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Accumulation of lipids by the oleaginous yeast *Yarrowia lipolytica* grown on carboxylic acids simulating syngas and carbon dioxide fermentation

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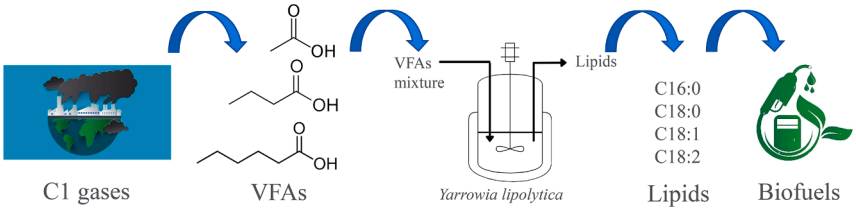
HIGHLIGHTS

- Lipid synthesis by oleaginous yeasts is a promising alternative to vegetable oils.
- *Y. lipolytica* can bioconvert mixed C_2-C_6 VFAs from gas fermentation into lipids.
- *Yarrowia lipolytica* shows preference for acetic acid over butyric and caproic acids.
- *Y. lipolytica* used the VFA mixture at a rate of 0.664 g/L·h with lipid accumulation.
- The lipid profile obtained shows its high suitability for biodiesel production.

GRAPHICAL ABSTRACT



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ABSTRACT

Volatile fatty acids (VFAs) can be considered as low-cost carbon substrates for lipid accumulation by oleaginous yeasts. This study demonstrates that a common mixture of VFAs, typically obtained from the anaerobic fermentation of C1-gases by some acetogenic bacteria, can be used in a second aerobic fermentation with the yeast *Yarrowia lipolytica* to obtain lipids as precursors of biodiesel. In the batch experiments, the preference of *Yarrowia lipolytica* W29 for acetic acid over butyric and caproic acids was demonstrated, with the highest consumption rate reaching 0.664 g/L·h. In the bioreactor experiments, the amount initial biomass inoculated, as well as the initial acid concentration, were found to have a significant influence on the process. Though the lipid content was relatively low, it can be optimized and further improved. Oleic, linoleic and palmitic acids accounted for about 80 % of the fatty acids in the lipids, which makes them suitable for biodiesel.

1. Introduction

The continuous increase in global industrial activities leads to more waste generation, and does also result in global warming and greenhouse effect. Biofuels, are receiving increasing attention from industries and governments in general as they are a promoting alternative to non-environmentally-friendly fossil fuels (Bao et al., 2021; Gao et al., 2017; Pereira et al., 2021). The most common biofuels include bioethanol, biogas, biohydrogen, and biodiesel, mainly (Kennes and Veiga, 2013). Biodiesel can be produced either from vegetable oils, which is the most typical process, or from microbial oils, which represents a more recent approach. These biofuels, derived from oleaginous microorganisms, e.g., fungi, microalgae, bacteria or yeasts, are considered as being fully sustainable and are becoming increasingly attractive, also due to their broad availability and accessibility (Leong et al., 2018). Nevertheless, although these biofuels have still many other advantages, one of their

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main challenges remains their limited cost-effective commercialization. Their production is largely studied at laboratory scale and the overall cost of the process is generally high (Bao et al., 2021; Leong et al., 2018). Even so, microbial oils and biodiesel are a highly attractive alternative compared to other, first and second generation, biofuels and conventional biodiesel production. It eliminates dependence on arable land, weather conditions, labour and high energy consumption; and microorganisms have a very short harvesting time and are able to grow on different substrates, even cheap ones, including waste (Robles-Iglesias et al., 2021).

In the present study, oleaginous yeasts were chosen as they exhibit several advantages over other organisms. Compared to microalgae, they are able to synthesize lipids from many different carbon sources and are not limited by physico-chemical factors; they have a much higher tolerance to metal ions than moulds; and lipid extraction is quite simpler than with bacteria (Bao et al., 2021). The non-conventional yeast *Yarrowia lipolytica* is characterized by its high ability to accumulate lipids, often reaching 30 % or more of its dry weight, thus representing an important biocatalyst for biodiesel production (Bao et al., 2021; Yook et al., 2019).

It is also important to note that this yeast species is able to degrade hydrophobic substrates such as alkanes, fats, oils and fatty acids very efficiently, and it can be grown using acetic acid and some other volatile fatty acids as carbon and energy source. The use of Volatile Fatty Acids (VFAs) for lipid synthesis with oleaginous microorganisms in general greatly reduces the costs of this microbial process, compared to other common substrates. So far, glucose or other similar carbon sources have generally been used, which may represent as much as about 80 % of the cost of the medium (Fei et al., 2011). Instead, VFAs, considered in this study, can be obtained from industrial waste, sludge, and even greenhouse gases, and their use represents an innovative and cost-effective approach, as they are very competitive substrates for biodiesel production (Fontanille et al., 2012). Due to the fact that oligosaccharides and sugars have so far widely been used for lipid synthesis by oleaginous microorganisms, there is as yet little information on the accumulation of these metabolites using other, low-cost, carbon sources such as VFAs, though it has been the focus of some recent studies in recent years (Fontanille et al., 2012; Gao et al., 2020; Park et al., 2021; Pereira et al., 2021; Llamas et al., 2020a; Morales-Palomo et al. 2022).

Furthermore, after an exhaustive literature search, it was found that there are hardly any studies on the synthesis of lipids from greenhouse gases. Anaerobic acetogenic microorganisms are able to grow on C1-gases (CO, CO_2/H_2, syngas) to produce mostly acetate and, occasionally, also longer chain VFAs and sometimes alcohols (Arslan et al. 2021; Fernández-Blanco et al. 2022; Fernández-Naveira et al. 2017a, Fernández-Naveira et al. 2017b, Fernández-Naveira et al. 2019). This process, known as acetogenesis, can thus yield fatty acids, and sometimes other metabolites, from the bioconversion of greenhouse gases. In such case, these gases should therefore no longer merely be considered as pollutants, but rather as raw materials (Fernández-Naveira et al., 2017a), as there are acetogenic bacterial species, such as *Clostridium* spp., among others, capable of synthesizing VFAs as raw materials for the subsequent production of lipids by oleaginous yeasts.

