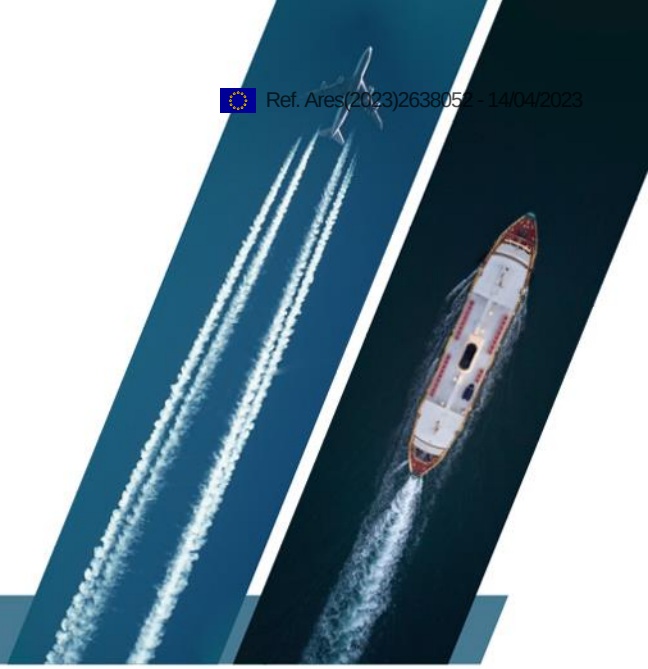
BioSFerA
Biofuel for biotravels



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Scale-up of TAG production

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Abbreviations

BBEPP	Bio Base Europe Pilot Plant (BioSFerA partner, Belgium)
CDW	Cell dry weight
CER	Carbon dioxide emission rate
CERTH	Centre for Research and Technology Hellas (BioSFerA partner, Greece)
CO_2	Carbon dioxide
DO	Dissolved oxygen
DSP	Downstream processing
ENVIPA	Environment Park (BioSFerA partner, Italy)
IBC	Intermediate bulk container
KPRT	Kuwait Petroleum Research and Technology (BioSFerA partner, Netherlands)
NH_4^+	Ammonium
O_2	Oxygen
OD	Optical density
OUR	Oxygen uptake rate
RQ	Respiratory quotient
TAG	Triacylglycerol

Contents

1 Executive Summary

This deliverable describes the development of a production process for microbial oil in the form of triacylglycerol (TAG) at pilot scale. This process includes (i) the cultivation of *Yarrowia lipolytica* which accumulates TAG in intracellular lipid bodies (executed in BioSFerA task 4.4) and (ii) the downstream processing (DSP) of the fermentation broth to extract and purify the TAG oil (executed in BioSFerA task 4.5). The insights obtained during the lab scale developments of this process within BioSFerA workpackage 3 were used as a starting point for this scale-up. The purified oil will be used for upgrading within BioSFerA workpackage 5 and its quality should thus meet the requirements for hydrotreatment.

First, two 150 L runs (BioSFerA-T4.4-F01 and BioSFerA-T4.4-F02) were performed, allowing the fine-tuning of the fermentation and DSP strategy established in BioSFerA workpackage 3 (described in BioSFerA deliverable 3.5). Having determined the best pathway for effective TAG production and purification at 150 L scale, BBEPP proceeded to the upscaling to 15 m³ (BioSFerA-T4.4-F03), thereby aiming to produce TAG for further treatment in BioSFerA workpackage 5. This report describes the results obtained in all three fermentations as well as the DSP of F02 and F03. A summary of the obtained results is given in Table 1. The broth of BioSFerA-T4.4-F01 was shipped to ENVIPA to assess steam explosion as an alternative strategy for cell disruption (described in BioSFerA deliverable 3.6).

Table 1: Summary of results obtained during the scale-up of the TAG production to pilot scale at BBEPP.

	Fermentation					DSP	
	Scale (L)	Volume (L)	CDW conc. (g/L)	TAG conc. (g/L)	TAG prod. (g/L/h)	TAG (kg)	Recovery (%)
BioSFerA-T4.4-F01	150	103	67	31	0.16	-	-
BioSFerA-T4.4-F02	150	95	64	25	0.10	0.69	29
BioSFerA-T4.4-F03	15000	6114	59	19	0.09	22	19

2 Introduction

Based on the knowledge obtained in BioSFerA task 3.5 and task 3.6, a feasible fermentation and DSP plan have been defined for the pilot scale runs.

2.1 Fermentation plan for TAG fermentation

The production of TAG is achieved by the fermentation of the yeast *Y. lipolytica* accumulating TAG intracellularly in lipid bodies. As found from the lab-scale experiments in BioSFerA task 3.5, the process ideally consists of a growth phase and a TAG production phase which is induced by nitrogen limitation (Figure 1). During the growth phase, nitrogen is an essential nutrient for the synthesis of several cell components such as proteins and nucleic acids. To reach high lipid titers during a fermentation process, cultivating high cell densities is necessary before switching to the lipid accumulation phase. Therefore, 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.

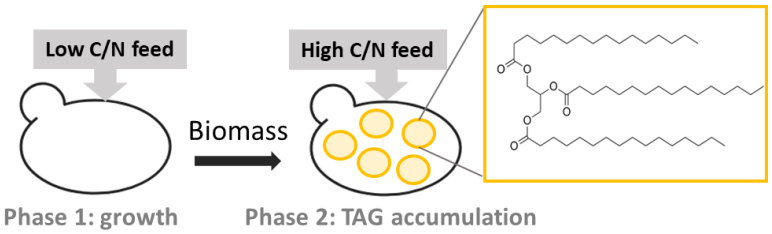


Figure 1: Representation of fermentation strategy for TAG production induced by nitrogen limitation.

As described in BioSFerA deliverable 3.5, the production of TAG from acetic acid was below the target. Further strain engineering advances will be required to enhance the flux of acetate towards TAG. Alternatively, glucose was assessed as the substrate instead of acetic acid in BioSFerA task 4.4. Using the *Y. lipolytica* DGA10 strain, which was engineered by CSIC for improved TAG production, showed higher production of TAG, namely 20 g/L TAG when glucose was employed as substrate instead of 10 g/L TAG on acetic acid. Hence, together with all involved BioSFerA partners, it was agreed to cultivate the *Y. lipolytica* DGA10 strain and use glucose as the carbon source during scale-up runs of BioSFerA task 4.4 as a safer approach to secure an adequate quantity of lipids for the lab and pilot scale activities in WP5. Key fermentation parameters for the production of TAG from glucose were investigated on lab-scale in BioSFerA task 3.5. As such, the resulting optimal conditions were used during the pilot-scale fermentation trials.

