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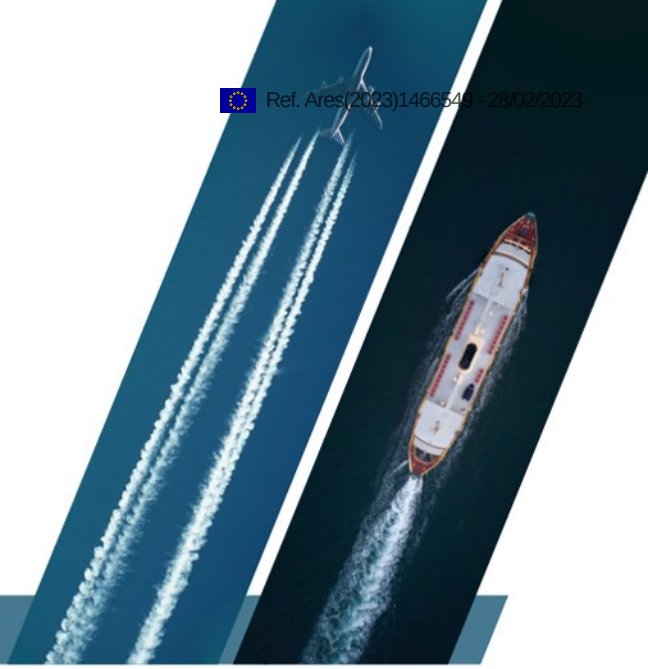
Biofuel for biotravels



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Deliverable 3.5

Optimization of acetate fermentation process parameters for C14 and C16-18 TAGs production (2nd stage)

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Abbreviations

AA	Acetic acid
BBEPP	Bio Base Europe Pilot Plant
CDW	Cell dry weight
DO	Dissolved oxygen
HPLC	High pressure liquid chromatography
GC-MS	Gas chromatography-mass spectrometry
Na-acetate	Sodium acetate
NH_4-acetate	Ammonium acetate
NaOH	Sodium hydroxyde
$(NH_4)_2SO_4$	Ammonium sulfate
OD_{600}	Optical density at 600 nm
SF	Shake flask
TAGs	Triacylglycerides
vv	Vessel volumes per minute
WT	Wild-type
YPA	Yeast extract peptone acetate
YPD	Yeast extract peptone dextrose

Contents

1 Executive Summary

Deliverable 3.5 “Optimization of acetate fermentation process parameters for C14 and C16-18 TAGs production (2nd stage)” is a public document of the BioSFerA project, delivered in the context of WP3 “Biological production of lipids from syngas at lab scale” and more specifically in the task 3.4 “Optimization of acetate fermentation process parameters for C14 and C16-18 TAGs production (2nd stage) using metabolically engineered oleaginous yeasts”.

This Deliverable contains only the public version of the results achieved in task 3.4. The rest of the results are presented in much more detail in the confidential version of D3.5.

The most suitable metabolically engineered oleaginous yeast strains developed by CSIC were tested and validated by CARTIF and BBEPP. For this purpose, not only batch and fed-batch fermentation processes but also continuous fermentations with cell recycle, fed with a diluted acetate solution and decoupled from the previous gas fermentation process, were carried out at lab scale using bench scale bioreactors of 1.5L and 7L.

Impact of process parameters, feed rates, growth rates and conversion rates (g/g), titers (g/L), volumetric productivity (g/L/h) as well as the fatty acid composition of TAGs produced were evaluated to select the most promising combinations in terms of economics and maximization of the performance of the overall process requirements for further upgrading at pilot plant scale in WP4.

Since the results of lipid production using acetic acid as the sole carbon source were not very high and did not meet expectations (despite having modified different process variables and using a modified *Yarrowia* strain obtained by the CSIC), different alternatives were proposed in order to increase the lipid production yield and reach the final target set for WP5 and for the subsequent purification and hydrogenation processes.

In this regard, two alternatives have been proposed:

-Use of glucose instead of acetate as an alternative carbon source to increase the yield of lipid production. *Yarrowia* metabolises glucose much more easily to generate lipids, as it is an ideal carbon source to generate pyruvate and then acetyl CoA, which is a key precursor involved in TAGs biosynthesis, and also under nitrogen starvation conditions the yield of lipid accumulation is much higher.

-Using a co-substrate such as glycerol together with acetate to facilitate the generation of TAGs (a TAG molecule consists of glycerol + three fatty acids).

In both cases, fermentation conditions were optimised at laboratory level to determine the most important parameters influencing lipid accumulation (pH, DO, C/N ratio).

Glucose is a better carbon source for lipid production than acetate, resulting in a titer of 20 g/L compared to 10 g/L for acetic acid using the modified strain *Y. lipolytica* DGA (CSIC-2). The use of glycerol as co-substrate together with acetate resulted in similar TAGs accumulation (%) but higher biomass concentration and therefore higher lipid titer (16.9 g/L).

The produced microbial TAGs mainly consist of fatty acids like oleate, (C18.1) stearate (C18) and palmitate (C16). A similar profile was observed for all cases, meaning that the difference in fermentation settings, the use of a different strain, and the use of glucose instead of acetate did not considerably change the product composition. This fact is crucial for the TAG hydrotreatment process which will be investigated in BioSFerA WP5.

2 Introduction

The main objective of WP3 is to develop a two-stage biological gas-to-liquid process for the conversion of CO2/CO/H2 produced by gasification into medium (C14) and long (C16-18) chain triacylglycerides (TAGs) that will be upgraded to biofuels.

Regarding the Task 3.4, the main aims are:

- To study the influence of the acetate concentration and medium composition in order to optimize the performance of the liquid fermentation stage and optimize the operating conditions for a high TAGs productivity.

- To optimise the operation conditions of the fermentation processes at lab scale: TAGs titer [50-100 g/L], productivity: [0.26 g/L/h]

The production of lipids from acetate has been described in different microbial species. This is a high yield process since acetate presents efficient conversion to acetyl-CoA in the cell cytoplasm by the acetyl-CoA synthetase. So far, the most efficient microorganisms in carrying out this conversion are the oleaginous yeasts such as *Yarrowia lipolytica* which have a well-suited metabolism for TAGs biosynthesis when grown under nutrient-limited conditions. In order to increase even more the lipid concentration, yield and acetate conversion, a metabolic engineering strategy of *Y. lipolytica* have been adopted (Task 3.2.2) to obtain recombinant strains capable of expressing the *dga1* gene from *Rhodosporidium toruloides*, the *dga2* gene from *Claviceps purpurea*, and deleting the *mfe1* gene which is involved in fatty acid β-oxidation in the peroxisome.

To fulfil the main aims of Task 3.4, Deliverable 3.5 considers to study the influence of the acetate concentration and medium composition in order to optimize the performance of the liquid fermentation stage and optimize the operating conditions for a high TAGs productivity. It is very important to optimize the fermentation parameters considering different variables since these process conditions largely affect the growth and productivity of the yeast strains.

Acetate fermentation for TAGs production was studied independently from gas fermentation, although a dilute acetic acid solution (3-6 %) was studied as feedstock, in order to mimic the low-strength acetic acid stream obtained from acetogenesis. This represents a challenge for high biomass and lipid production. Work was performed with the *Y.lipolytica* wild-type (WT) strain and with the modified obese strain DGA (CSIC-2) provided by CSIC.

Several parameters have been controlled in bioreactor fermentation: pH, DO, feed flows, stirring, temperature, C/N ratio, etc. The operation in three different modes has also been studied: batch, fed-batch and continuous. As reference parameters, indicative of fermentation quality, TAG content (%), TAG concentration (g/L) and TAG productivity (g/L/h) were considered.

This document will therefore explain the main developments and results obtained.

3 Shake flasks experiments (CARTIF)

In order to test whether the oleaginous yeast strain *Y. lipolytica* W29 (WT) is able to grow in a culture medium containing acetate as the sole carbon source, a growth assay in shake flasks was carried out, testing different concentrations of acetate in the medium (10, 30, 50 and 100 g/L). The oleaginous yeast *Y. lipolytica* WT was provided as an active culture on a yeast extract-peptone-dextrose (YPD) (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract) agar plate by CSIC.

The starter culture for the experiments was set up by inoculating the strain from an active YPD agar plate. The medium contained 50 mL of YPD to rapidly accumulate cell density. After 24 h, 2.5 mL of the cultures were transferred to 250 mL shake flask (100 mL total volume) cultures containing variable concentrations of sodium acetate, 1.34 g/L ammonium sulfate, and 1.7 g/L YNB-AA-AS to adapt the cells and synchronize growth.

The cultures were kept in an incubator with orbital shaking at 30°C and 200 rpm for 3 days and the optical density was measured at 600nm with a spectrophotometer, as an indicator of microbial growth. The following figure shows the results of the growth assay (Figure 1):

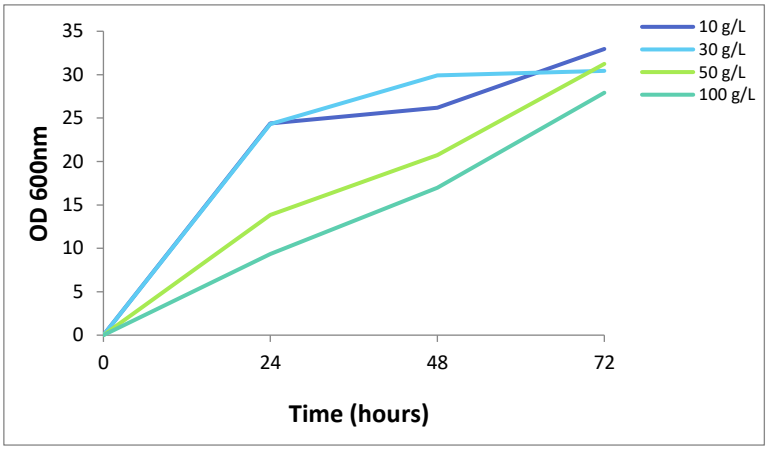

Figure 1: Growth of *Y.lipolytica* WT using different acetate concentrations.

Y. lipolytica W29 WT strain was able to grow well in all acetate concentrations tested, indicating that it is highly tolerant to acetate concentrations and is able to metabolise this compound efficiently to produce biomass.

4 Shake flasks experiments (BBEPP)

4.1 Cultivation conditions and sampling

All shake flask cultivations were performed at 28 °C and flasks were stirred at 200 rpm in an incubator with an orbit of 1.9 or 2.5 cm for 250 or 500 mL shake flasks, respectively. The biomass growth was determined by my measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer. The pH was measured using semi-quantitative color indicator strips.

