. As the substrate concentration increased, the lag phase in the growth curve of *R. toruloides* was also longer, up to the point where complete inhibition was observed, at 21.1 g/L, which can lead to the yeast's cell death.

The maximum growth rate of the yeast was 0.03 h^{-1}, which was the same for all the experiments at concentrations below 16 g/L.

It is interesting to observe how concentrations between 15 and 20 g/L show a similar growth rate, but at different times, i.e. with gradually longer lag phases at higher acetic acid concentrations. When comparing Figs. 2 and 3, the relationship between biomass growth and acetic acid consumption is evident. The higher the amount acetic acid consumed, the higher the yeast's growth rate.

3.3. Effect of the acetic acid concentration on the production of lipids

After each experiment in bottles, the amount of lipids accumulated in

Table 1
Acetic acid (HAc) consumption rates and growth yields ($Y_{(X/S)}$) at different initial acid concentrations.

HAc initial concentration (g/L)	HAc consumption rate (g/L day^{-1})	$Y_{(X/S)}$ (g/gC)
14.2	7.85	0.286
15.4	8.04	0.265
18.2	6.87	0.183
19.0	6.93	0.210
21.1	0	0

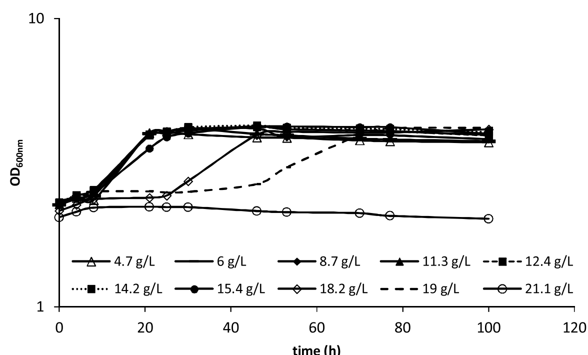

Fig. 3. Growth curves of the *R. toruloides* yeast at different initial concentrations of acetic acid, with the OD data represented on a logarithmic scale.

Table 2
Yield of lipid production ($Y_{(L/S)}$) in *R. toruloides* at different initial acetic acid (HAc) concentrations.

HAc initial concentration (g/L)	$Y_{(L/S)}$ (g/gC)
4.7	0.008
6.0	0.008
8.7	0.015
11.4	0.021
12.4	0.028
14.2	0.035
15.4	0.047
18.2	0.020
19.0	0.028
21.1	0

the system was determined by gas chromatography. Fig. 4 shows the amount lipids obtained in the experiments (% g/g) and the concentration of lipids (g/L) as a function of the initial acetic acid concentration. The highest value achieved for the lipid content is 17.8 % per gram dry weight of the strain. This value corresponds to an initial amount of acetic acid of 15.4 g/L and the above-mentioned higher consumption rate of 8.04 g/L day^{-1}. As for the lipid concentration, the maximum value recorded was 0.73 g/L, also corresponding to the initial acetic acid concentration of 15.4 g/L. Besides, Fig. 4 shows the inhibitory effect of acetic acid, observing a constant increasing behaviour at concentrations from 5 to 16 g/L, while at higher concentrations, the lipid content and concentration decreased.

Considering the inhibitory effect of high amounts of VFA on yeast cultures, low VFA concentrations are more extensively used in the literature. Xiang-Feng and co-workers cultured *R. toruloides* using acetic acid as the sole carbon source for the production of lipids [18]. They grew the yeast using initial acetic acid concentrations of 5, 10 and 20 g/L, obtaining the highest amount of lipids in the experiment with 20 g/L, corresponding to 23.5 % (g/g) [18]. This result is similar to the value of 17.8 % (g/g) obtained in our experiment. Other studies where *R. toruloides* was grown on crude glycerol and glucose show different results compared to our study. The following results were obtained when using 20 g/L glucose or other carbon sources such as crude glycerol as substrate: lipid yields (g/L) for glucose and crude glycerol of, respectively, 5.58 and 6.2 g/L, and lipid contents (%) of 36.3 and 41.7 %, respectively [35]. This can be compared to the present research in which the maximum values reached 0.73 g/L and 17.8 %.

Table 2 shows the lipid yield for each concentration of acetic acid. The highest yield was 0.047 g/gC, which is reached for the initial acid concentration of 15.4 g/L. These are generally lower values than reported by Xian-Feng and co-workers, whose highest result was 0.277 g/gC for an initial substrate concentration of 5 g/L, using acetic acid as the sole carbon source [18].

According to the literature, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and other unsaturated fatty acids and free fatty acids represent the majority of fatty acids formed by *R. toruloides* [18]. These components are desirable in biodiesel composition, especially oleic acid (C18:1), as indicated by Hoekman and co-workers in a review on biodiesel composition, properties and specifications [36].