2.2 DSP plan for TAG extraction and purification

In BioSFerA task 3.6, several DSP techniques for TAG isolation have been evaluated on lab scale and an optimal route was established consisting of the following process steps:

1. Biomass concentration and water removal by filtration: to reduce the working volume and increase the efficiency of other process steps.
2. Cell disruption by homogenization: higher pressure and increased number of passes improve cell rupture. However, on a larger scale the amount of passes is limited because of economical and technical considerations.
3. Solvent extraction.
4. Removal of the water phase containing the cell debris: centrifugation was required to get phase separation and allow decantation the water phase.
5. Evaporation of the solvent: the relatively low boiling point of ethyl acetate will allow for easy evaporation and recovery of the TAG.

This strategy will be scaled up to 150 L and 15 m³ pilot-scale using industrially relevant equipment. As such, the feasibility of the process on a larger scale can be assessed and purified TAG can be obtained for further testing.

3 Scale-up of TAG fermentation to 150 L

3.1 Process overview

First, BBEPP scaled the production of TAG by *Y. lipolytica* to 150 L pilot-scale fermentation bioreactors (Figure 2). All online parameters (temperature, DO, stirring speed, aeration, pH, acid addition) were continuously monitored (data not shown). The temperature was set at 28 °C. The level of dissolved oxygen (DO) was controlled by adjusting the stirring speed and aeration. The pH was controlled via addition of H_2SO_4 or NaOH. Antifoam was added when excessive foaming was observed.

Figure 2: Pilot-scale 150 L fermentation bioreactors for TAG production at BBEPP.

The fermentation inoculum was prepared from *Y. lipolytica* stock cultures stored at – 80 °C. More specifically, the first preculture consisted of 1.5 mL of stock culture inoculated in 500 mL shake flasks filled with 100 mL medium. The second preculture consisted of 2 L shake flasks filled with 100 mL medium and 3 mL of the first preculture. The latter was used to inoculate the main fermentation. An overview of the process is presented in Figure 3.

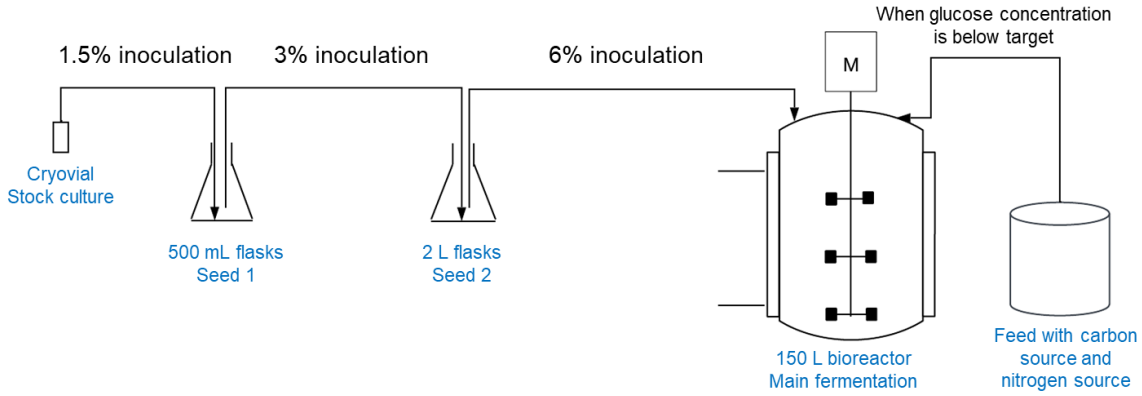


Figure 3: Process overview of BioSFerA-T4.4-F01 and BioSFerA-T4.4-F02.

3.2 TAG production on glucose in fed-batch fermentation (BioSFerA-T4.4-F01)

The fermentation conditions for the first 150 L fermentation were based on the optimized process developed in BioSFerA task 3.4. The results obtained from the fermentation samples, including the cell dry weight (CDW), ammonium (NH_4^+), and TAG concentration are presented in Figure 4.

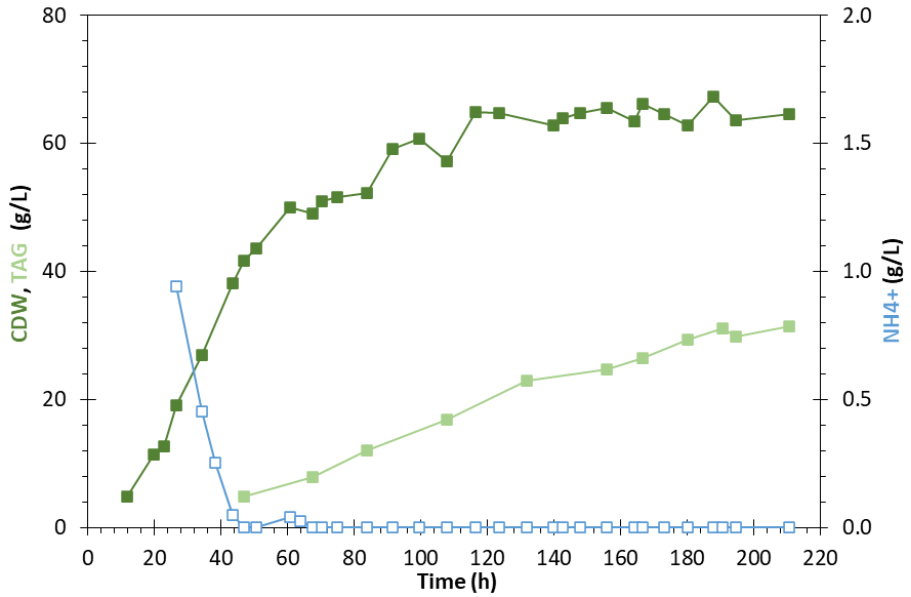


Figure 4: Offline data BioSFerA-T4.4-F01

When the glucose concentration dropped below the target concentration, the feed containing carbon and nitrogen source was initiated. When nitrogen became depleted after 44h, the production phase was initiated. At this point, also the control of DO and pH was adjusted to decrease by-product formation. Conclusively, after 188 h (8 d) of fermentation, a biomass concentration of 67 g/L CDW was reached containing 49% TAG or 31 g/L TAG with a productivity of 0.21 g/L/h during the second phase. Although the target titer of 50 g/L TAG and productivity of 0.26 g/L/h set in the GA were not reached, this fermentation exceeded the best results obtained within BioSFerA task 3.4. This can be attributed to the advanced control and more stable conditions in the pilot-scale fermenter. In addition, the influence of the key process parameters identified in BioSFerA task 3.4 was confirmed.