The aim of this study was to assess the behavior and potential of the yeast *Y. lipolytica* to grow and synthesize lipids from VFA mixtures as sole carbon source and in a culture medium typical from the anaerobic fermentation of syngas or carbon dioxide into C2-C6 carboxylic acids by acetogenic bacteria, as an innovative strategy for C1 gas valorization.

2. Material and Methods

2.1. Microorganism, culture conditions and inoculum preparation

The wild type yeast strain *Yarrowia lipolytica* W29 was used in all the experiments. It was originally grown and maintained on Yeast Extract-Peptone-Dextrose (YPD) agar plates, prepared with an aqueous medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and 20 g/L agar. *Y. lipolytica* was inoculated in this solid medium and grown in a thermostated room, at 33 °C, for approximately 24 h. Then, the plates with the reactivated yeast were kept in the refrigerator.

Besides, liquid culture medium was also prepared with (per liter distilled water): 10 g yeast extract, 20 g peptone, and 20 g dextrose. This medium was used to inoculate single colonies of the yeast from the plates, described above, and the strain was grown at 33 °C, with constant agitation, to obtain the preculture to be used in the experiments. After growing overnight, the precultures were harvested through centrifugation and the supernatant was decanted. Then, they were washed with 0.9 % NaCl, harvested by centrifugation again (decanting the supernatant). Finally, a small volume of a few millilitres distilled water was added to the pellets in the centrifuge tubes and the optical density (OD_{600nm}) was measured to estimate the amount inoculated biomass. All culture media and material were autoclaved at 120 °C for 20 min before use, in order to avoid any possible contamination.

The yeast was also stored in glycerol, in plastic tubes of 2 mL, mixing 0.5 mL of glycerol 80 % and 1 mL of the aqueous culture medium with the yeast. Then, they were frozen and stored at −80 °C.

2.2. Batch cultures

The batch experiments were carried out in 250 mL flasks. The culture medium had the following composition (per liter distilled water): yeast extract (YE), 2.30 g; NH_4Cl, 0.275 g; KCl, 0.125 g; KH_2PO_4, 1.17 g; K_2HPO_4, 5.07 g; $NaHCO_3$, 6.00 g; $MgSO_4 \times 7\ H_2O$, 0.46 g; $CaCl_2 \times 2\ H_2O$, 0.05; NaCl, 1 g; cysteine-HCl, 0.48 g; $Na_2S \times 9\ H_2O$, 0.54 g. This medium is similar to acetogenic media used for C1 gas fermentation. Each bottle contained 50 mL aqueous medium. Different amounts of a substrate mixture of even-chain fatty acids were added to each bottle, using specific ratios, based on unpublished results from C1-gas fermentation as well as recently published studies (Fernández-Naveira et al., 2017b, 2019). Afterwards, the pH was adjusted to 6.0, and then the bottles were sterilized by autoclaving at 120 °C for 20 min. These bottles were inoculated to reach an optical density of 0.5. Prior to inoculation, the seed cultures are always collected by centrifuging at 4000 rpm for 10 min, and the pellets are washed with 0.9 % NaCl, in order to remove any remaining nutrients, present in the YPD medium. The flasks were placed in a thermostated room at 30 °C and agitated at 150 rpm on an orbital shaker (Infors HT, Bottmingen, Switzerland).

2.3. Bioreactor cultures

Experiments in Stirred Tank Reactors (STR) were carried out in 2 L BIOFLO 120 fermentors (Eppendorf), containing 1.0 L of the growth medium described above. Since Na_2S and cysteine-HCl seemed to have no significant effect on the fermentation, they were omitted in the bioreactor studies. The medium contained 5 mL of trace metal solution, with the following composition (per liter distilled water): nitriloacetic acid, 3.50 g; $MgSO_4 \times 7\ H_2O$, 6.00 g; $MnSO_4{\cdot}H_2O$, 1.50 g; $(NH_4)_2Fe(SO_4)_2 \times 6\ H_2O$, 0.90 g; $CoCl_2 \times 6\ H_2O$, 0.40 g; $ZnSO_4 \times 7\ H_2O$, 0.38 g; $CuCl_2 \times 2\ H_2O$, 0.04; $NiCl_2 \times 6\ H_2O$, 0.05 g; Na_2SeO_4, 0.3 g; $Na_2MoO_4 \times 2\ H_2O$, 0.03 g; $FeSO_4 \times 7\ H_2O$, 0.2; $CoSO_4 \times 7\ H_2O$, 0.36; $CuSO_4 \times 5\ H_2O$, 0.02; $KAl(SO_4)_2 \times 12\ H_2O$, 0.04; H_3BO_3, 0.02 g. In order to be able to accurately determine the C/N ratio, which is known to affect lipid accumulation in yeasts, yeast extract was omitted and the amount of ammonium chloride (N source) in the medium was adjusted depending on the desired C/N ratio on starting the experiments. Besides, different amounts of an acid mixture were added, depending on the study to be carried out. Before starting the experiment, the medium was autoclaved at 120 °C for 20 min. Prior to inoculation of the medium with the required amount of microbial seed culture, 2.5 mL of a vitamin solution from a sterile stock solution was added as well. The vitamin solution had de following composition (per liter distilled water): biotin, 2.00 mg; folic

acid, 2.00 mg; pyridoxine-HCl, 10.00 mg; thiamine-HCl, 5.00 mg; riboflavin, 5.00 mg; nicotinic acid, 5.00 mg; Ca-D-pantothenate, 5.00 mg; vitamin B_{12}, 0.10 mg; p-aminobenzoic acid, 5.00 mg; (±)-α-lipoic acid, 5.00 mg.

The automated bioreactors are equipped with a pH sensor, a temperature controller, an aeration system and a mechanical stirring system with two propellers. During cultivation, aeration was maintained at 0.5 slpm, the agitation at 250 rpm, the temperature at 30 °C and the pH was adjusted to 6.0 by adding either hydrochloric acid (HCl) 1 M or potassium hydroxide (KOH) 1 M. The parameters were maintained constant throughout the studies. In some experiments, sequential additions of the VFA mixture were performed, in semi-continuous mode to assess the effect on the yeast and lipid accumulation.