4.2 *Y. lipolytica* W29 (WT) growth on acetate

For preliminary experiments, BBEPP used the commercially available wild-type (WT) strain *Y. lipolytica* W29. This oleaginous yeast was provided as an active culture on an agar plate by CARTIF. In order to verify the ability of *Y. lipolytica* WT to assimilate acetic acid, an exploratory growth experiment was conducted. Therefore, 500 mL shake flasks were filled with 100 mL of YPA medium (Annex 1) and inoculated with 3 mL of a growing *Y. lipolytica* WT culture in YPD medium (Annex 1). Samples were taken at regular intervals. The results are presented in Figure 2.

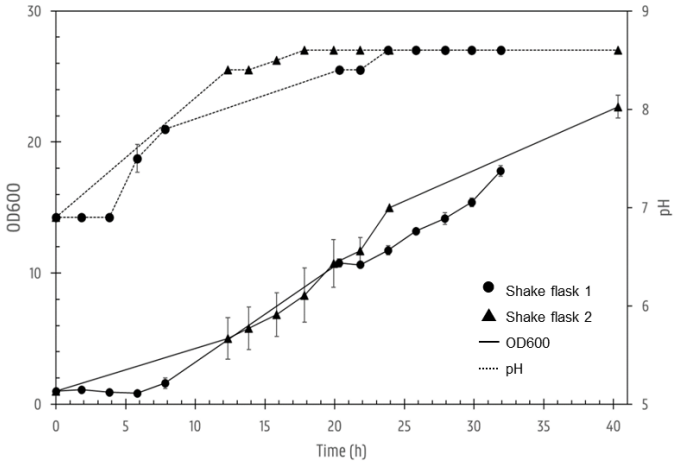

Figure 2: Results of growth and pH for the cultivation of *Y. lipolytica* WT on YPA medium.

The *Y. lipolytica* WT strain successfully consumed the acetate present in the medium. Indeed, acetic acid can be directly transformed into acetyl-CoA, a key molecule in the central metabolism (as described in BioSFerA Deliverable 3.3). The biomass increased to an OD_{600} of 23 with a maximum specific growth rate of 0.12 h^{-1}. Moreover, while acetate was consumed, an increase in pH was observed up to 8.5. This can be explained by the fact that upon acetate consumption, a proton has to be imported into the cell to maintain its charge balance, which causes the pH of the medium to rise.

4.3 *Y. lipolytica* DGA (CSIC-2) growth on gas fermentation effluent

To validate the BioSFerA coupled fermentation concept, BBEPP performed an additional shake flask experiment in which the growth of *Y. lipolytica* on acetate produced by *M. thermoacetica* was evaluated. The modified *Y. lipolytica* DGA (CSIC-2) strain prepared by CSIC within BioSFerA Task 3.2 was used.

Gas fermentation effluent, containing 3% acetate, was obtained from the continuous fermentation with cell-recycle performed within BioSFerA Task 3.3, (as described in BioSFerA Deliverable 3.4). This was compared to the standard medium (YPA, control) as well as the effluent with additional nutrients.

It was observed that biomass production of *Y. lipolytica* CISC-2 was similar. It could thus be concluded that no inhibiting compounds were present in the gas fermentation effluent stream, showing that no acetate purification is required and that direct coupling of the two fermentation processes, as envisioned in the BioSFerA concept, is feasible.

5 Fermentations at 1.5 L scale (CARTIF)

5.1 Equipment and fermentation set-up

CARTIF investigated the production of TAG by *Y. lipolytica* in bench-scale 1.5 L fermentation bioreactors (Bionet and Applikon) with an initial volume of 0.5 L (Figure 3). Bioreactor cultures were maintained at a temperature of 28 °C and a pH of 7.0 through the addition of 10 wt% sulfuric acid. The aeration rate was 1 vvm and the DO level was maintained at 20% through agitation. 100 µL 20 vol% Antifoam 204 (Sigma-Aldrich) were added at 6 and 24 h after inoculation to prevent foaming.

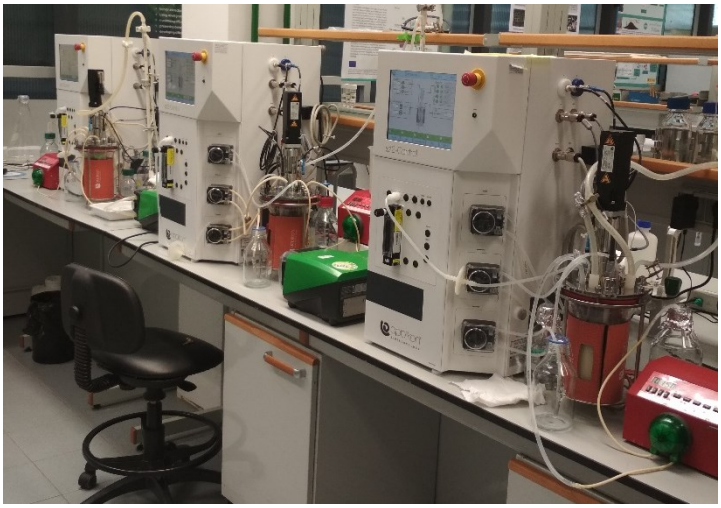

Figure 3: Bench-scale 1.5 L fermentation bioreactors for TAGs production at CARTIF.

Fermentation samples were collected at different time intervals in order to evaluate parameters such as growth rate, lipid titer (g/L), lipid productivity (g/L/h), total lipid content (%), fatty acid composition of produced TAGs, acetate consumption (g/L) and citrate production (g/L).
The biomass production was assessed by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer and by gravimetrical determination of the cell dry weight (CDW) (g/L). The TAG concentration was determined by gas chromatography-mass spectrometry (GC-MS) analysis. The concentrations of acetate, glucose, citrate, and succinate were measured by HPLC analysis. All these methods are described in more detail in the Annexes.

5.2 Batch fermentations with *Y. lipolytica* W29 (WT) on acetic acid

In order to study in more depth the characteristics of the different process conditions, different fermentations were carried out in bioreactors using acetate as a carbon source and following a batch strategy, the simplest one.

First, a preliminar design of experiments was carried out to study the influence of three parameters that are of great importance during the fermentation process: initial acetate concentration in the culture medium, dissolved oxygen and pH. For each of these parameters, two reference values (low and high) were selected. They are described in more detail in the following Table 1:

Parameter	Low level	High level
pH	5.5	8
C_{Ac}^0 (g/L)	15	50
DO (%)	0	20

Table 1: Parameters tested in the study.

The trials were carried out using 1.5 L lab-scale bioreactors (Bionet) and the fermentation conditions were kept constant with the following parameters:

-Strain: *Y. lipolytica* W29 (WT)
-Working volume of the reactor: 0.8 L
-Operation mode: Batch, no cell recycling
-Inoculum size: 5%, OD_{600nm} 1.0.
-Temperature: 28 ºC
-Agitation: 600 rpm
-Time: 96 hours

The initial medium was composed of 30 g/L sodium acetate, 2.5 g/L yeast extract, 4.2 g/L yeast nitrogen base (without amino acids and ammonium sulfate), and 2.4 g/L ammonium sulfate (C/N molar ratio of 20). The pH and DO levels were monitored using a pH probe and a DO probe.

During the different fermentations, pH control was carried out using a solution of 10% (v/v) glacial acetic acid and a solution of 2M NaOH.

Figure 4 shows a picture of the bioreactors used for the fermentations.

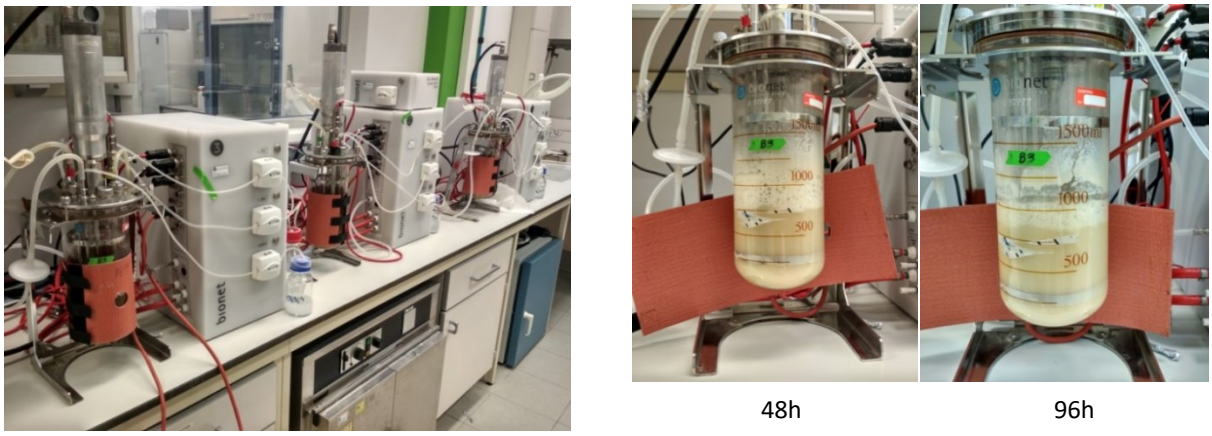

Figure 4: Detail of bench-scale bioreactors used at CARTIF.

The following Table 2 shows the results of the different fermentations carried out.

Name	pH	DO (%)	Acetate (g/L)	CDW (g/L)	TAGs (g/L)	TAGs/CDW (%)	qTAGs (g/L/h)
1	5.5	20	15	7.93	1.418	19.85	0.014
2	5.5	20	50	8.53	1.358	16.29	0.014
3	8	20	15	13	3.04	12.79	0.031
4	8	20	50	17.4	3.479	19.45	0.036
5	8	0	15	1.198	0.1	8.55	0.0011

Table 2: Summary of fermentations testing different values of pH, acetate and DO.

As can be seen, 3.48 g/L lipids were achieved with a productivity of 0.036 g/L/h, although these values were far from the target values (50-100 g/L and 0.26 g/L/h, respectively). Graphs of the evolution of biomass production, lipid titer and lipid content during F4 are shown below in the Figures 5 and 6.