Interestingly, BBEPP has implemented precise off-gas analysis with the pilot scale bioreactors featuring continuous measurement of the outlet gas O_2 content, CO_2 content, O_2 uptake rate (OUR), CO_2 emission rate (CER), and respiratory quotient (RQ) (Figure 5 and Figure 6). This gives advanced insights into the fermentation process. As such, it was observed that the OUR and CER rise during growth, while during the TAG production phase, the CER and OUR remain stable with a slight decrease and a higher RQ.

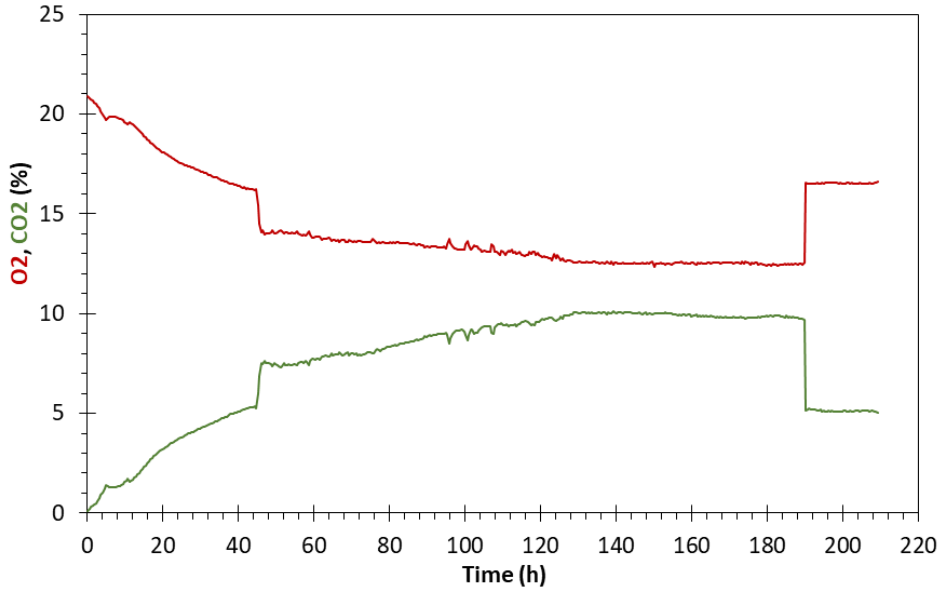


Figure 5: O_2 (O2) and carbon dioxide (CO2) content in the off-gas of BioSFerA-T4.4-F01

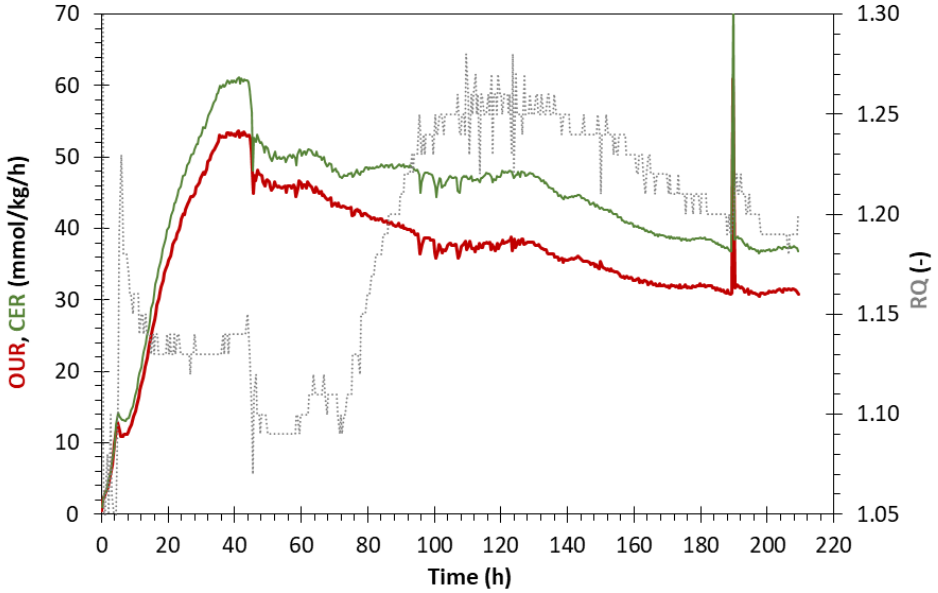


Figure 6: OUR, CER, and RQ of BioSFerA-T4.4-F01.

3.3 TAG production on glucose in fed-batch fermentation - repetition (BioSFerA-T4.4-F02)

For the second fermentation run at 150 L, similar conditions were used as for F01 with some minor adjustments. As such, the initial glucose concentration was increased so less fed-batch addition would be needed and the final volume could be lower. Moreover, the pH control was adjusted. The results are presented in Figure 7.

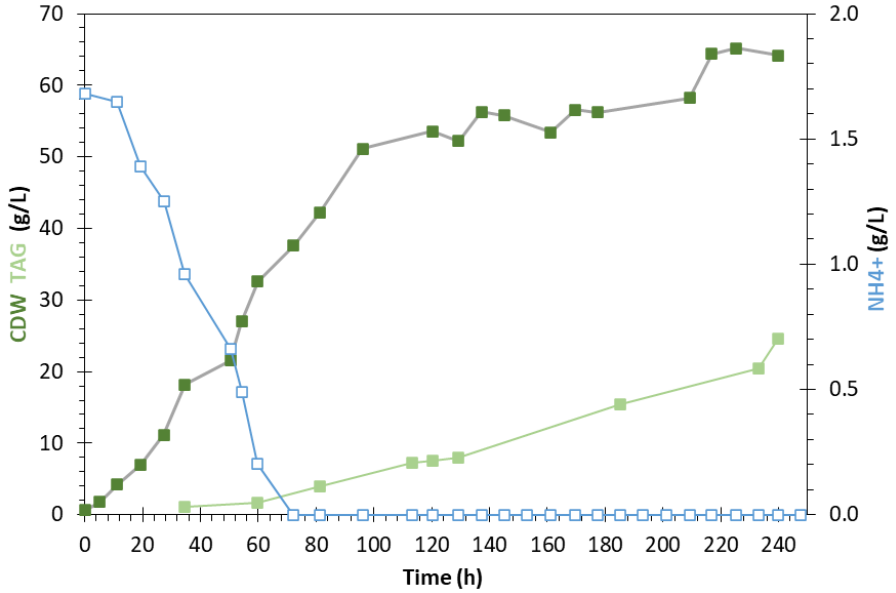


Figure 7: Offline data of BioSFerA-T4.4-F02.