2.4. Analytical methods

2.4.1. Biomass determination

Yeast growth was followed and determined spectrophotometrically. Therefore, the optical density of a 1 mL sample was measured at a wavelength of 600 nm (OD_{600nm}), using also a blank with distilled water. The optical density values allowed to plot the different growth curves.

2.4.2. VFA analyses

A high-performance liquid chromatograph (HPLC) was used in order to determine the consumption of the different acids *vs* time during the fermentation. The HPLC was equipped with two different detectors, i.e., a diode array detector and a refractive index detector, maintained at 50 °C. In each case, 1 mL samples were withdrawn, either from the flasks or from the bioreactors. Those samples were centrifuged for 5 min at 7000 rpm in an Eppendorf tube. The biomass settled down at the bottom of the Eppendorf tube, while the aqueous supernatant was injected in the HPLC, after filtering through a 0.22 μm polytetrafluoroethylene (PTFE) filter. A solution of sulphuric acid, 0.005 M, was used as mobile phase, with a flow rate of 0.80 mL/min. A 20 μL sample volume was injected, at 45 °C, using an Agilent Hi-Plex H Column.

2.4.3. Ammonium determination

The determination of ammonium was carried out spectrophotometrically, at a wavelength of 635 nm. The procedure consisted in using 600 μL of the previously centrifuged sample and adding 240 μL and 360 μL of phenol and sodium hypochlorite, respectively. The sample was vortexed and absorbance was measured after 45 min. Ammonium concentrations could be determined by means of a previously plotted calibration line.

2.4.4. Determination of lipid content

The determination of lipid content was carried out following the method described by Larroude and co-workers (Larroude et al., 2018). This procedure consists of three stages: sampling, lyophilization and then lipid analysis.

2.4.4.1. Sampling and lyophilization. Samples for lipid analyses were first centrifuged for 10 min at 4000 rpm. Then, the supernatant was discarded and 1 mL distilled water was added to each tube, before storing in the freezer until the analyses. Before the analyses, the samples were lyophilized for 24–48 h.

2.4.4.2. Lipids analysis. The lipids were quantified after direct transesterification of the fatty acids obtained. From 10 to 30 mg of freeze-dried biomass, the total lipids accumulated in the cells are extracted with a solution of methanol and sulphuric acid (40:1) in order to convert them into their methyl esters. A gas chromatograph (GC) (Thermo Fisher Scientific, Madrid, Spain) was used for their quantification. It was equipped with a flame ionisation detector (FID) and an Agilent vf-23 ms column (60 m × 0.25 mm × 0.25 μm). The carrier gas was helium and the initial oven temperature was 120 °C, which was held constant for 1 min, then increased by 25 °C/min up to 200 °C, and later increased again by 4 °C/min up to 230 °C, which was maintained for another additional minute. In order to identify the fatty acids, a comparison was made with fatty acid methyl ester (FAME) solutions prepared in the laboratory. In addition, an internal standard method was used to quantify the lipids by adding 25 mg of commercial C12:0 converted to FAME.

3. Results and discussion

3.1. Toxicity of the acid mixtures on the growth of Y. Lipolytica

Since the main goal was to assess the ability and efficiency of the yeast to grow and accumulate lipids in acetogenic media collected from acetogenic C1-gas (e.g., syngas, CO_2) fermentation, typical ratios of C2-C6 acids from such fermentations were used, for later further optimization research on C1-gas valorization. The ratios were then chosen based on a preliminary acetogenic gas fermentation test (data not shown) and on recent, published, C1-gas fermentation studies (Fernández-Naveira et al. 2017b; Fernández-Naveira et al. 2019). The three sole VFAs present in such anaerobic fermentation, produced through the Wood-Ljungdahl pathway, are even acids, i.e., acetic (C2), butyric (C4) and caproic (C6) acids, tested here as carbon sources for *Y. lipolytica*. Taking into account the amounts of acids obtained in the acidogenic fermentation, a toxicity study was carried out in five different flasks with different total concentrations of acids ranging from 6 to 16 g/L and with acid ratios of 0.81:0.14:0.05 for acetic, butyric and caproic acid, respectively. Furthermore, it should be noted that the initial and maximum concentrations used were also chosen based on the research carried out by Robles-Iglesias et al. (2021), who studied the effect of different acetic acid concentrations on the growth of the oleaginous yeast *Rhodosporidium toruloides*. From the afore mentioned study it was concluded that at initial concentrations above 15 g/L, *R. toruloides* underwent significant inhibitory effects resulting in different rates of acetic acid consumption and prolonged lag phases. Thus, the experiments with *Y. lipolytica* were carried out in batch assays, with different initial acids concentrations in the culture medium described in the previous sections. Besides, the initial pH of all experiments was adjusted to 6.0.

Fig. 1 shows the optical density measured at 600 nm (OD_{600nm}) in batch cultures with different initial acid concentrations. It can be seen that *Y. lipolytica* was able to grow quite fast in all five assays, with hardly any lag phase (less than 7 h). Furthermore, although all the growth curves are quite similar, as the concentration increases, the growth rate slightly increases as well, as summarized in Table 1. Furthermore, it should be noted that the maximum biomass production is reached in all cultures 32 h after inoculation, with the highest optical density (OD_{600nm} = 18) being observed for the highest initial acid concentration of 16 g/L (Fig. 1).

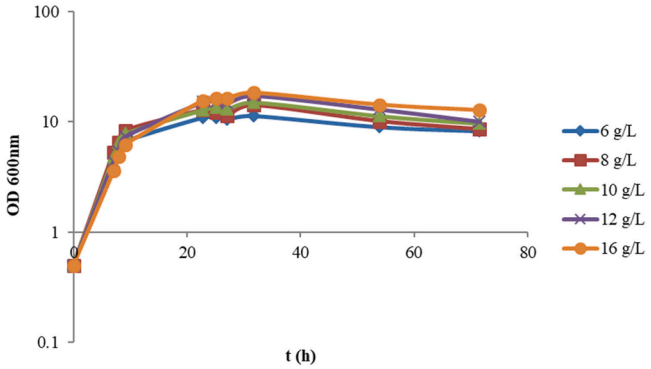

Fig. 1. Growth of *Y. lipolytica* measured as OD_{600nm}, represented on a logarithmic scale, and at different initial concentrations of the mixture of acids.