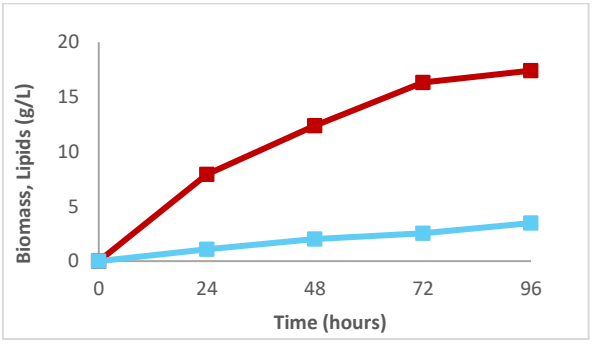

Figure 5: Time course of biomass production (red) and lipid titer (blue) in F4.

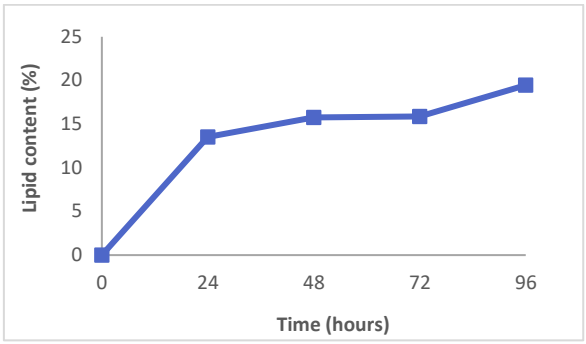

Figure 6: Time course of lipid content in F4.

Acetate consumption and citrate production were also monitored during the fermentations. Figure 7 shows the concentration of both compounds.

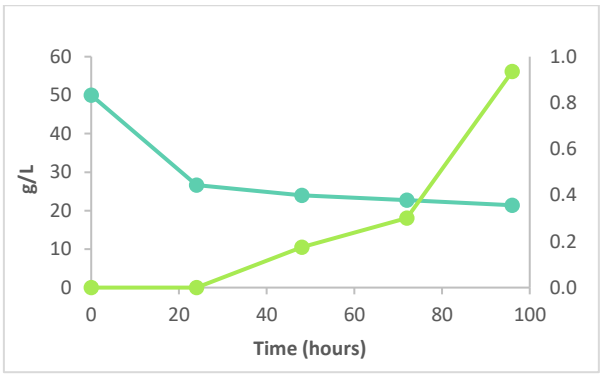

Figure 7: Evolution of acetate (dark green), citrate (light green) concentrations in F4. Left axis represents acetate concentration (g/L) and right axis represents citrate concentration (g/L).

With regard to the lipid profile of fatty acids, no significant differences were detected between the fermentations carried out. The major lipids were C16:0, C18:1 and C18:0. An example of F4 is shown in Figure 8. It can be seen how C18:2 decreased over the course of fermentation while C18:0 increased.

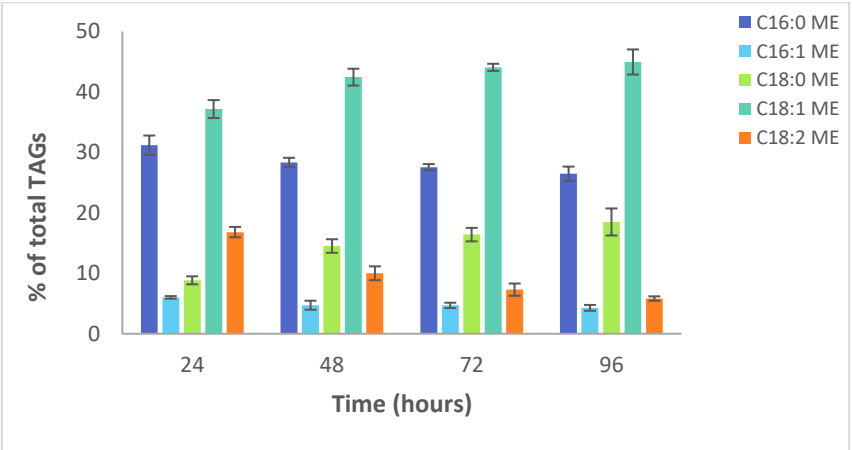

Figure 8: Fatty acid distribution obtained in F4.

In addition, it was observed that DO is an essential condition for the correct growth of *Y.lipolytica* WT since in the absence of aeration there was very limited growth and lipid production was practically non-existent.

Subsequently, the best performing conditions were used and replicated for a longer period of time (168 hours), as most of the fermentations showed a tendency to increase lipid production over time. Results of these fermentations are shown below in Table 3:

Name	pH	DO (%)	AA (g/L)	CDW (g/L)	TAGs (g/L)	TAGs/CDW (%)	qTAG (g/L/h)
6	8	20	15	17.38	5.91	33.84	0.036
7	5.5	20	50	26.77	3.58	13.36	0.025

Table 3: Summary of the results of the second round of fermentations.

The best results in terms of lipid content (33.84 %) and lipid titer (5.91 g/L) were obtained with the conditions of pH 8, 15 g/L acetate and DO 20%, for 168 hours. A pH of 5.5 may be too low for a fermentation with sodium acetate, especially in high concentration (50 g/L). Acetate would change to its acid form and could be harmful to the cells.

Graphs of the evolution of biomass production, lipid titer and lipid content during F6 are shown below in Figures 9 and 10.

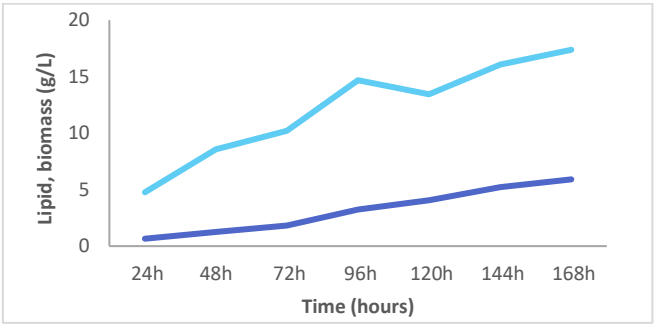

Figure 9: Time course of biomass production (light blue), lipid titer (dark blue) in F6.

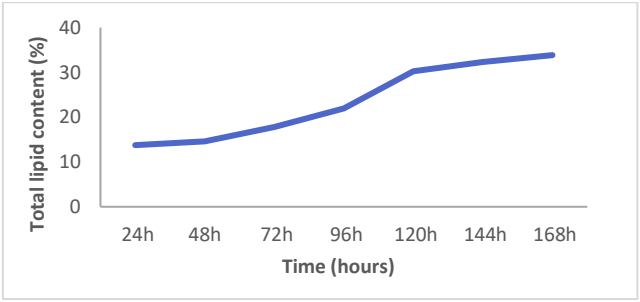

Figure 10: Time course of lipid content in F6.

Acetate consumption and citrate production were also monitored. Figure 11 shows the concentration of both compounds.

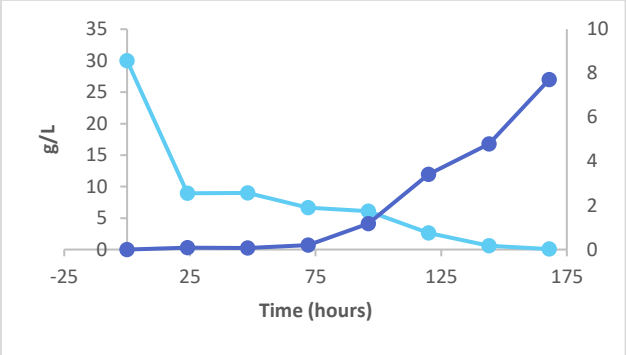

Figure 11. Evolution of acetate (light blue), citrate (dark blue) concentrations in F6. Left axis represents acetate concentration (g/L) and right axis represents citrate concentration (g/L).

The lipid profile achieved was similar to the previous ones, as shown in Figure 12. Measurement of C14 fatty acids was also included in these analyses (it can be seen that the percentage of fatty acids of this type is very low).

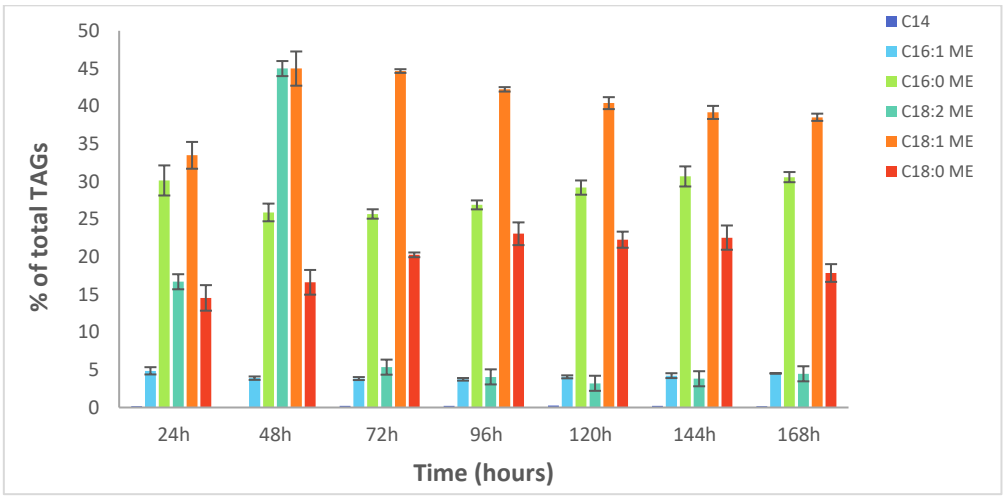

Figure 12: Fatty acid distribution obtained in F6.

With these results, the basis for optimising the fermentation process was established. The next step was to investigate new modes of operation (continuous with cell recycling) as well as to study the influence of feed solutions (different C/N ratios depending on the fermentation stage) on lipid production.

5.3 Continuous fermentations (with cell recycle) with *Y. lipolytica* WT and DGA (CSIC-2) on acetic acid

Fermentation following a continuous strategy was applied using *Y. lipolytica* W29 (WT) and *Y. lipolytica* DGA (modified) strains. The fermentations were carried out in 1.5 L bioreactors (Applikon). The method of operation was based on an initial fed-batch fermentation stage and a successive continuous fermentation stage. The initial volume of medium was 0.5 L. Substrate was fed until a final volume of 1.5 L was reached (fed-batch operation). At this point, substrate was continued to be fed and continuous operation was started, maintaining a constant volume inside the reactor through continuous extraction of a cell-free broth stream. Some parameters were kept constant during fermentation: temperature (28 °C), stirring (600 rpm), inoculum size (10 %, OD 600 nm = 1), and initial volume (0.5 L). The culture medium was Hu medium (composition shown in the Annexes).