After 240 h (10 d) of fermentation, a biomass concentration of 64 g/L CDW was reached containing 38% TAG or 31 g/L TAG with an overall productivity of 0.10 g/L/h TAG. These results are below what was obtained during F01, this could be attributed to the lower control of pH at the beginning of the fermentation, which prolonged the duration of the growth phase which is ended by nitrogen limitation. While in F01 the growth phase ended after around 50 h, it took around 70 h in F02. The off-gas data show similar trends (Figure 8 and Figure 9).

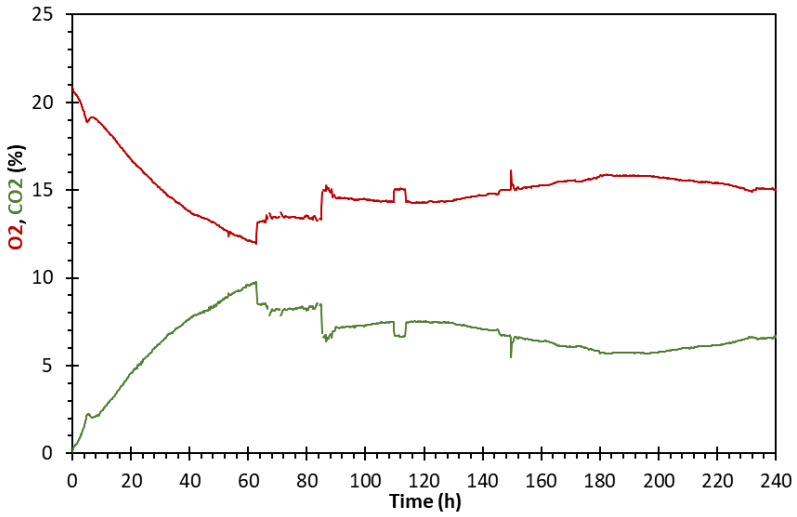


Figure 8: O_2 and CO_2 content in the off-gas of BioSFerA-T4.4-F02.

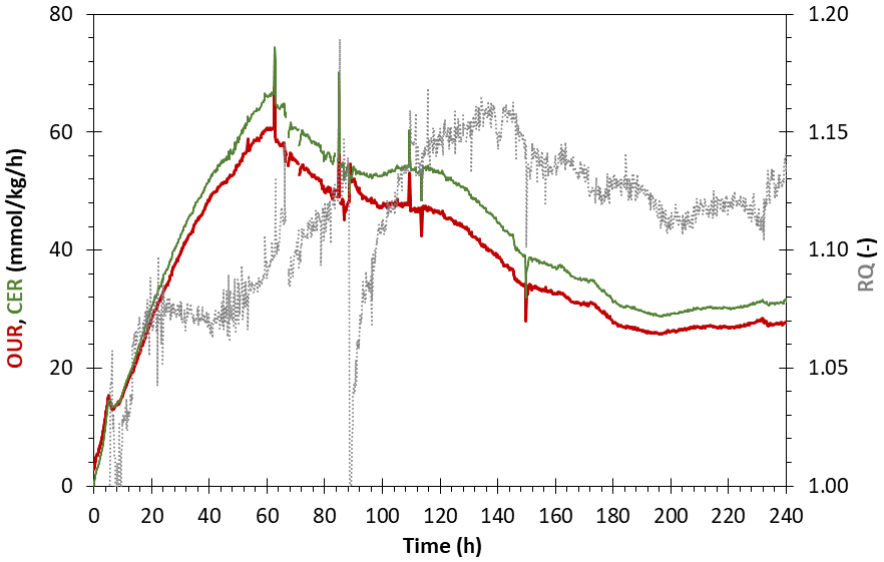


Figure 9: OUR, CER, and RQ of BioSFerA-T4.4-F02.

4 Scale-up of TAG purification to 150 L

4.1 Process overview

Based on the insights obtained during the lab scale experiments in WP3 (described in BioSFerA deliverable 3.6), a protocol was developed for the DSP of TAG at pilot scale. The process consists of six operations, as presented in Figure 10. The equipment used for the different steps is shown in Figure 11.

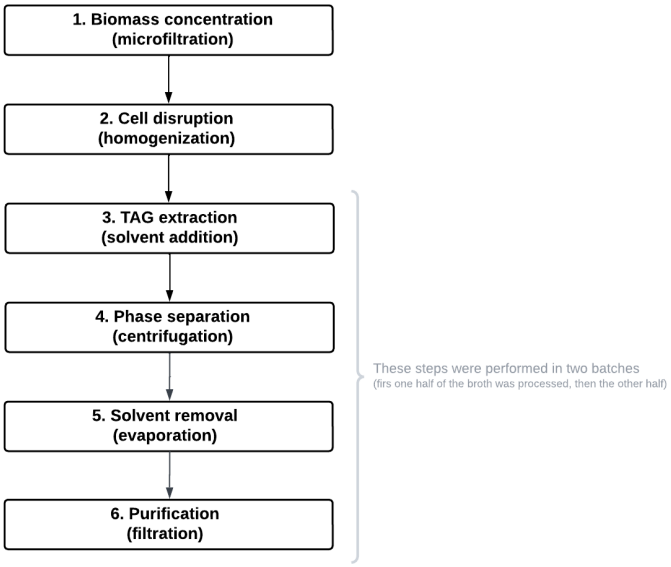


Figure 10: Process overview of DSP operations for the extraction and purification of TAG from 150 L scale fermentation.

4.2 Starting material: Broth from BioSFerA-T4.4-F02

The fermentation broth of BioSFerA-T4.4-F02 was used for the first TAG DSP process at pilot-scale. As described in Section 3.3, 95 L of fermentation broth was produced containing 60 g/L CDW of glucose-grown *Y. lipolytica* cells containing 42% TAG. Hence, 2.38 kg of TAG was present in the starting material.