Table 1
Lag phase (h), growth rate (h^{-1}) and substrates consumption rate (g/L·h) obtained from each assay with different volatile fatty acids concentrations.

Tital initial concentration of acids (g/L)	Lag phase (h)	Growth rate (h^{-1})	Consumption rate (g/L·h)
6	less than 7	0.158	Acetic: 0.310 Butyric: 0.134 Caproic: 0.083
8		0.224	Acetic: 0.357 Butyric: 0.197 Caproic: 0.090
10		0.238	Acetic: 0.502 Butyric: 0.181 Caproic: 0.087
12		0.255	Acetic: 0.601 Butyric: 0.190 Caproic: 0.088
16		0.275	Acetic: 0.664 Butyric: 0.171 Caproic: 0.063

With regard to the substrates, i.e., acids, used in this experiment, several studies have proven the ability of various oleaginous yeasts to grow in the presence of mixtures of mainly short chain VFAs, such as acetic, propionic and butyric acids, as well as the ability to grow in the presence of individual acids. However, it is important not to neglect other isomers and longer chain acids such as iso-butyric, valeric, iso-valeric and caproic acids, among others, as they can sometimes represent more than 30 % of the total VFAs in anaerobic digestion (Llamas et al., 2020b), and, as indicated above, caproic acid has been found in C1-gas fermentation together with acetic and butyric acids (Fernández-Naveira et al., 2017b).

Fig. 2 shows the acid consumption trends obtained with different total initial amounts of acids, ranging from 6 g/L up to 16 g/L. Reaching toxicity levels may trigger both inhibited yeast growth and subsequent poor lipid production. Each of the graphs clearly shows the lag phases experienced by the yeast at different concentrations of acids. Besides, the different consumption curves allow to calculate the substrates consumption rates, which could vary depending on the initial concentration.

As appears in Fig. 1, the lag phases for cell growth were very short and less than 7 h. The same was observed for the profiles of acids consumptions, and at the initial concentrations tested in these assays with *Y. lipolytica*, i.e., up to 16 g/L, the substrates concentrations started dropping already a few hours after inoculation. This agrees with the results reported by Robles-Iglesias and co-workers (Robles-Iglesias et al. 2021), who reported a constant, short, lag phase of about 10 h up to initial concentrations of acetic acid of 16 g/L, while at higher concentrations the lag phase was prolonged, even exceeding 53 h when working at concentrations above 19 g/L.

With the three acids used in this study, some differences in VFA consumption could be observed. In the five bottles, it could be seen that all three fatty acids were metabolised simultaneously. By extrapolating acid consumption data, it was determined that 6 and 8 g/L acid concentrations needed less than 8 and 10 h to consume all acids; 10 and 12 g/L required around 24 h of fermentation, and 16 g/L required about 33 h after inoculation. Besides, different consumption rates (Table 1) were identified for each one of the acids present in the mixture. In all cases, acetic acid showed the fastest consumption rates, followed by butyric acid and finally caproic acid. These results highlight the preference of the oleaginous yeast *Y. lipolytica* for acetic acid over longer-chain VFAs, which can be explained by the different metabolic fates of these acids after their initial assimilation; e.g., only acetate is able to be directly cleaved into acetyl-CoA. These results are also consistent with some previous recent research (Gao et al. 2017; Llamas et al., 2020a; Morales-Palomo et al. 2022).

The maximum consumption rate of acetic acid reached in this experiment was 0.664 g/L·h, found at the highest initial substrate concentration. It should also be noted that in the case of acetic acid, the consumption rate tends to increase as the concentration increases, which may be justified by the absence of inhibition despite the presence of potentially more toxic longer chain acids (i.e, butyric, caproic acids). However, the consumption rates for butyric and caproic acids did not seem to undergo such a same trend, remaining roughly constant, around 0.1–0.2 g/L·h, in the case of butyric acid and not exceeding 0.1 g/L·h in the case of caproic acid, for which a slight decreasing trend can even be seen as the concentration increases, which may be attributed to the higher toxicity of this longer chain fatty acid. Even so, analysing the consumption rates and taking into account the total concentration of all three acids, it could be concluded that the overall rates increased as the total concentration of acids increases, as was also the case with the growth rate. Comparison with other research has shown that, for example, Llamas et al. (2020a), in their study based on the screening of oleaginous yeasts for lipid production from VFAs, also found that for the yeast *Y. lipolytica*, growth rates increased as the concentration of VFAs increased, similarly as in the present study. They also studied other oleaginous yeasts, such as *Cyberlindnera saturnus, Cutaneotrichosporon curvatum*, among others, for which it was more difficult to find a clear trend, though the rates still seemed to increase with increasing substrate concentrations, over the range of values tested. However, for the