The substrate solution to be fed contains a carbon source, acetic acid in most cases, and a nitrogen source, ammonium sulfate. During the fed-batch stage, a relative low C/N ratio was used so that there was sufficient nitrogen to ensure the growth of the microorganisms. In the continuous stage, the nitrogen concentration was decreased so that the ratio became, in most cases, C/N = 102. Limiting the presence of nitrogen in the medium results in cell stress and thus in increased TAGs accumulation.

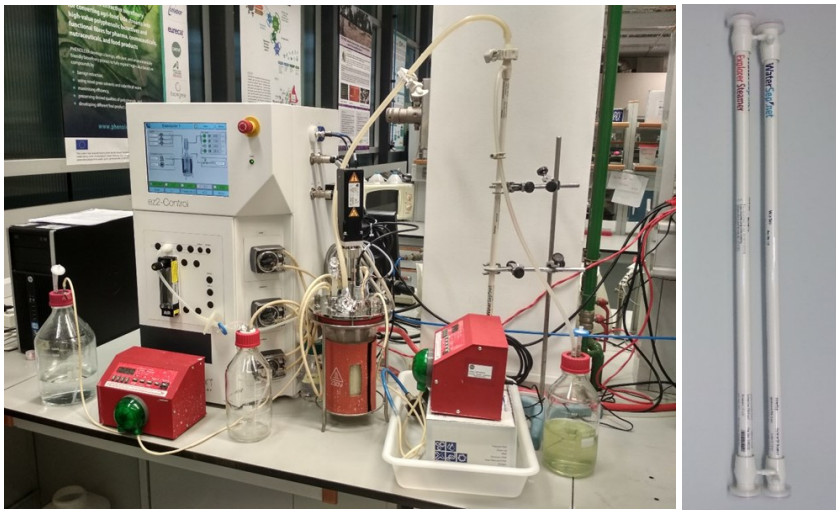

Figure 13: 1.5 L bioreactor set-up for continuous mode. Hollow fiber cartidges 24", 0.5mm ID (Watersep, Sartorius) used.

Figure 13 shows the bioreactor configuration for continuous operation. The substrate solution is continuously pumped and introduced into the bioreactor. A cell solution is removed from the bioreactor also in continuous operation and sent to a tangential microfiltration membrane (Watersep Explorer 24, Sartorius). The retentate stream, which contains the cells, is recirculated into the bioreactor. The permeate stream is withdrawn at a flow rate such that the volume in the bioreactor remains approximately constant. Thanks to the stirring system, the composition of the permeate stream is at all times similar to the composition inside the bioreactor. This way of operating allows the processing of large volumes of substrate, thereby lengthening the fermentation process and thus presumably obtaining a higher concentration of product.

Regarding the chemical analysis, in each fermentation the liquid samples were characterized by HPLC determining the concentration of the components present, among them acetate, citrate, glucose and glycerol. In addition, the biomass concentration (g/L) was determined gravimetrically and with this information and the GC analysis the TAGs content (%) was determined.

TAGs were analysed by GC-MS to determine the TAGs profile and total concentration.

Table 4 shows a summary of the results of the fermentations that have been performed with the continuous mode on acetic acid or derivatives. First, the wild-type strain *Y. lipolytica* W29 was used (F1). Then, modified strain *Y. lipolytica* DGA was employed and the effect of different parameters was studied:

- Different substrate feeding flow rate (F2 and F3).
- Modification fo the carbon source in the form of sodium acetate (F4) and acetic acid combined with glycerol (F5).
- Modification of the nitrogen source in the form of ammonium acetate (F6).
- Combined strategy: increase in carbon source concentration, decrease in dissolved oxygen and decrease in pH (F7).
- Decrease in the presence of nitrogen (F8).
- Combination of different carbon sources to increase biomass growth (F9).

	Feed 1	Feed 2	Feed flow (mL/min)	DO (%)	pH	Biomass (g/L)	Citrate (g/L)	TAGs (%)	TAGs (g/L)	TAGs yield (g/g), Prod (g/L/h)
F1	3 % AA 5 g/L $(NH_4)_2SO_4$ C/N=13	3 % AA 0.65 g/L $(NH_4)_2SO_4$ C/N=102	0.3	30	7	7.9	n.d.	29.1	2.3	0.20
F2	3 % AA 5 g/L $(NH_4)_2SO_4$ C/N=13	3 % AA 0.65 g/L $(NH_4)_2SO_4$ C/N=102	0.25	30	7	23.0	n.d.	22.6	5.2	0.14, 0.04
F3	3 % AA 5 g/L $(NH_4)_2SO_4$ C/N=13	3 % AA 0.65 g/L $(NH_4)_2SO_4$ C/N=102	0.88-1.6	30	7	9.8	n.d.	25.2	2.5	0.06, 0.03
F4	3 % NaAc 5 g/L $(NH_4)_2SO_4$ C/N=13	3 % NaAc 0.65 g/L $(NH_4)_2SO_4$ C/N=102	0.4-0.6	30	7	25.9	n.d.	24.0	6.2	0.13, 0.04
F5	3 % AA 3 % glycerol 5 g/L $(NH_4)_2SO_4$ C/N=26	3 % AA 3 % glycerol 0.65 g/L $(NH_4)_2SO_4$ C/N=204	0.4-0.6	30	7	67.5	11.8	25.1	16.9	0.28, 0.10
F6	3 % AA 5 g/L NH_4Ac C/N=17	3 % AA 0.4 g/L NH_4Ac C/N=113	0.3	30	7	16.4	0.1	24.9	4.1	0.14, 0.07
F7	6 % AA 6 % glycerol 18 g/L $(NH_4)_2SO_4$ C/N=14	6 % AA 6 % glycerol 2.6 g/L $(NH_4)_2SO_4$ C/N=100	0.15	30→5	From 7 to 3 (fast)	26→16.2	0.8	12.0	1.9	0.04, 0.03

F8	3 % AA 3 % glycerol 0.65 g/L $(NH_4)_2SO_4$ C/N=204	3 % AA 3 % glycerol C/N = inf	0.09-0.12	30→5	From 7 to 2.5 (slow)	27.8	0.4	24.1	6.7	0.27, 0.06
F9	- (100 g/L glucose in basal medium)	6 % NaAc 6 % glycerol	0.1	30→10	7	90.5	31.2	21.9	19.8	0.177, 0.261

Table 4: Summary of fermentations in continuous mode on acetic acid carried out at CARTIF.

DO: dissolved oxygen, AA: acetic acid, $(NH_4)2SO_4$: ammonium sulfate, NaAc: sodium acetate, NH_4Ac: ammonium acetate, Prod: productivity.

Acetate fermentations with *Y. lipolytica* WT strain

The **F1** fermentation consisted of a preliminary dilute acetic acid (3 %) fermentation trial with wild-type commercial strain of *Y. lipolytica* W29. A C/N = 13 was used in first stage of the fermentation and C/N = 102 in the second stage. The feed flow rate, 0.3 mL/min, was such that fed-batch stage lasted 2-2.5 days, followed by continuous operation until the end of the fermentation. Total biomass production was moderate, 7.9 g/L, with a TAGs content of 29.1 % and thus a TAGs concentration of 2.3 g/L, far below the target. The fatty acid profile can be seen in Figure 14. In this case the most abundant were C18:1 and C18:0, while in the batch operation they were C18:1 and C16:0. In this case C16:0 me was decreasing along the fermentation while C18:0 was increasing. TAGs yield was defined as the amount of TAGs produced over the amount of substrate consumed, including acetate, glycerol and glucose. The maximum TAGs yield during F1 was 0.20 g/g.

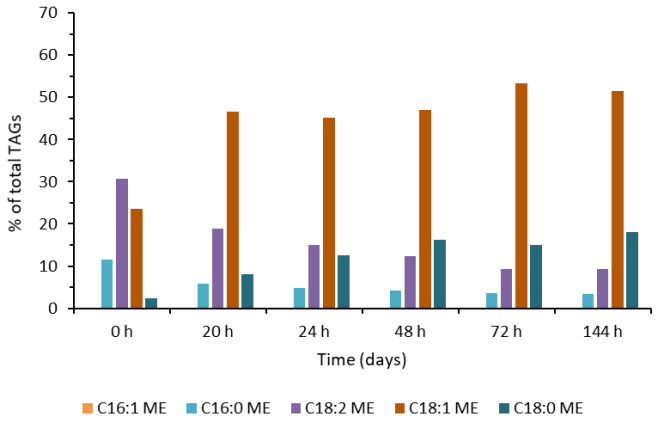

Figure 14. Fatty acid distribution obtained in F1.

Acetate fermentations with *Y. lipolytica* DGA strain: effect of substrate feed flow

F2 and **F3** fermentations were carried out with the obese modified strain *Y. lipolytica* DGA provided by CSIC, obtaining better results than in F1. The effect of two feed rates, 0.25 mL/min (low and constant) and 0.88-1.6 mL/min (high and variable), was studied. The biomass concentration resulted in 23.0 g/L for low flow and 9.8 mL/min for high flow. Consequently, a higher substrate inlet did not result in a higher

biomass concentration. One possible reason is the necessary supply of alkaline medium in order to neutralize the acidity of the acetic acid that is continuously introduced at a higher flow rate. The alkaline solution could contribute to a dilute in the nutrients and in the product. Figure 15 and Figure 16 show the fatty acid profile, very similar in both cases. Compared to wild-type strain, C16:1 was also formed in these fermentations, and C16:0 became the second most abundant instead of C18:2. The most abundant remained C18:1. The maximum TAGs yield was 0.14 g/g for F2 and 0.06 g/g for F3, while maximum productivity was 0.04 g/L/h for F2 and 0.03 g/L/h for F3, far from the objectives (0.6 g/g and 0.55 g/L/h).

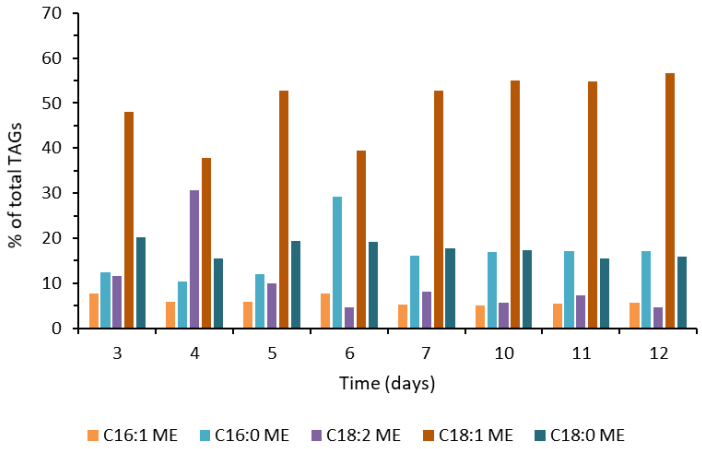

Figure 15: Fatty acid distribution obtained in F2.