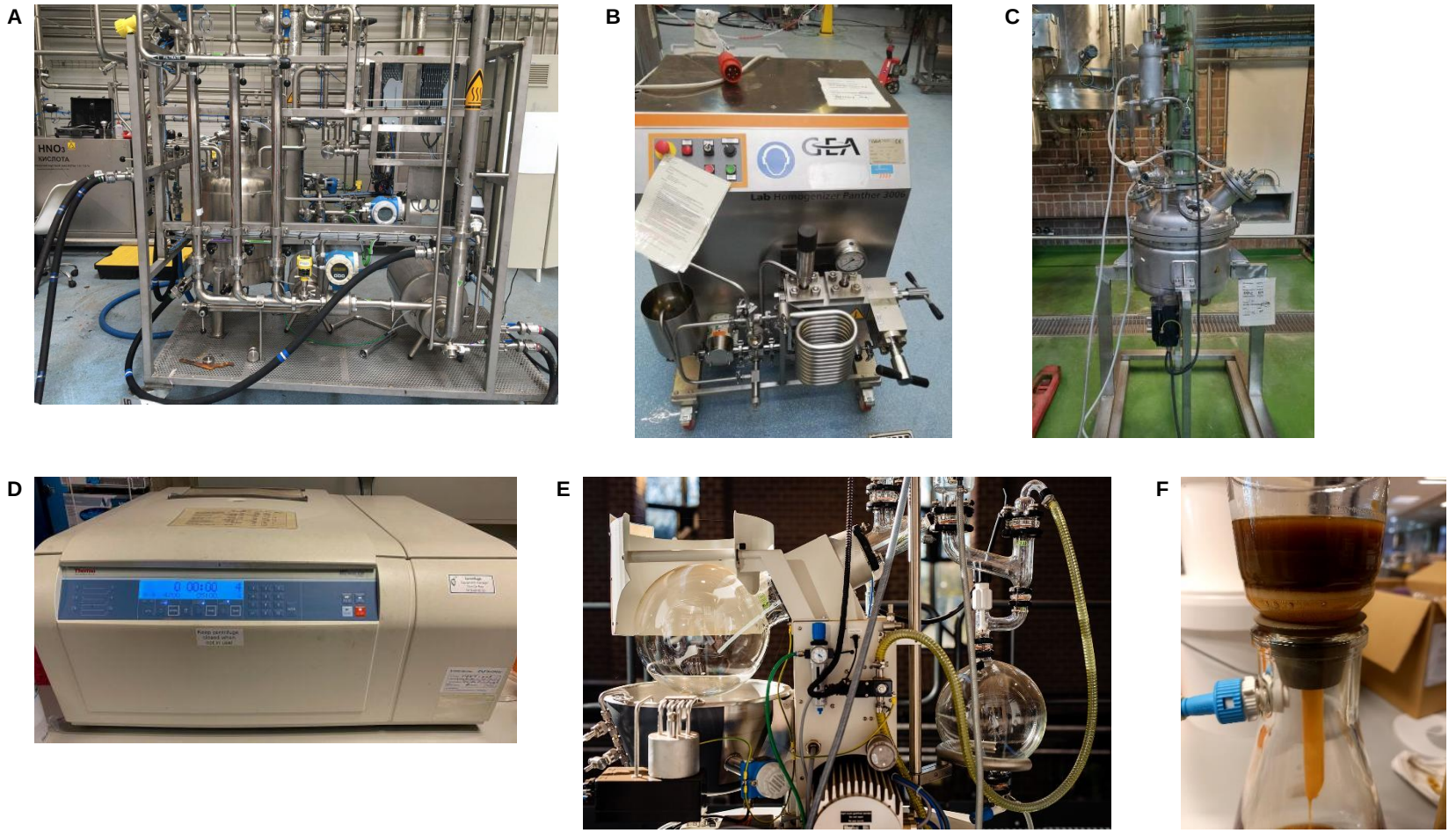


Figure 11: Overview of some of the equipment used during the scale-up of TAG purification to 150L: Three candle filter (A), Panther homogenizer (B), R80 chemical reactor (C), lab centrifuge (D), industrial rotavapor (E), Buchner filter (F).

4.3 Step 1: Biomass concentration by microfiltration

To concentrate the biomass, microfiltration was performed using a three-candle filter equipped with ceramic membranes. As such, the biomass was concentrated from 95 L to 55 L. In addition, diafiltration was performed to wash the yeast cells.

4.4 Step 2: Cell disruption by homogenization

To disrupt the cells, high-pressure homogenization was performed. To ensure a scalable strategy, two passes were performed at 1000 bar. The resulting broth was divided into two batches for solvent extraction.

4.5 Step 3: TAG extraction by solvent addition

The homogenized broth was divided into two batches for solvent extraction. The first batch of 22 kg was transferred to a chemical reactor. Then, the appropriate amount of extractant was added. The temperature was increased and the mixture was stirred for TAG extraction. After extraction, the mixture was cooled to let it settle. A similar strategy was applied for the second batch.

4.6 Step 4: Oil separation by centrifugation

When harvesting the broth after the solvent extraction, it settled into two phases (Figure 12). The heavy phase consisting of the cell debris and water was discarded, while the light phase containing TAG and solvent was centrifuged to remove the emulsion. The light phase was used in the following step.

Figure 12: Two phases after solvent extraction

4.7 Step 5: Oil recovery by evaporation

The solvent phase was transferred to the industrial rotavapor for evaporation to recover the TAG. During the evaporation, brown solids appeared (Figure 13). These could originate from some leftover cell debris.

Figure 13: Sample after evaporation showing brown solids (left) which settled after centrifugation (right).

4.8 Step 6: Oil purification by filtration

The brownish solids could easily be removed by dead-end filtration to get purified oil. The solids comprised 18 w% of the total volume. Finally, 0.32 kg and 0.38 kg of oil could be obtained from batches 1 and 2, corresponding to an overall recovery of 27% and 32%, respectively. The oil had a density of 0.905 kg/m³ and solidified at room temperature (Figure 14). A sample of the oil was shipped to KPRT for analysis. The results of these analyses will be reported in BioSFerA deliverable 5.1.

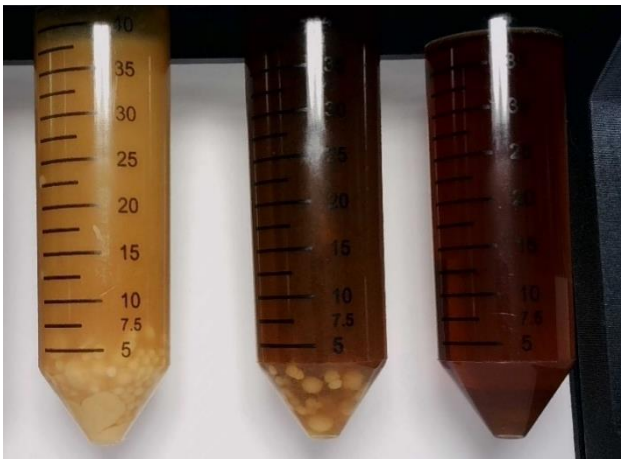


Figure 14: Pictures of the TAG product obtained from the broth of BioSFerA-T4.4-F02 at 4 °C, 20 °C, and 50 °C (from left to right).

5 Scale-up of TAG fermentation to 15 m³

5.1 Process overview

After BBEPP scaled the production of TAG by *Y. lipolytica* to 150 L, the process was further scaled to 15 m³ in a pilot-scale fermentation bioreactor (Figure 15). All online parameters (temperature, DO, stirring, aeration, pH, etc.) were continuously monitored (data not shown). The temperature was set at 28 °C. The level of DO was controlled by adjusting the stirring speed and aeration. The pH was controlled via addition of H_2SO_4 or NaOH. A drop of antifoam was added when excessive foaming was observed.