The fatty acids content in our experiment and with different culture strategies are shown in Table 3. The lipid profile obtained shows a high amount of oleic acid (C18:1), dominant at low initial acetic acid concentrations, reaching up to 50 %. A high amount of palmitic acid (C16:0) is also observed, which increases as the initial acetic acid concentration increases, reaching up to 35 %. Generally, the lipid composition observed was as follows: C18:1 > C16:0 > C18:2 > C18:0 > Others. Within the category of other fatty acids produced by this yeast, apart from those already listed, the main identified compounds are linolenic acid (C18:3) and myristic acid (C14:0), produced in small quantities.

Comparing our produced lipids profile with those of other studies [18,35,37], some differences stand out. In this study, the dominant compound found in all experiments was C16:0, with values above 25 % in all cases. For the C18:0 compound, the values obtained in this research are generally slightly lower than in the literature, except for the assays performed with 60 g/L crude glycerol (Table 3). For the C18:1 lipid, we observed values very similar to other reported experiments, except for the study with glycerol, which shows significantly lower values. The amounts of C18:2 found in our study are slightly lower than those reported by others.

Overall, the lipids composition obtained in our experiment indicates

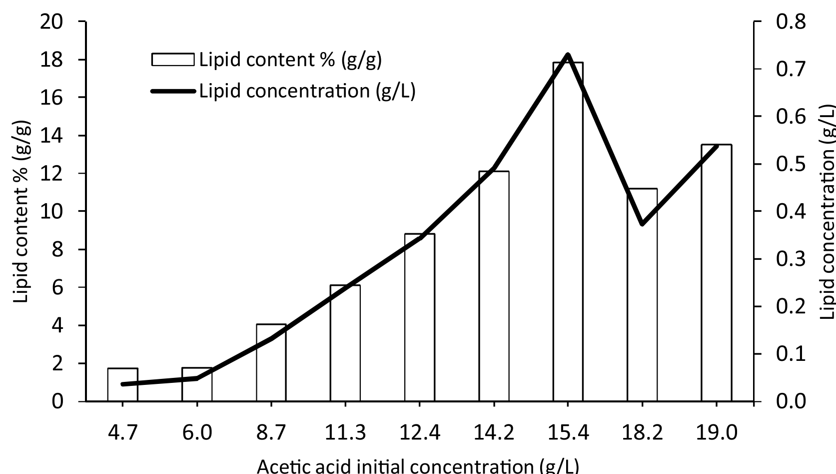

Fig. 4. Effect of the initial acetic acid concentration on the lipid content and lipid concentration.

Table 3
Fatty acids composition of *R. toruloides* corresponding to different culture strategies.

Culture Strategy	Relative amount of total fatty acids (%, g/g)					
	C16:0	C18:0	C18:1	C18:2	Others	Reference
8.7 g/L Acetic acid	26.2	4.8	50.0	13.7	5.3	This study
11.4 g/L Acetic acid	30.2	7.1	50.6	7.8	4.3	This study
12.4 g/L Acetic acid	32.7	7.4	49.8	7.0	3.1	This study
14.2 g/L Acetic acid	34.7	9.2	40.8	11.2	4.1	This study
15.4 g/L Acetic acid	35.5	7.6	42.0	8.8	6.2	This study
18.2 g/L Acetic acid	36.0	8.6	34.4	12.3	8.8	This study
19.0 g/L Acetic acid	34.1	11.9	34.7	11.6	7.7	This study This study
4.0 g/L pure Acetic acid	15.6	8.2	49.5	13.7	13.1	[18]
20.0 g/L pure Acetic acid	16.6	16.1	38.3	16.5	12.5	[18]
20.0 g/L Glucose	20.3	17.0	42.1	10.0	10.5	[18]
60.0 g/L pure Glycerol	22.2	5.7	21.3	39.5	11.3	[35]
20.0 g/L Glucose	20.0	14.6	46.8	13.1	5.5	[37]

that feeding *R. toruloides* with a medium rich in acetic acid produced by *A. woodii* has a high potential for use in the manufacture of biofuels, e.g., biodiesel.

4. Conclusions

CO_2 emissions can efficiently be bio-converted to acetic acid by the anaerobic bacterium *A. woodii*. The acetic acid generated can then further be accumulated in the form of lipids by the yeast *R. toruloides*. During the bio-conversion process into lipids, some acetic acid inhibition was observed at concentrations above 15 g/L, above all in terms of lag phase, showing a lag phase of up to 50 h for 19 g/L acetic acid compared to not more than 10 h at concentrations below 15 g/L. The yields obtained in terms of production of lipids is still somewhat low. Additional research related to this process aiming at further improving the production would be helpful. On the other side, this process is highly favourable in terms of lipid profile, obtaining over 40 % accumulation of the C18:1 compound, which is one of the main compounds looked for to produce biodiesel.

CRediT authorship contribution statement

Raúl Robles: Investigation, Data curation, Methodology, Writing original and revised drafts; **María C. Veiga**: Supervision, Resources, Reviewing drafts; **Christian Kennes**: Conceptualization, Funding acquisition; Supervision; Project administration; Methodology; Data curation; Reviewing drafts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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