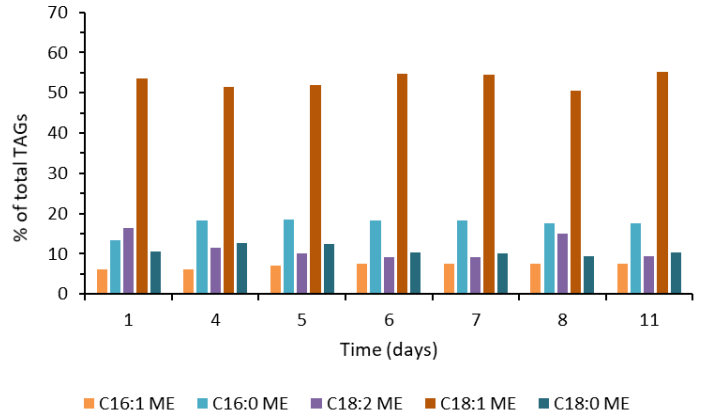

Figure 16: Fatty acid distribution obtained in F3.

Acetate fermentations with *Y. lipolytica* DGA strain: effect of different carbon sources

In view of the low concentration reached in F3, in the **F4** fermentation it was decided to supply sodium acetate (3 %) instead of acetic acid, in order to reduce the supply of alkaline solution. The ratios C/N were the same as in F1, F2 and F3. The feed flow selected was intermediate between the flow rates of F2 and F3, being a variable flow rate between 0.4-0.6 mL/min. The modified strain was used again and

the highest biomass concentration so far (25.9 g/L) was obtained with a TAGs content similar to that previously achieved, 24 % (6.2 g/L).

According to CSIC suggestion, the effect of the addition of glycerol as co-substrate together with acetic acid was studied. The **F5** fermentation was carried out with 3 % acetic acid, 3 % glycerol, and with C/N ratios of 26 and 204. There was a marked increase in biomass production, reaching 67.5 g/L. The lipid content was similar to that of other fermentations, 25.1 %, but the lipid concentration was high, due to the high biomass content, reaching 16.7 g/L. For the first time, citrate was detected in the fermentation, a by-product, reaching a concentration of 11.8 g/L. Fatty acid profile distribution of F4 and F5 can be seen in Figure 17 and Figure 18. The distribution feeding with sodium acetate was very similar to the previous distributions. The distribution using glycerol differed from the previous ones in the first days of fermentation. At the beginning of fermentation, the major fatty acid was C18:2 instead of C18:1, which was the second. After 2 days all fatty acids had a similar concentration, although C18:0 was the major and C16:1 the minor one. In the last days the distribution was almost identical to the previous fermentations. TAGs yield was 0.13 g/g for F4 and 0.28 g/g for F5, much higher than in F2 and F3. TAGs productivity was similar to the previous ones in F4 (0.04 g/L/h) and higher in F5 (0.10 g/L/h).

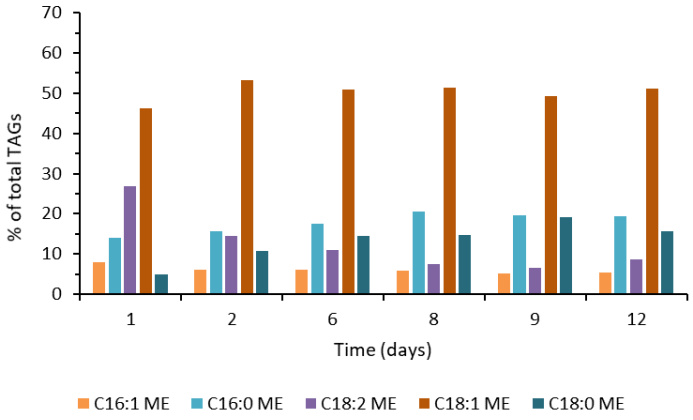

Figure 17: Fatty acid distribution obtained in F4.

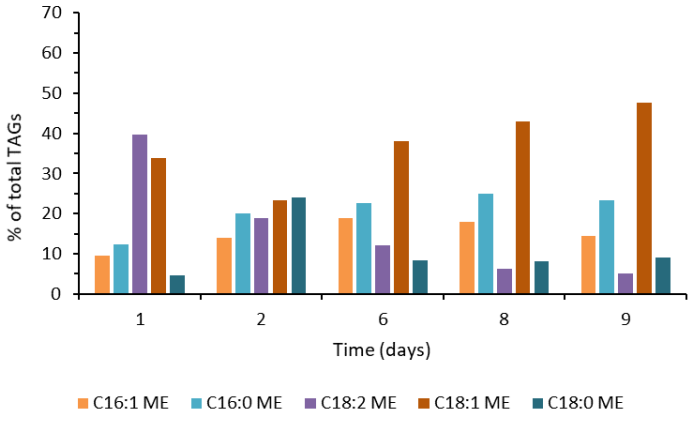

Figure 18: Fatty acid distribution obtained in F5.

Acetate fermentations with *Y. lipolytica* DGA strain: effect of a different nitrogen source

In the **F6** fermentation, the effect of a different nitrogen source was studied, adding ammonium acetate instead of ammonium sulfate. The carbon source was 3 % acetic acid and the C/N ratios were 17 and 113. Substrate flow rate was 0.3 mL/min, similar to F1 and F2. Biomass production was not higher than that obtained so far, reaching a concentration of 16.4 g/L, a very low citrate content of 0.1 g/L, and a lipid percentage similar to that obtained so far, 24.9 % (4.1 g/L). TAGs yield was 0.14 g/g, similar to F2, and TAGs productivity was 0.07 g/L/h, lower than the one obtained with glycerol. Fatty acid profile distribution can be seen in Figure 19. The distribution was similar to the previous ones but with a higher concentration of C18:0, being the second most abundant after C18:1.

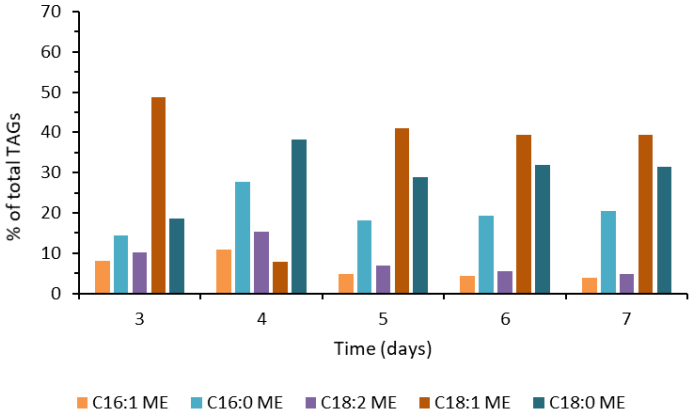

Figure 19: Fatty acid distribution obtained in F6.

Acetate fermentations with *Y. lipolytica* DGA strain: effect of increasing the concentration of the carbon source, decreasing pH and decreasing dissolved oxygen

In the next fermentation, **F7**, a mixture of acetic acid and glycerol was again used as a carbon source. In this case, the concentrations were increased with respect to F5, being 6 % acetic acid and 6 % glycerol. The C/N ratios were maintained at 14 and 100. A low feed flow rate of 0.15 mL/min was selected. Both DO and pH were made to decreased sharply from 30 to 5 % and from 7 to 2.8, respectively. Biomass concentration reached a value similar to that attained at high glucose concentration, but subsequently declined from 26 to 16.2 g/L. The percentage of TAGs was quite low, 12.0 5, so that the modification of the variables does not seem to be the best option for even an increase in TAGs. Another possible reason for the reduced percentage of TAGs is an excessive presence of nitrogen in the medium, since a higher C/N ratio (204) was used in F5 comparing with F7 (100). TAGs yield and productivity were very low, 0.04 g/g and 0.03 g/L/h, respectively. The fatty acid profile distribution of F7, seen in Figure 20, was similar to previous ones such as F4 and F5.

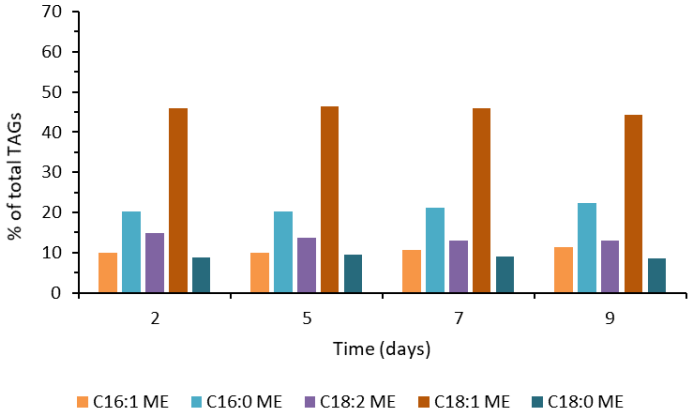

Figure 20: Fatty acid distribution obtained in F7.

Acetate fermentations with *Y. lipolytica* DGA strain: effect of decreasing the concentration of nitrogen, decreasing pH and decreasing dissolved oxygen

The next fermentation, **F8**, was carried out on 3 % of both acetic acid and glycerol. In addition, the nitrogen content was decreased in order to increase TAGs. For this purpose, a C/N ratio = 204 was established in the first feeding and an infinite ratio (zero nitrogen content) in the second feeding. DO was decreased in the second stage, and although the pH was also decreased, this change was performed gradually, which did not cause excessive damage to the cells. Consequently, the biomass concentration only increased, reaching a value of 27.8 g/L. The TAGs content was similar to the usual 24.1 %, which resulted in a concentration of 6.7 g/L. The biomass content was lower than in F5, which could be due to the inadequacy of lowering the pH in the presence of acetic acid, since it then remains in acid form and could be toxic to some degree. TAGs yield was the highest 0.27 g/g, very similar to the yield in F5 (0.28 g/g). Productivity was 0.06 g/L/h, lower than in F5 (0.10 g/L/h). Fatty acid profile distribution of F8 can be seen in Figure 21. The distribution was similar to F6, being C18:0 the second most abundant after C18:1.