Figure 15: Pilot-scale 15 m³ fermentation bioreactors for TAG production at BBEPP.

The fermentation inoculum was prepared from *Y. lipolytica* stock cultures stored at – 80 °C. More specifically, the first preculture consisted of 1.5 mL of stock culture inoculated in a 500 mL shake flask containing 100 mL medium. The second preculture consisted of 2 L shake flasks filled with 500 mL medium and 15 mL of the first preculture. The medium contained the carbon source used in the fermentation. Afterwards, 5 L of this second preculture was used to inoculate seed 3, which was cultivated in a 150 L fermentation bioreactor. The broth obtained from this seed fermentation was then completely used for the inoculation of the main fermentation. An overview of the process is shown at Figure 16.

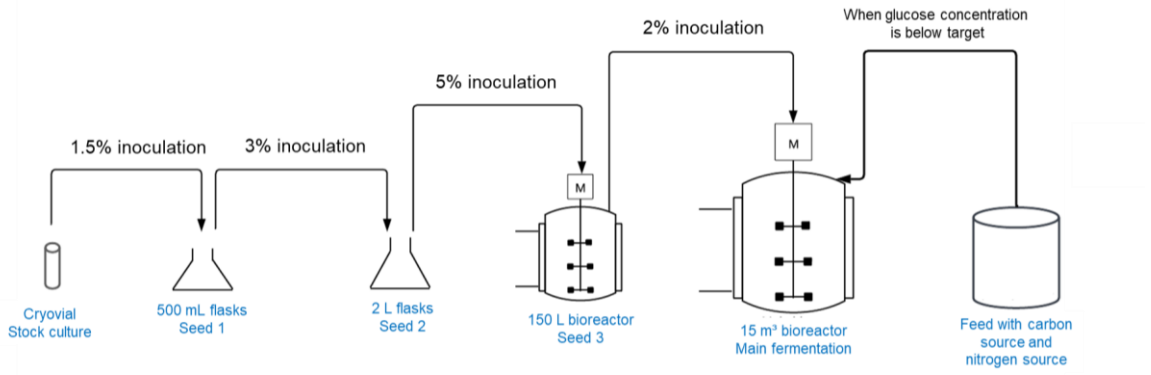


Figure 16: Process overview of BioSFerA-T4.4-F03.

5.2 TAG production on glucose in fed-batch fermentation (BioSFerA-T4.4-F03)

5.2.1 Seed 1 and seed 2 in shake flasks

The first seed step was performed in 500 mL flasks containing 100 mL of medium. One cryovial was transferred as inoculum to each shake flask. After 16 h of cultivation, the target OD was met and the seed was used as inoculum for the next stage. The second seed step consisted of ten 2 L shake flasks containing 500 mL of medium. Each shake flask was inoculated with 15 mL of seed 1. After 12 h of cultivation, the target OD was met, and the seed was used as inoculum for the next stage. No contamination was detected during the microscopic screening of the stained cells at the end of seed 1 and seed 2, as shown in Figure 17.

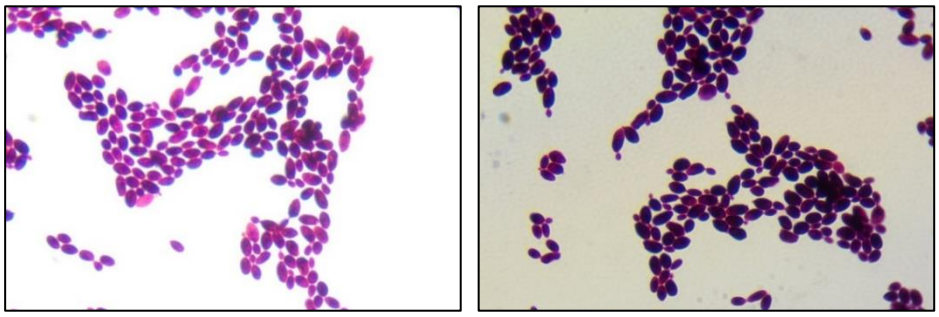


Figure 17: Microscopic picture (1000x) of Gram-stained *Y. lipolytica* cells at the end of seed 1 (left) and seed 2 (right).

5.2.2 Seed 3 in 150 L bioreactor

Seed 3 was performed in a 150 L fermentation bioreactor. The fermentation medium was used with shake flasks from seed 2 as inoculum. Growth started immediately leading to an OD of 240 and CDW of 54 g/L after 48 h (Figure 18). This exponentially growing seed culture was used to inoculate the main fermentation at 15 m³ scale. No contamination was detected after Gram-staining. A microscopic picture at the end of seed 3 is shown in Figure 19.

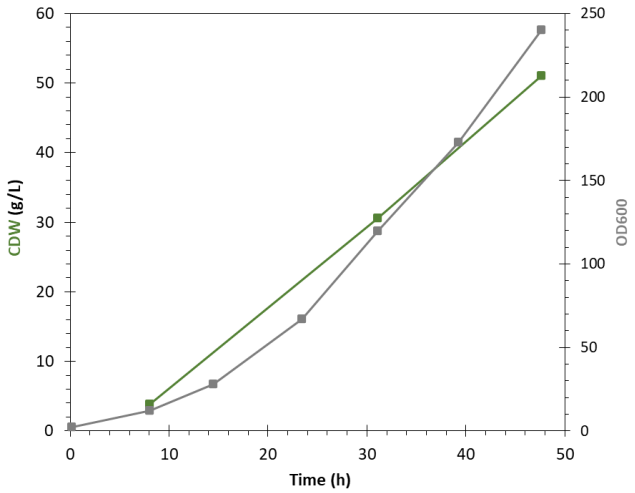


Figure 18: Offline data of seed 3 of BioSFerA-T4.4-F03

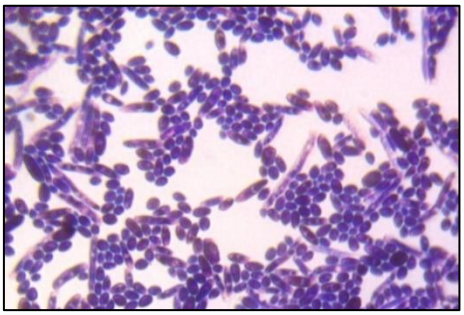


Figure 19: Microscopic picture (1000x) of Gram-stained *Y. lipolytica* cells at the end of seed 3.

5.2.3 Main fermentation in 15 m³ bioreactor

The main fermentation was performed in the 15 m³ fermentation bioreactor. The offline data is shown in Figure 20.