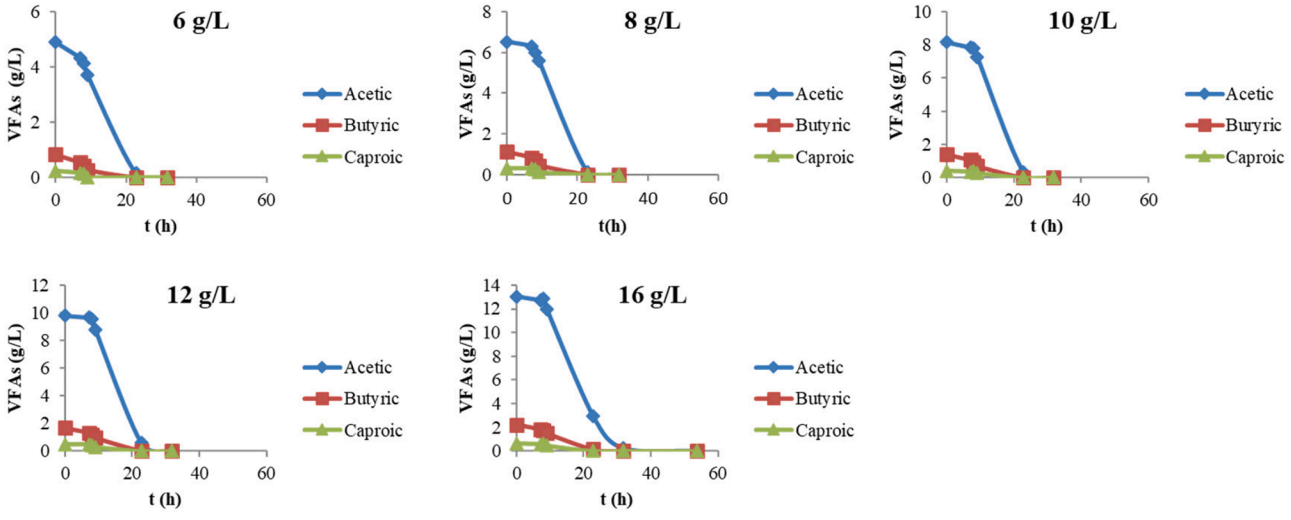

Fig. 2. Consumption of the three acids by *Y. lipolytica* at different total initial acid concentrations.

oleaginous yeast *Rhodotorula toruloides*, the growth rates decreased with increasing VFA concentrations (Robles-Iglesias et al. (2021), and the same was observed for the substrate consumption rate, as it also tended to decrease with increasing acetic acid concentrations.

3.2. Growth, acids consumption and lipid accumulation by Y. Lipolytica in stirred tank reactor (STR) studies

After having carried out the experiments in bottles and demonstrated the ability of *Y. lipolytica* to grow in the presence of a mixture of even chain VFAs typical of the acetogenic fermentation of acetogenic bacteria (e.g., *Clostridium* spp.), the next step consisted in performing a series of bioreactor studies in order to establish suitable conditions for lipid production from even chain C2-C6 fatty acids. Thus, the *Y. lipolytica* strain was grown in bioreactors as described in Material and Methods. In these bioreactor experiments it was decided to remove yeast extract from the culture medium, in order to be able to accurately determine the C/N ratio on starting the assays as well as during the course of the experiment. The C/N ratio is known to be a key parameter, affecting the accumulation of lipids. Yeast extract is a possible nitrogen source, though poorly characterised and defined. Following a similar approach, Egermeier et al. (2017), also decided to eliminate this compound in their research focused on characterising the metabolism of different strains of *Y. lipolytica* on glycerol and at variable pH values, in order to work with less complex and better defined media.

In the first test, a mixture of acids was used, at the same ratio as in the batch studies, and at a total concentration of 10 g/L. In addition, during the preparation of the bioreactor medium, the required amount of NH_4Cl was added so as to reach an initial C/N ratio higher than 40. A zero-time sample was taken in order to determine, analytically, the actual C/N ratio and an actual value of 56 was obtained. In this case, the goal was to work with a relatively high ratio as it has been reported that values above 40 and below 80 are quite suitable for efficient lipid production with most oleaginous microorganisms (Gao et al., 2020). On the other hand, a given amount biomass was inoculated in order to obtain an initial optical density (OD_{600nm}) similar as in the batch studies, i.e., around 0.6.

Fig. 3 shows the optical density measured at 600 nm and the combined consumption over time of the three acids, i.e., acetic, butyric and caproic acids. It can be seen that, despite maintaining similar conditions to those used in the bottle experiments, *Y. lipolytica* faced problems in growing in the bioreactor and, consequently, it did not manage to start assimilating the acids as substrates. This could have been due to the toxic effect of the acids under the operating conditions used in this experiment and also to the absence of yeast extract, which is known to stimulate growth and could have affected the start-up procedure. The stimulating effect of yeast extract on inoculum development and growth of microorganisms, including bacteria, yeasts or fungi, has been reported and may have different effects even depending on the characteristics of preparation of that yeast extract and its composition (Barrette et al. 2001).

In order to find out the reason for this initial failure of biomass growth, and manage to start-up the reactor and draw conclusions, it was decided to dilute the concentration of acids in the reactor, by adding more culture medium, without acids, while also inoculating more biomass into the system. Besides the possible toxic effect of fatty acids, among others in a poorer medium without yeast extract, the inoculum size has also been observed to be able to affect the start-up phase of reactors for lipids production. For example, in a previous study, it has been shown that changing (i.e., increasing) the inoculum size may promote better microbial growth and also initiate lipid production in a more efficient way (Juanssilfero et al., 2018). On the other hand, it was decided to lower somewhat the concentration of VFAs, as above a certain threshold value, they can inhibit cell growth and lipid accumulation, depending also on the characteristics and composition of the culture broth. Furthermore, although in this study the concentration of acids was reduced while maintaining the same ratio between the different acids present in the mixture, it should be noted that *Y. lipolytica* was reported to show a higher resistance to acetic acid than other longer chain acids (Park et al., 2021).

Thus, after diluting the concentration of acids through the addition of more sterilised culture medium, an initial acid concentration of 6.8 g/L was obtained. In addition, the STR was inoculated in such a way that the initial optical density reached a value of 2. These data can be seen in Fig. 4. Besides, the concentration of ammonium was measured again to accurately recalculate the initial C/N ratio. In this respect, it was found that the C/N ratio once the yeast was inoculated (t = 0) was 39, somewhat lower than in the previous trial, as a result of the decrease in the concentration of acids, after dilution of the medium.

The efficient growth of *Y. lipolytica* during the first 90 h of culture after this new start-up phase as well as the consumption of acids can be seen in Fig. 4. In this case, in spite of presenting a rather long lag phase of 40 h, compared to 7 h in the experiments in bottles, it could be observed that under these new conditions the strain was able to efficiently grow with acids as the only carbon source and without yeast extract.