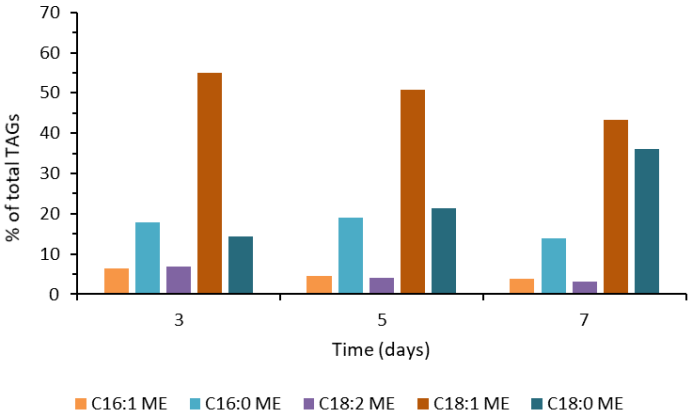

Figure 21: Fatty acid distribution obtained in F8.

Acetate fermentations with *Y. lipolytica* DGA strain: combination of different carbon sources to increase the biomass

In the last fermentation, **F9**, 100 g/L of glucose in the basal medium was used as initial carbon source in order to increase the cell growth during the early stage of the fermentation and provide sufficient biomass for an efficient lipid accumulation in the second stage of the fermentation. After the first growth stage, a 6 % sodium acetate (instead of acetic acid) and 6 % glycerol solution were added, without any nitrogen, on day 2. The pH was maintained during fermentation at 7 as acetate is present. The highest biomass concentration until now was obtained, 90.5 g/L, so the strategy was adequate. The highest citrate concentration was also obtained, 31.2 g/L. As for the TAGs content, it was 21.9 %, resulting in a concentration of 19.8 g/L. It should be noted that this percentage may be underestimated, since the centrifugation process prior to analysis did not achieve a good separation of the cells. An important part of the cells remained afloat due to the high TAGs content, so it was complicated to remove the supernatant trying to keep the cells for analysis. This can be seen in Figure 22, as well as the degree of turbidity of the supernatant. The yield of TAGs was 0.177, not as high as in F5 and F8 probably due to citrate co-production. The productivity was indeed the highest so far, 0.261 g/L/h. Fatty acids profile distribution of F9 (Figure 23) was intermediate between fermentations before, being the most abundant C18:1, the second one C16:0 and, very close to it, C18:0.

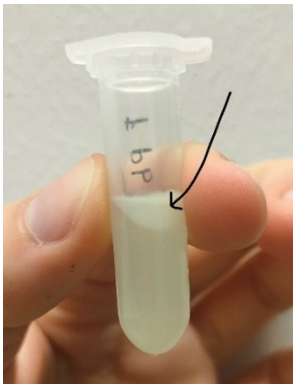

Figure 22: Floating cells after centrifugation.

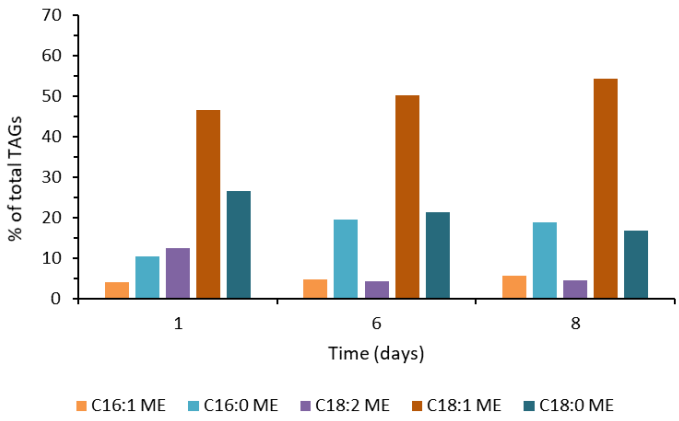

Figure 23: Fatty acid distribution obtained in F9.

5.4 Continuous fermentations (with cell recycle) with *Y. lipolytica* WT and DGA (CSIC-2) on glucose

Since the target TAGs concentration was not reached despite having modified different process variables and using the modified *Yarrowia* obese strain obtained by the CSIC, an alternative was proposed in order to increase the lipid production yield and reach the final target set for WP5 and for the subsequent purification and hydrogenation processes.
It was decided to use glucose as main carbon source instead of acetic acid. *Yarrowia* metabolises glucose much more easily to generate lipids, as it is an ideal carbon source to generate pyruvate and then acetyl CoA, which is a key precursor involved in TAGs biosynthesis. For this purpose, fermentation conditions were optimised at laboratory level to determine the most important parameters influencing lipid accumulation (pH, DO, C/N ratio).

Experiment carried out following this strategy are described in Table 5:

	Strain	Feed 1	Feed 2	Feed flow (mL/min)	DO (%)	pH	Biomass (g/L)	Citrate (g/L)	TAGs (%)	TAGs (g/L)	TAGs yield (g/g), Prod (g/L/h)
F10	DGA	60 g/L glucose 10 g/L $(NH_4)_2SO_4$ C/N=13	60 g/L glucose 1.3 g/L $(NH_4)_2SO_4$ C/N=102	0.2	30→5	7	22.9	n.d.	30.8	7.0	0.11, 0.08
F11	DGA	400 g/L glucose 66 g/L $(NH_4)_2SO_4$ C/N=13	400 g/L glucose 10 g/L $(NH_4)_2SO_4$ C/N=204	0.15	30→5	From 7 to 3 (fast)	23→11.3	n.d.	6.6	0.7	0.07, 0.03
F12	WT	400 g/L glucose 66 g/L $(NH_4)_2SO_4$ C/N=13	400 g/L glucose 10 g/L $(NH_4)_2SO_4$ C/N=204	0.15	30→5	From 7 to 3 (fast)	27→20.1	n.d.	4.7	0.9	0.002, 0.02

Table 5: Overview of fermentations in continuous mode on glucose carried out at CARTIF.

In fermentation **F10**, glucose was used as the sole carbon source, with a concentration of 60 g/L. The C/N ratios were 13 and 102. Due to the higher carbon concentration in the feed, a low fee flow rate of 0.2 mL/min was selected. The effect of decreasing dissolved oxygen in the TAGs accumulation stage from 30 to 5 % was studied. The biomass concentration was similar to that obtained by operating with acetic acid or sodium acetate, being 22.9 g/L. The percentage of TAGs was higher than that obtained so far, reaching 30.8 %, resulting in a total TAGs concentration of 7.0 g/L. TAGs yield and TAGs productivity reached 0.11 g/g and 0.08 g/L/h, respectively, both similar to working with acetate.

The next step was to increase the glucose concentration to try to increase the biomass concentration and thus the TAGs concentration. Fermentation was performed with modified strain (**F11**) and wild-type strain (**F12**), comparing both strains. The feeding solution contained 400 g/L glucose as carbon source. The C/N ratios were 13 and 204, so 66 and 10 g/L ammonium sulfate were supplied. The lowest feed flow rate used so far was selected due to the high concentration (0.15 mL/min). The decrease in DO in

the second stage was maintained, going from 30 to 5 %. In addition, the pH value in the second stage was rapidly decreased from 7 to 3, thinking that this would result in a higher lipid accumulation. Since the carbon source was glucose, it was possible to lower the pH, but this was not convenient with acetic acid because it is toxic in its acid form. The sharp decrease in pH had a negative effect on both fermentations as it resulted in a decrease in biomass concentration from 23 to 11.3 g/L (F11) and from 27 to 20.1 g/L (F12). This also occurred in F7. The percentage of TAGs was also lower than in any other fermentation, being 6.6 % (F10) and 12.0 % (F11). This low TAGs accumulation could be associated with a high presence of nitrogen in the medium due to a high nitrogen concentration in the feed solution. It is concluded that maintaining C/N ratios at values such as 14 (first stage) and 102 (second stage) is key as long as the nitrogen concentration in the medium is not excessive, because in that case the strain is not able to accumulate a high percentage of TAGs. There were no significant differences between the two strains, although the WT strain produced more biomass also resulted in a lower TAGs concentration. TAGs yield and productivity were very low in both fermentations, as can be seen in Table 5. Fatty acid profile distribution of F10, F11 and F12 can be seen in Figure 24, Figure 25 and Figure 26, respectively. Working with a lower substrate concentration the most abundant fatty acids were C18:1 and C18:0 (F10), while in F11 and F12 the most abundant were C18:1 and C16:0. No significant differences in the distribution between DGA and WT were found.

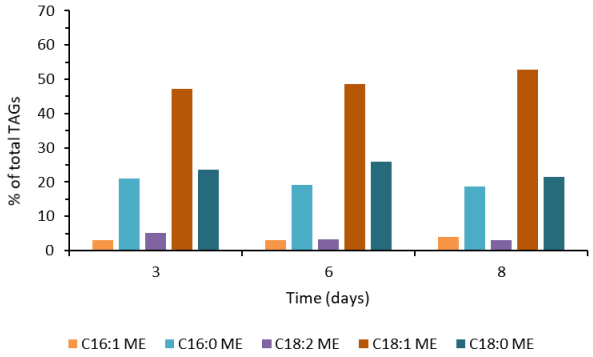

Figure 24: Fatty acid distribution obtained in F11.

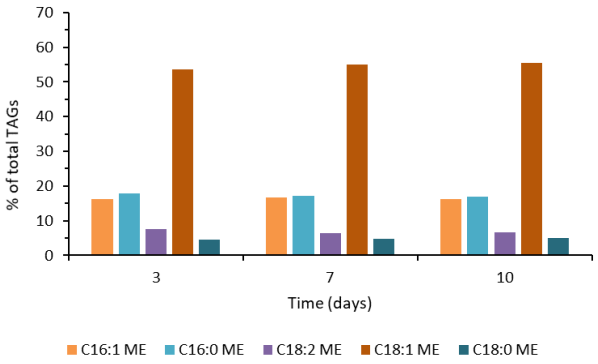

Figure 25: Fatty acid distribution obtained in F12.

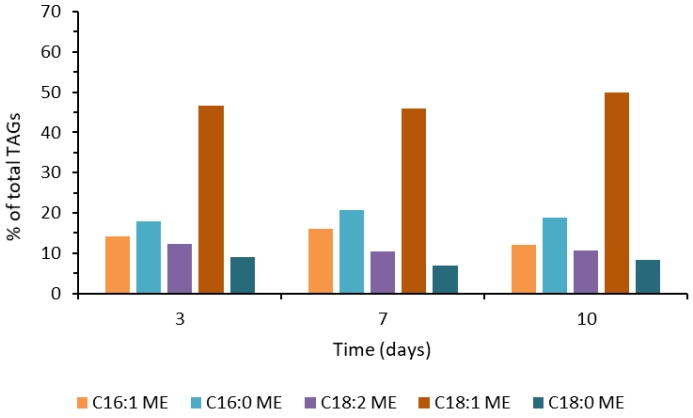

Figure 26: Fatty acid distribution obtained in F13.