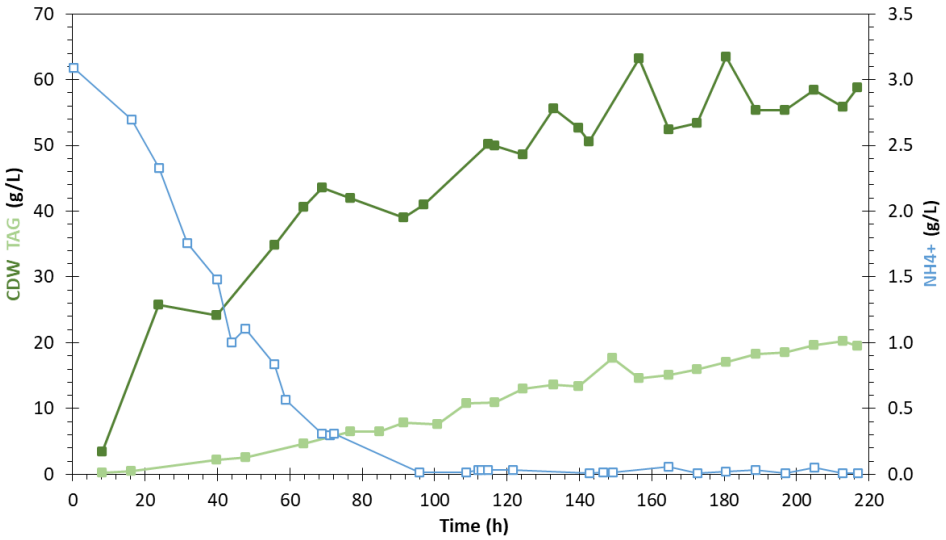


Figure 20: Offline data of the main fermentation of BioSFerA-T4.4-F03

In the first 8 h the culture showed little growth and the glucose was not being consumed at a high rate. After 16 h, the glucose concentration decreased in a manner that can be interpreted as a sign of accelerating growth together with the steeper increase in optical density and CDW. However, after 44 h, the consumption of glucose and increase in biomass concentration slowed down again. The performance of the culture on 15 m³ scale was thus inferior compared to the performance at 150 L scale and lab scale. This could be attributed to changes in several parameters inherently related to scale-up, such as variations in inoculum development, hydrostatic pressure, mixing, cooling, aeration, foaming.

Biomass production was increasing up until a stagnation of growth was observed with OD values around 220 and CDW in the range of 50-65 g/L. TAG production was observed from the start of the fermentation and the cells significantly started to accumulate the lipids from the point of nitrogen limitation with a productivity of 0.12 g/L/h resulting in 19 g/L TAG end concentration. A maximum OUR of approximately 42 mmol/L/h was obtained during batch growth. Afterward, the OUR showed first a sudden then a gradual decrease and it stabilized around 26 mmol/L/h in the last three days.

The fermentation was performed until DSP was scheduled to start, although the final fermentation volume was not reached yet. After 216 h of fermentation, the feed was stopped, thereby ending at a final volume of 6.1 m³ and a

final TAG concentration of 19 g/L. The sterility of the fermentation was checked regularly by performing a Gram-staining. No contamination was observed until the end of the fermentation, as shown in Figure 21.

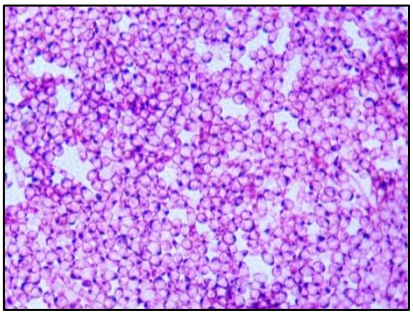


Figure 21: Microscopic picture at the end of the main fermentation. The picture shows cells after Gram-staining (magnification 1000x).

6 Scale-up of TAG purification to 15 m³

6.1 Process overview

The fermentation broth of BioSFerA-T4.4-F03 performed in the 15 m³ bioreactor was handled for the extraction and purification of the TAG. The process was designed similarly to the DSP at 150 L scale, thus as presented in Figure 10. More specifically, the biomass was first concentrated on the HARI ceramic tangential flow filter unit to obtain a higher cell density and diafiltration was performed to wash the cells (step 1). This concentration step improves homogenization efficiency in the next step and decreases the total processing volume. The concentrated biomass was divided into two batches, which were each homogenized separately by the Rannie homogenizer (step 2). Both batches passed the homogenizer to break the cells and release TAG. Then, the broth was transferred to the ATEX R5400 reactor where solvent and co-solvent were added to extract the TAG (step 3). The water phase was removed by centrifugation (step 4), while the solvent phase was maintained in the R5400 to evaporate (step 6). Finally, the remaining concentrated oil was filtered to obtain clarified TAG (step 6). An overview of the process performed at 15 m³ scale is illustrated in Figure 22 and pictures of the used pilot-scale equipment are shown in Figure 23.