Once this was confirmed, it was decided to set up a second bioreactor in order to determine whether the factor that most influenced growth and substrate consumption, among others, was the initial amount of inoculated biomass or the initial amount of acids present in the medium. With this in mind, this second reactor was operated with an initial acid concentration of approximately 10 g/L, an initial optical density of 2 and a similar initial C/N ratio which appeared to reach 36, as accurately determined analytically. The experiment lasted a total of 400 h, during which samples were taken daily to measure the optical density and acid

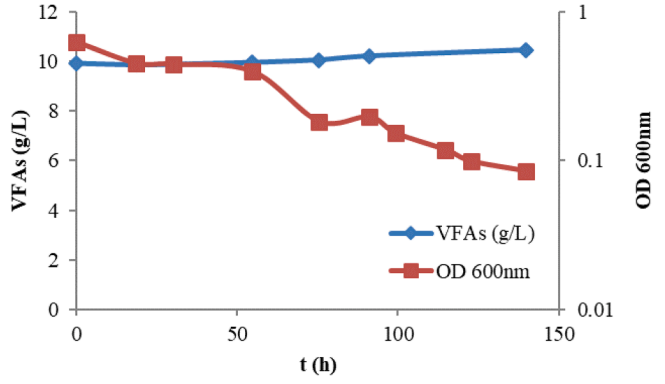

Fig. 3. Growth of *Y. lipolytica* represented on a logarithmic scale and measured as OD_{600nm}, and VFAs consumption in the STR with 10 g/L initial acids and initial OD_{600nm} = 0.6.

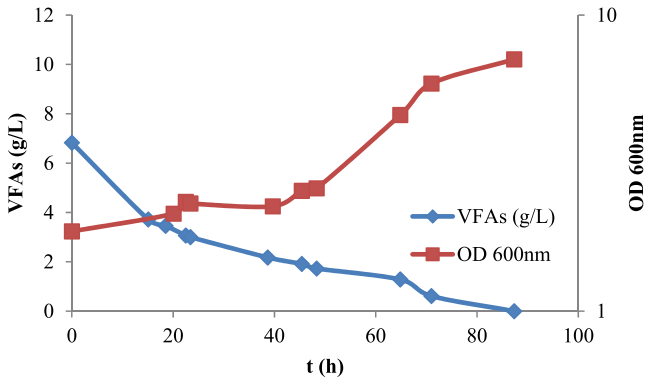

Fig. 4. Growth of *Y. lipolytica* represented on a logarithmic scale and measured as OD_{600nm}, and VFAs consumption in the STR with 6.8 g/L initial acids and initial OD_{600nm} = 2.

consumption. A total of five successive acid additions was applied, as increased amounts of substrates are expected to favour the accumulation of more lipids, while the concentration of nitrogen will progressively decrease as a result of its assimilation. At each new substrate supply, samples were also taken to check the consumption of NH_4Cl (nitrogen source) by *Y. lipolytica* and thus see the progressive increase of the C/N ratio throughout the experimental run. The total amount of acids consumed was 37.7 g/L, at the end of the fifth substrate addition. These results can be seen in detail in Fig. 5.

Fig. 5a shows that in this experiment the lag phase was shorter than in the previous bioreactor test (see Fig. 4), being in this case shorter than 40 h, as the optical density at t = 40 h was higher than 4. This could be explained by the higher activity of the inoculum in the preculture. Indeed, in this assay, a highly active yeast culture, in exponential growth phase was used, expected to be able to grow better and faster in this medium with a higher concentration of VFAs, compared to the previous trial (Fig. 4) in which re-inoculation had been necessary. Before going into the explanation of the growth curve in more detail, it is worth mentioning again that the nitrogen source used is of great importance for both cell growth and subsequent lipid synthesis. In fact, nitrogen depletion in the presence of an excess carbon source has been reported to greatly stimulate the synthesis of lipids. In this study, it was decided to remove yeast extract from the medium, which would be a possible source of organic nitrogen, and to use only NH_4Cl, an inorganic source of nitrogen that is very common for promoting cell growth (Bao et al., 2021). Though some yeasts may only use specific nitrogen sources (e.g, either NH_4^+ or NO_3^-) (Estévez et al. 2005), *Y. lipolytica* was confirmed to efficiently assimilate ammonium chloride. Taking this into account, once the oleaginous microorganism exhausts most of the nitrogen for growth and once nitrogen is no longer available, cell proliferation will drop while the oleaginous yeast continues to assimilate the carbon source, which is now directly directed to lipid synthesis through the accumulation of triacylglycerols (TAGs) intracellularly, in the form of lipid bodies (Beopoulos et al. 2009). Besides biomass growth shown in Fig. 5a, Fig. 5b shows the profile of acids consumed over time. Each of the five successive acid additions were done approximately when the available acids present in the culture medium reached near exhaustion and had dropped to roughly only 2 g/L.

Comparison of Fig. 5a and 5b indicates that *Y. lipolytica* started to grow simultaneously with the initial consumption of the acid mixture. The figures show that each new substrate addition corresponded to some drop in the optical density values. This was due to the formation of rather significant amounts of foam in the culture medium and the partial dilution of the medium as a consequence of the additions of acids as well as some KOH in order to maintain the pH constant at its original value of 6. Related to this, it should be noted that, occasionally, when too much foam was formed, it was decided to modify the stirring rate, which was reduced to 150 rpm, when the addition a few drops of paraffin oil (anti-foaming agent) was not sufficient to remove all foam. The extent of foam formation was, however, progressively less pronounced after each addition and cell growth appeared also to cease after the third addition, remaining approximately constant from 140 h of fermentation onwards. This trend was in line with what was explained in the previous paragraph; reaching a point where the yeast *Y. lipolytica* stops growing and uses the carbon source probably mainly for the accumulation of TAGs. Besides, after sampling for ammonium measurement, before each acid addition, it was found that at time t = 49 h, just before the second acid addition, NH_4Cl had already been fully consumed by the yeast, thus reaching a C/N ratio near ∞.