6 Fermentations at 2 L scale (CSIC)

6.1 Equipment and fermentation set-up

CSIC investigated the production of TAGs by *Y. lipolytica* in bench-scale 2 L fermentation bioreactors (Sartorius Stedim) with an initial volume of 1 L. Bioreactor cultures were maintained at a temperature of 30 °C and a pH of 7.0 through the addition of 2M H_2SO_4. The aeration rate was 1 vvm and the DO level was maintained at 20% through agitation. Antifoam 204 (Sigma-Aldrich) was added when necessary to prevent excessive foaming.

Figure 27: Bioreactors of 2 L at CSIC.

6.2 Fed-batch fermentations with *Y. lipolytica* DGA (CSIC-2) on glucose

CSIC developed a lipid production experiment in a bioreactor with the *Y. lipolytica* DGA (CSIC-2) strain using glucose as substrate. The operating conditions were similar to those described by Friedlander et al. (2016). The experiment was run for 120 hours in a fed-batch mode reaching finally a concentration of 50 g/L biomass with 54% lipid content.

An overview of the fermentation results is shown in the following Figures 28 and 29.

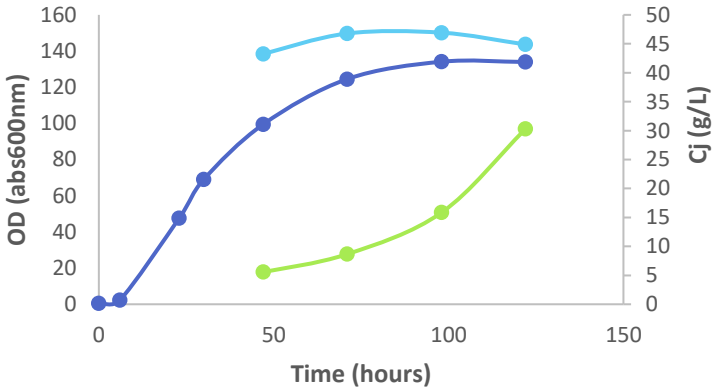

Figure 28: Evolution of biomass in terms of DO and Cx in a fed-batch experiment (total glucose 400 g/L) in a 2 L bioreactor. Dark blue (OD_{600}). Light blue (biomass, g/L). Green (lipids, g/L).

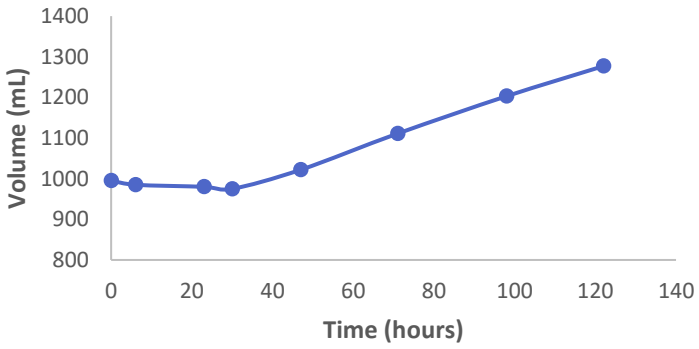

Figure 29: Evolution of volume in the 2 L bioreactor.

Interestingly, the accumulated lipids had an upward trend at the end of the experiment suggesting that higher lipid concentrations could be achieved if fermentation continued.

Furthermore, fluorescence images showing lipid accumulation over time were also obtained by staining with the fluorescent neutral lipid dye BODIPY 493/503 (Thermofisher), Results are shown in Figure 30.

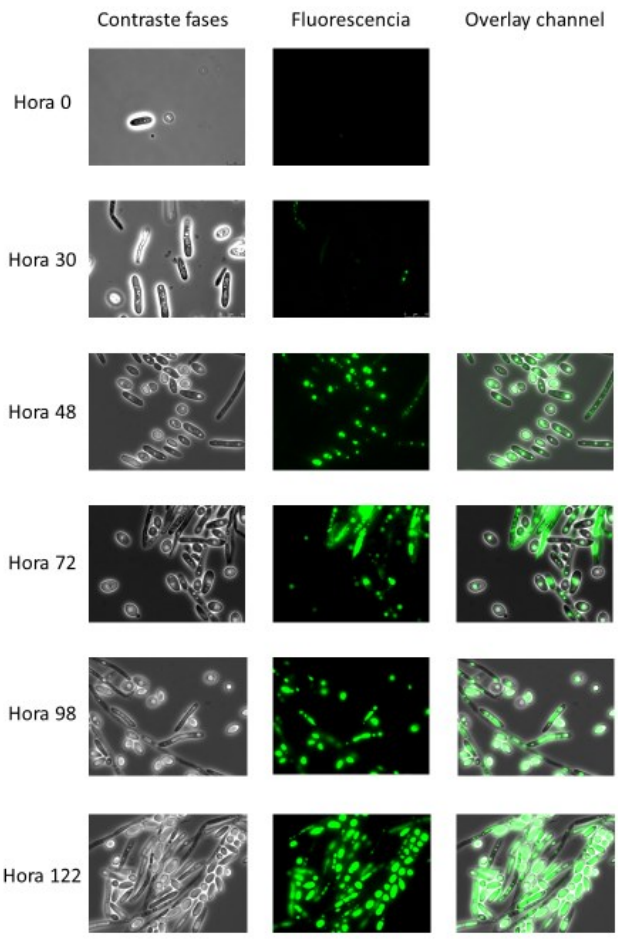

Figure 30: Fluorescence microscopy images showing the content of lipids.

With these results it can be concluded that the modified *Y. lipolytica* DGA strain (CSIC-2) is able to produce and accumulate a large amount of lipids using glucose as a carbon source, similar to the fermentation conditions previously described in the literature.

7 Fermentations at 7 L scale (BBEPP)

7.1 Equipment and fermentation set-up

BBEPP investigated the production of TAG by *Y. lipolytica* in bench-scale 7 L fermentation bioreactors with an initial volume of 3 L (Figure 2). All online parameters (temperature, DO, stirring speed, aeration, pH, acid addition) were continuously monitored. The temperature was set at 28 °C. The level of DO was controlled by adjusting the stirring speed (200 – 1200 rpm) and aeration (0.2 – 2 vvm). The pH was controlled via the addition of H_2SO_4 or NaOH, unless when pH-static feeding with acetic acid was applied. A drop of antifoam was added when excessive foaming was observed.

Figure 31: Bench-scale 7 L fermentation bioreactors for TAGs production at BBEPP.

Each fermentation consisted of a growth phase and a TAG production phase which was induced by nitrogen limitation (Figure 32). During the growth phase, nitrogen is an essential nutrient for the synthesis of several cell components such as proteins and nucleic acids. In order to reach high lipid titers during a fermentation process, cultivating high cell densities is necessary before switching to the lipid accumulation phase. In order to achieve this, sufficient nitrogen should be fed to the fermenter during the growth stage. Afterward, the exhaustion of nitrogen in the medium stimulates the production of TAG via de novo lipid synthesis.

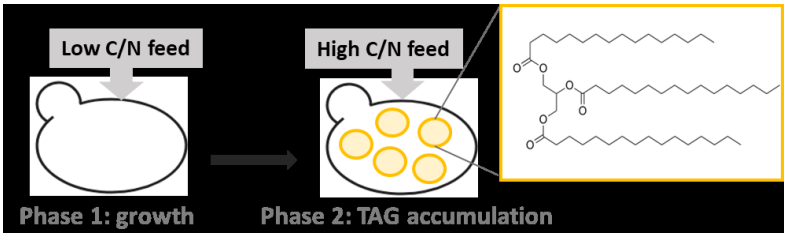

Figure 32: Representation of fermentation strategy for TAG production induced by nitrogen limitation.

Samples were collected at regular time intervals and different analyses were performed to determine key process values. The biomass production was assessed by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer and by gravimetrical determination of the CDW (g/L). The TAG concentration was determined by GC-MS analysis. The concentrations of acetate, glucose, citrate, and succinate were measured by HPLC analysis. The concentration of ammonium (NH_4^+) was measured with an ion-selective electrode or with colorimetric test strips for concentrations below 0.5 g/L. In this public report, BBEPP included the main outcome and conclusions of all 15 performed fermentations.

7.2 Fermentations with *Y. lipolytica* W29 (WT)

Since at the beginning of the execution of BioSFerA Task 3.4 no modified strain from BioSFerA Task 3.2 was available yet, the *Y. lipolytica* WT was used for the first fermentations. Although this strain is known to only synthesize a low amount of TAG, valuable insights can be gained as concerns the development of a fermentation strategy for the use of acetic acid. More specifically, the influence of nitrogen feeding as well as the type of nitrogen source were investigated.

7.2.1 Fed-batch fermentations on acetic acid

The addition of acetic acid was performed by pH-static feeding. As acetate is consumed by the culture, the pH of the fermentation increases, hence, acetic acid addition is required to maintain the pH at 6.8. As a result, acetic acid feeding occurs automatically upon consumption of the acetate. In such a stable system, acetate is continuously supplied to the culture while its concentration in the broth remains constant (Kiefer et al. 2020). Four fed-batch fermentations were performed with different acetic acid feed solutions.

It was observed that optimization of nitrogen feeding clearly improved the robustness of the fermentation. However, by using the *Y. lipolytica* WT, only 2 g/L TAG was accumulated, corresponding to a lipid content of 5%. In contrast, nitrogen limitation triggered the formation of citrate as a by-product. Its production should be avoided so the carbon flows to TAG instead.

7.2.2 Continuous fermentation with cell-recycle on acetic acid

7.2.2.1 BioSFerA-T3.4-BBEPP-F05: acetic acid and NH_4-acetate

An alternative fermentation mode, namely continuous fermentation with cell-recycle, was tested. This allows for continuous feeding of a dilute acetic acid stream (3% acetate), as it is produced during the gas fermentation in the BioSFerA concept (BioSFerA Deliverable 3.4). Large volumes can be fed while maintaining a constant operating volume and retaining the cells as well as the product in the bioreactor. It was concluded that the continuous cell-recycle operation mode is indeed a valid strategy to use a dilute acetate stream while reaching a high biomass productivity. This has further been investigated by CARTIF.