Focusing now exclusively on Fig. 5b, it can be seen that the consumption slopes become less steep as the fermentation time progresses. For each of the five additions, from the first to the fifth one, the consumption rates encompassing the mixture of the three acids are: 0.299 g/L·h, 0.211 g/L·h, 0.137 g/L·h, 0.07 g/L·h, 0.06 g/L·h. Even the highest, initial, consumption rate in this aerobic bioreactor is quite lower than that obtained in the bottle experiments, also for a concentration of 10 g/L acids, which reached 0.584 g/L·h in those bottle experiments. However, although both cell growth and acid consumption were much more favoured in the batch experiments, the value of 0.299 g/L·h, observed in the bioreactor, was still significantly higher than values reported in other similar studies. For example, Morales-Palomo and co-workers (Morales-Palomo et al. 2022), who carried out batch experiments with the oleaginous yeast *Y. lipolytica* ACA DC 50109, observed that acetic acid led to the highest consumption rates, with the maximum rate being 0.19 g/L·h in one of the tests carried out in a real digestate and an initial acid mixture concentration of 15 g/L. The value obtained in the present research is significantly higher than the one achieved in that study, especially when taking into account that in the latter the result was only calculated in terms of highest consumption rate for acids individually. In batch experiments with *Y. lipolytica* W29, Pereira et al. (2021) found the highest consumption rate, of 0.38 g/L·h, for an initial acetate concentration of 5 g/L, supplied as the only carbon source. On the other side, for studies in two-stage batch cultures, also carried out with *Y. lipolytica* W29, and in all cases using acid mixtures as carbon source (acetate, propionate, butyrate) and glucose as co-substrate (20 g/L), the highest rate obtained was 0.28 g/L·h for a total VFAs concentration of 6 g/L. Finally, in batch culture studies, but carried out in a stirred tank bioreactor, with 6 g/L VFAs mixture and glucose as co-substrate (20 g/L), the highest acids consumption rates were 0.28 g/L·h for *Y. lipolytica* W29 and 0.264 g/L·h for *Y. lipolytica* NCYC 2904. These last two consumption rates are similar to the maximum value obtained in our experiment.

The fatty acid composition of the lipids obtained during cultivation in the third bioreactor (section 3.2) was determined by gas chromatography (GC) after esterification. Fig. 6 shows the amount of lipids obtained in this bioreactor experiment (% g/g) in relation to the concentration of VFAs consumed. The highest lipid content reached is 13.2 % per gram dry weight, for a concentration of 36.9 g/L acids consumed. Fig. 6 clearly shows that increasing lipid contents were obtained as the amount acids consumed by the strain increased, as a result of the successive semi-continuous substrate additions. This could be corroborated by carrying out a statistical study (Minitab Statistical Software), where a

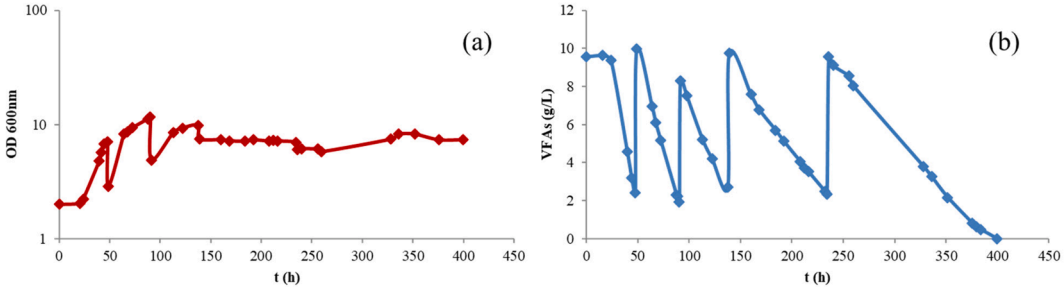

Fig. 5. (a) Growth of *Y. lipolytica* represented on a logarithmic scale obtained during cultivation in the STR with 10 g/L initial total acids and initial $OD_{600nm} = 2$; (b) VFAs consumption by *Y. lipolytica* during cultivation in the STR with 10 g/L initial acids and initial $OD_{600nm} = 2$.

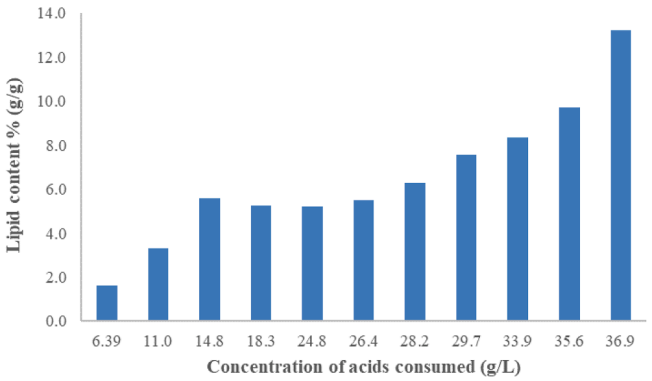

Fig. 6. Effect of the amount of acids consumed by *Y. lipolytica* on the lipid content.

Pearson correlation value of 0.889 was obtained when comparing these two variables, which means that they are highly correlated and, furthermore, the "p" value obtained was p less than 0.05, which means that there is a 100 % probability that these variables are correlated (Obilor et al. 2018). It is highly probable that additional research on optimizing the culture conditions (on-going studies) and increasing the total amount substrate (VFAs) supplied and assimilated will increase the lipid content.

The use of VFAs for lipid synthesis using oleaginous yeasts is considered a very promising alternative to vegetable oils nowadays. However, it is necessary to take into account the possible inhibitory effect when exceeding a certain concentration of acids in the culture medium. Because of this, it is common to find a large number of studies in the literature working with low concentrations of acids and even using co-substrates. In fact, although in the present study the total concentration of acids consumed was close to 40 g/L, different limited substrate additions were carried out in semi-continuous mode, permanently remaining below possible threshold inhibitory values (Fig. 5b), in order to avoid toxic effects. Pereira et al. (2021) carried out a study based on analysing the factors that affect lipid synthesis by *Y. lipolytica* from VFAs, studying the mode of operation and the effect of co-substrates, among others. It can be highlighted that in their bioreactor experiments, where, besides using a mixture of VFAs (6 g/L), in this case 2 g/L acetate, 2 g/L propionate and 2 g/L butyrate, they also used glucose (20 g/L) in the culture medium, they reached a lipid content of 11.5 % per gram of dry weight (see supplementary material), which is comparable, though slightly lower, to our study, in which we supplied acids as single carbon sources. Considering that the enhancement of cellular growth, through addition of glucose, leads to an increase of lipids production (Pereira et al., 2021), the results obtained in the present work are quite promising. Besides, this is the first study assessing the potential to use even chain C2-C6 carboxylic acids from C1-gas (e.g., syngas, CO_2) valorization. Table 2 shows these and other parameters of growth and lipid production, which are of interest for comparison with research of Pereira et al. (2021) (see supplementary material), in which one of the strains used is the same as in the present work, i.e. *Y. lipolytica* W29. As for the lipid concentration, the maximum value reached was 0.30 g/L, corresponding to a concentration of 36.9 g/L acids consumed. This value is higher than the one obtained in one of the bioreactor trials described by Pereira et al. (2021), i.e., 0.20 g/L. However, in the same experiment, the authors also managed to reach concentrations higher than 1 g/L under conditions of higher volumetric mass transfer of oxygen, a factor that therefore seems to greatly affects lipid accumulation. It should also be mentioned that in the study of Pereira et al. (2021), the culture medium used was quite different from the present study, as it contained, among others, yeast extract and also YNB (without amino acids and ammonium sulfate), the latter being highly optimized for yeast growth and activity by providing nutrients reported in the literature to have been developed specifically for yeast cultures. In contrast, in our research focusing on C1-gas valorization, a culture medium optimized for the growth of anaerobic *Clostridium* spp. was tested, because the future prospects are the conversion of pollutant gases into lipids (biodiesel) through a first anaerobic fermentation with acetogenic bacteria and a second aerobic fermentation carried out with oleaginous yeasts, based on the bioconversion of the VFAs obtained in the first stage, into lipids. When checking the literature (data not shown), it can be observed that *Y. lipolytica* is a species considered to efficiently accumulate lipids, though the strain used in this work, i.e., *Y. lipolytica* W29, does actually generally accumulate lower amounts than other strains. Thus, further studies will now also be planned and started with other species and other genera, in order to optimize these already promising results.

Table 2
Values of biomass yield ($Y_{X/S}$), lipid yield ($Y_{L/S}$), lipid content and lipid concentration obtained in *Y. lipolytica* W29 using a mixture of VFAs as the sole substrate mixture.[a]

Carbon source	Culture mode	Initial ratio C/N	Lipid content % (g/g)	$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)	Lipid concentration (g/L)
VFAs (6.39 g/L)	Fed-batch	36	1.6	0.34	0.005	0.04
VFAs (11.0 g/L)			3.3	0.23	0.008	0.08
VFAs (14.8 g/L)			5.6	0.19	0.011	0.16
VFAs (18.3 g/L)			5.3	0.13	0.007	0.12
VFAs (24.8 g/L)			5.2	0.09	0.004	0.11
VFAs (26.4 g/L)			5.5	0.09	0.005	0.13
VFAs (28.2 g/L)			6.3	0.08	0.005	0.14
VFAs (29.7 g/L)			7.6	0.06	0.005	0.14
VFAs (33.9 g/L)			8.4	0.07	0.006	0.19
VFAs (35.6 g/L)			9.7	0.07	0.007	0.23
VFAs (36.9 g/L)			13	0.06	0.008	0.30

[a] DCW = Dry Cell Weight.

Finally, the lipid composition is provided in Table 3. Most of the accumulated fatty acids are of 16 and 18 carbons-length, highly similar to vegetable and soybean oil chains. Specifically, the final lipid composition obtained, in decreasing order, was as follows: oleic (C18:1) > linoleic (C18:2) > palmitic (C16:0) > stearic (C18:0) > Others. However, it should be noted that linoleic acid, which is very suitable for biodiesel production, becomes the predominant acid after having fed and consumed 18 g/L fatty acids, reaching in that case up to 38 %. In contrast, linoleic acid starts to dominate at the beginning of cultivation, with a maximum percentage of 50 % for 6 g/L substrate consumed, and then it starts to decrease, in contrast to C18:1. As for palmitic acid, the percentages are in all cases close to 20 %, being also very similar to some already reported values in the literature. Finally, the C18:0 percentages achieved in the present study are somewhat lower than those obtained in

Table 3
Relative fatty acid composition of *Y. lipolytica* at different concentrations of consumed VFAs.

Concentration of acids consumed (g/L)	Relative amount of total fatty acids (%, g/g)				
	C16:0	C18:0	C18:1	C18:2	Others
6.39	16.1	0.0	26.5	49.7	7.7
11.0	15.4	0.0	37.0	41.7	5.9
14.8	19.5	7.1	31.9	33.9	7.7
18.3	18.1	7.6	35.3	32.4	6.5
24.8	17.7	8.4	37.6	30.1	6.2
26.4	19.6	8.0	36.0	30.6	5.9
28.2	18.4	8.4	37.2	28.9	7.2
29.7	17.7	9.4	36.4	29.3	7.2
33.9	18.8	9.8	36.2	27.5	7.7
35.6	18.1	11.0	35.0	26.3	9.5
36.9	19.1	9.2	37.8	27.4	6.4

other published studies.

In general, despite the need to improve the efficiency of lipid production from an acetogenic bacterial medium using oleaginous yeasts, the lipid composition achieved in our research (mostly unsaturated fatty acids) indicates that metabolites of great industrial interest, in this case lipids, can be obtained from different acids present in a mixture, thus conforming a technique with great potential for the manufacture of biofuels, e.g., biodiesel.

4. Conclusions

The production of lipids by oleaginous yeasts (microbial oils) is a promising alternative to vegetable oils. Besides, carboxylic acids are a more cost-effective alternative compared to other, more conventional, substrates, such as sugars. This study is the first one proving the ability of *Y. lipolytica* to assimilate even chain C2-C6 fatty acids from C1-gas valorization, reaching similar growth and lipid patterns as with sugars and optimized media for yeasts. Thus, additional optimization of aspects such as, among others, the culture conditions or the yeast strain, warrants the successful further development of the proposed technology.

CRediT authorship contribution statement

Cecilia Naveira-Pazos: Investigation, Data curation, Writing – review & editing. **María C. Veiga:** Supervision, Resources, Funding acquisition, Writing – review & editing. **Christian Kennes:** Conceptualization, Supervision, Funding acquisition, Project administration, Methodology, Data curation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127649.

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