7.3 Fermentations with *Y. lipolytica* DGA Leu- (CSIC-1)

Y. lipolytica accumulates lipids under nitrogen-depleted conditions. However, the previous fermentations showed that most of the carbon was converted to citrate. Indeed, the production of citrate by *Y. lipolytica* WT has previously been reported in literature. Yuzbasheva et al. (2019) observed a citrate titer of 50 $g.L^{-1}$ on glucose under nitrogen-depleted conditions. In contrast, by using a metabolically engineered *Y. lipolytica* strain in a similar strategy, Hu et al. (2016) achieved a final lipid content of 61%, while almost no citrate was produced. Hence, genetic engineering efforts could potentially promote the carbon flux toward lipid accumulation. As a result of BioSFerA Task 3.2, CSIC provided a first recombinant strain: *Y. lipolytica* DGA Leu- (CSIC-1).

7.3.1 Fed-batch fermentations on acetic acid

The lipid accumulation potential of the recombinant strain *Y. lipolytica* CISIC-1 was tested by performing two fed-batch fermentations on acetic acid and resulted in a 22% increase in lipid content. However, since this first strain was L-leucine autotrophic, regular additions of L-leucine were required. In this respect, it should also be noted that the supplementation of L-leucine could have a beneficial effect on lipid synthesis. Blazeck et al. (2014) demonstrated that the supplementation of L-leucine to an auxotrophic *Y. lipolytica* mutant triggers lipid accumulation similar to nitrogen depletion.

7.4 Fermentation with *Y. lipolytica* DGA (CSIC-2)

The L-leucine auxotrophic *Y. lipolytica* CSIC-1 showed promising results, yet caused major problems as concerns process control because of the frequent depletion of L-leucine in the fermentation medium. Therefore, a second version of the strain was received from CSIC, namely *Y. lipolytica* DGA (CSIC-2) which was prototrophic for L-leucine.

7.4.1 Fed-batch fermentations on acetic acid

Three fed-batch fermentations were executed to optimize the fermentation of *Y. lipolytica* CSIC-2 on acetic acid. First, the influence of a different C/N ratio during the lipid accumulation phase was assessed. Furthermore, also the effect of DO was evaluated. As described by Qiao et al. (2015), citrate formation can be decreased by limiting the DO during the production phase.

The production of TAG from acetic acid could be improved up to 10 g/L TAG by utilization of the *Y. lipolytica* CSIC-2 strain and optimization of several key process conditions, including the fermentation mode, feeding strategy, nitrogen source, C/N ratio, pH, and DO. Furthermore, by-product formation could indeed be reduced and a productivity of 0.06 g/L/h was reached.

7.4.2 Fed-batch fermentations on glucose

Since the BioSFerA target TAG concentration was not reached on acetate, glucose was assessed as an alternative substrate aiming to produce more TAG, which will be required for the scale-up within BioSFerA WP4 and WP5.

Five fed-batch fermentations were performed using the *Y. lipolytica* CSIC-2 strain with glucose. In addition, the influence of the key process parameters was further investigated, including pH. As described by Zhang et al. (2019), a lower pH tends to stimulate the production of lipids and decrease citrate by-product formation.

By cultivating the *Y. lipolytica* CSIC-2 strain on glucose with the same fermentation strategy as previous fermentations on acetic acid, lipid production was improved up to 14 g/L TAG, which corresponds to more than 23% of the total cell mass, at an overall productivity of 0.07 g/L/h. Hence, the modified strain showed better lipid production on glucose than on acetic acid, as was indicated by CSIC. In addition, by further optimization, lipid accumulation on glucose reached 20 g/L TAG. Furthermore, a lower pH control reduced citrate production significantly. Yet, it should be noted that although this low pH works for TAGs

production on glucose, this strategy cannot be used with acetate as this carboxylate is likely to become inhibitory when present in the acid form.

7.5 TAG fatty acid profile

Finally, for all TAG samples analyzed by GC-MS also the fatty acid profile was determined. A similar profile was observed for all cases, meaning that the difference in fermentation settings, the use of a different strain, and the use of glucose instead of acetate did not considerably change the product composition. This is crucial for the TAG hydrotreatment process which will be investigated in BioSFerA WP5.

8 Conclusions

Conclusions CARTIF:

- The use of a continuous fermentation mode with cell recycling using hollow fiber membranes to recirculate the cells while removing exhausted culture medium from the bioreactor resulted in increased lipid production.
- Acetate fermentations with the modified strain *Y. lipolytica* DGA resulted in a TAGs content of approximately 25% but with low biomass concentration (20-25 g/L) and lipid titer (6.2 g/L).
- Acetate fermentations with the modified strain *Y. lipolytica* DGA using glycerol as a co-substrate resulted in similar TAGs accumulation (%) but higher biomass concentration (67.5 g/L) and therefore higher lipid titer (16.9 g/L).
- Glucose fermentation does not provide more TAGs if the nitrogen concentration is not low enough.
- Glucose fermentation with stress conditions such as low DO (5%) and low pH (2.8) during the second stage of the fermentation resulted in higher TAGs accumulation (31%) with no by-product accumulation.
- In order to stimulate the lipid production during the lipid accumulation phase of the glucose fermentation, the change in pH must be gradual or low from the beginning. Abrupt change in pH (from 7 to 2.8) causes a decrease in cell viability, resulting in low biomass and lipid content at the end of the fermentation.

Conclusions BBEPP:

- The growth of *Y. lipolytica* was observed on gas fermentation effluent, thereby supporting and demonstrating the BioSFerA concept.
- Nutrient limitation, especially nitrogen, was successfully applied to induce TAG production.
- The *Y. lipolytica* W29 strain accumulated a minor amount of 5% TAG when using acetate as the carbon source.
- The modified *Y. lipolytica* DGA strain from CSIC has improved performance in terms of lipid production compared to the wild-type strain.
- The most important fermentation conditions for optimizing TAG production are nitrogen feeding, DO control, and pH control.
- Glucose is a better carbon source for lipid production than acetate, resulting in a titer of 20 g/L compared to 10 g/L for acetic acid using *Y. lipolytica* DGA, with the same fatty acid composition for both carbon sources.
- The optimized fermentation conditions obtained in this study will be used for the development of a scale-up TAG fermentation process in WP4.

9 Future actions

In order to improve the results obtained under WP3 CARTIF will continue studying the influence of key fermentation parameters, focusing on the use of acetic acid and glycerol as co-substrate for lipid production. Furthermore, very low or no N content in feed will be used to increase TAGs accumulation.

The findings will be reported in an updated version of D3.5.

More specifically, the following lab-scale trials will be carried out:

-Perform fermentations with a continuous feed of acetic acid plus glycerol in a higher concentration (6 - 10 %) to increase biomass production and then lipid production.

-Use glucose as carbon source in the basal medium in order to increase the biomass concentration at early stages of fermentation.

The aim of these additional lab-scale fermentation trials using acetate as main carbon source is to fine-tune the lipid production protocol and then scale up the process in a 150 L bioreactor at BBEPP during the first months of next year, in order to validate the BioSFerA concept (Task 4.4).

10 References

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Annexes

Annex 1: Media compositions

YPD medium

Yeast extract-peptone-dextrose (YPD) medium was acquired from BD (Difco™). It consists of:

- Yeast extract, 10 $g.L^{-1}$
- Peptone, 20 $g.L^{-1}$
- Glucose, 20 $g.L^{-1}$

YPA medium

Yeast extract-peptone-acetate medium (YPA) has been used for the cultivation of *Y. lipolytica* strain in shake flasks.

- Yeast extract, 10 $g.L^{-1}$
- Peptone, 20 $g.L^{-1}$
- Sodium acetate, 30 $g.L^{-1}$

Hu et al., (2016) medium

Hu medium has been used for the cultivation of *Y. lipolytica* strain in bioreactor.

- Yeast extract, 2.5 $g.L^{-1}$
- Yeast nitrogen base (without amino acids and ammonium sulfate), 4.2 $g.L^{-1}$
- Sodium acetate, 30 $g.L^{-1}$
- Ammonium sulfate 2.4 $g.L^{-1}$

Annex 2: Analytical methods

HPLC analysis

In the analysis method for the quantification of acetate, glucose, glycerol, citrate and succinate concentrations a mobile phase with a dilution of 0.005M H_2SO_4 in water was used. Once this mixture was prepared, it was filtered with cellulose nitrate membrane filters of 0.45 µm pore size, and then degassed in the ultrasonic bath equipment, in order to eliminate the gases present in the liquid that could obstruct the filling of the column. The flow rate, after being optimized to reduce the total time of each test, was set at 0.6 mL/min. The column used was Hi-Plex-H (7.7x300 mm, 8 µm) of Agilent equipped with the appropriate guard column. The temperature used in the RID detector was 35 ºC, while the column temperature was set at 50 ºC. The total analysis time for each sample was 30 minutes.

Lipid extraction and quantification

Lyophilized biomass (around 50 mg) was resuspended in 1 mL of methanol and H_2SO_4 (97.5% methanol and 2.5% H_2SO_4) with an internal standard. The samples were incubated at 80 °C for 1.5 h. The reaction that takes place is a transesterification of TAGs with methanol in presence of an acid catalyst. This reaction is necessary for the subsequent detection of methyl esters of fatty acids in the gas chromatograph. The reaction was stopped by the addition of 1.5 mL H_2O. Then 0.5 mL of hexane was added and the mixture was vigorously stirred. The upper phase was recovered after centrifugation for 5

min to 2500 r.p.m. 100 µL was collected and placed in glass vials for subsequent analysis. Methyl esters of fatty acids dissolved in hexane were analyzed on a gas chromatograph coupled to a mass spectrometer (GC–MS) QP2010 Shimazdu. A column HP-5MS (30 m long, 0.25 mm internal diameter and 25 µm of film) was used.

The conditions for the analysis were as follows: it was used helium with a flow of a 1.2 mL/min as a carrier gas, with a Split-ratio 80:1. The injector temperature was 260 °C and the interface temperature was 310 °C. The oven followed this program: initial temperature of 150°C for 2 min, a ramp of 8 °C/min to 210 °C, and a ramp of 3 °C/min to 220 °C and finally 8ºC/min to 290 ºC and held 1min. The fatty acids were identified by comparison with the methyl esters of fatty acids of standard commercial sample (Supelco FAME 37 mix). The total quantification of fatty acids was carried out following the method of standard internal pattern using 100 µg of heptadecanoic acid C17:0 (Sigma).