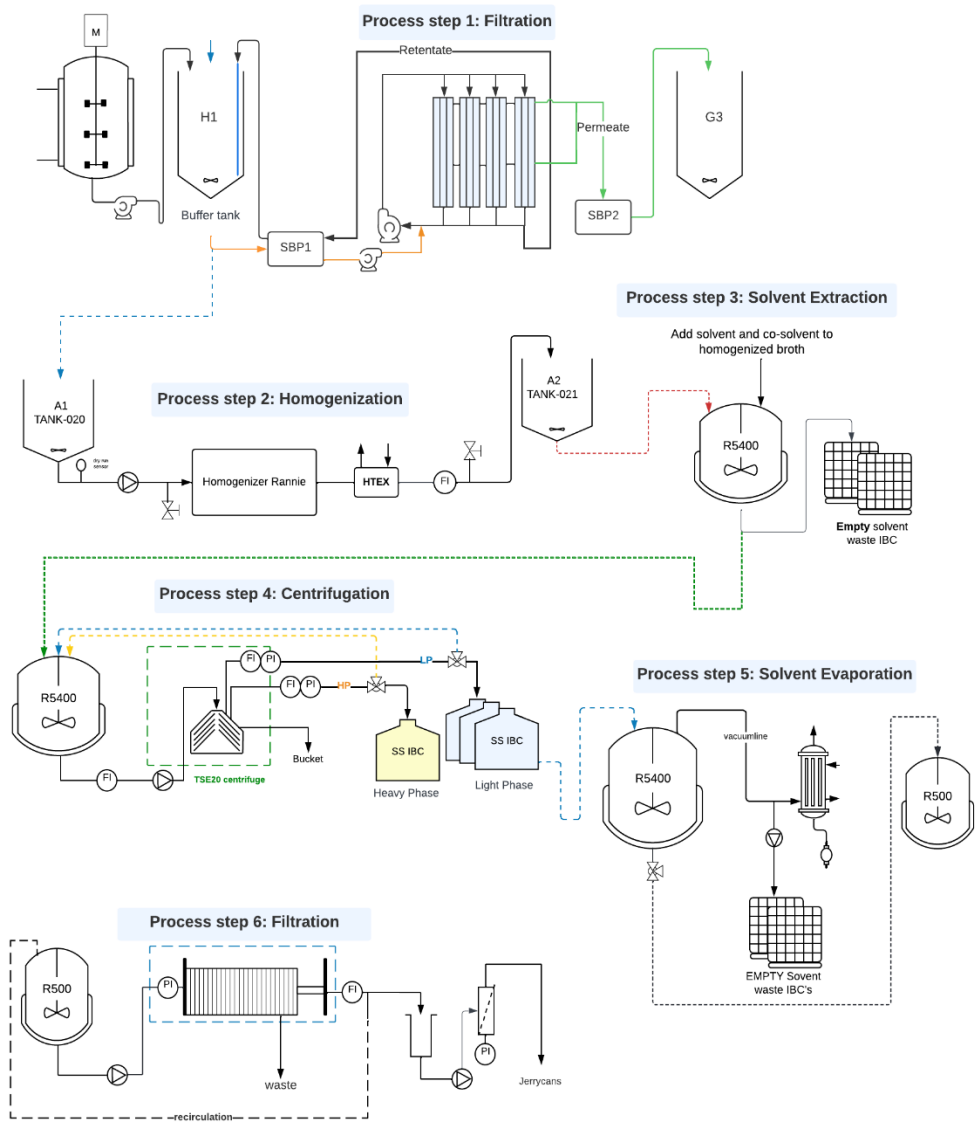


Figure 22: Process overview of the DSP of BioSFerA-T4.4-F03.

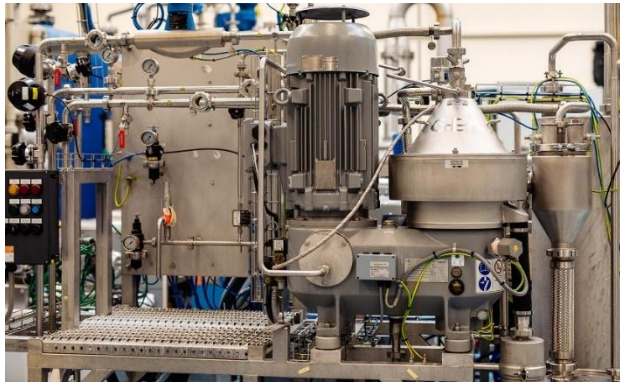
A 

B 

C 

D 

E 

F 

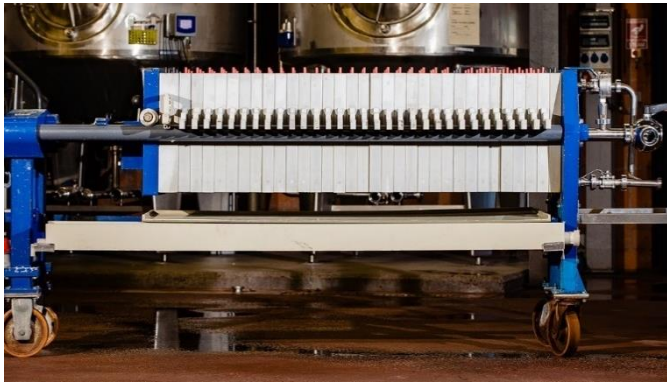
G 

Figure 23: Overview of some of the equipment used during scale-up of TAG purification to 15 m³: Hari ceramic filter (A), Rannie homogenizer (B), R5400 chemical reactor (C), A1 and A2 storage tanks (D), vacuum pump (E), Westfalia TSE20 centrifuge (F), Schenk chamber filter press (G).

6.2 Starting material: Broth from BioSFerA-T4.4-F03

The broth from BioSFerA-T4.4-F03 was used as starting material. During this fermentation 6.1 m^3 of culture was obtained consisting of 59 g/L CDW containing 32% TAG, thus 116 kg TAG in total.

6.3 Step 1: Biomass concentration by filtration

The broth from BioSFerA-T4.4-F03 was first pumped from the fermentation bioreactor into a buffer tank. Foam formation was observed during this transfer (Figure 24). As a result, 5750 kg broth was harvested and 5925 kg of RO water was added to rinse out the bioreactor. This resulted in a TAG concentration of 11 g/L. The addition of water can be considered as the first diafiltration step.

Figure 24: Foam in buffer tank after harvest of BioSFerA-T4.4-F03.

The diluted broth was concentrated to 7555 kg before the start of the second diafiltration. Next, two continuous diafiltrations were executed. After diafiltration, the broth was finally concentrated to 4077 kg of retentate. An overview of the process targets and results of the first step is given in Table 2.

Table 2: Mass balance of biomass concentration by microfiltration of BioSFerA-T4.4-F03-DSP (step 1).

Type	Parameter	Unit	Target	Actual value
Input	Broth	kg	10 000	5750
	RO Water	L	15000	15000
Output	Retentate	kg	4000	4077

6.4 Step 2: Biomass disruption by homogenization

The concentrated biomass was divided into two batches, which were each homogenized separately on the Rannie homogenizer. For batch 1 (2018 kg), only one pass was performed because a leak occurred during processing. For batch 2 (1871 kg), two passes were performed, as planned. An overview of the process targets and results of the first step is given in Table 3.

Table 3: Mass balance of cell disruption by homogenization of BioSFerA-T4.4-F03-DSP (step 2).

Type	Parameter	Unit	Actual value
BATCH 1			
Input	Retentate	kg	2018

	RO Water	L	0
Output	Homogenized broth	kg	1997
BATCH 2			
Input	Retentate	kg	1871
	RO Water	L	50
Output	Homogenized broth	kg	1914

Table 4 presents an overview of the homogenization efficiency of both batches. It is noted that almost twice as much TAG is liberated from the cells in batch 2 compared to batch 1. It can thus be concluded that multiple passes through the homogenizer are accompanied by an increase in homogenization efficiency. This was also observed during the lab-scale experiments, as described in BioSFerA deliverable 3.6.

Table 4: Homogenization efficiencies of BioSFerA-T4.4-F03-DSP (step 2).

	Passes	Free TAG (kg)	Total TAG (kg)	Efficiency
Batch 1 homogenized broth	1	5.86	50.34	12%
Batch 2 homogenized broth	2	15.23	49.57	31%

6.5 Step 3: Solvent extraction

The TAG in the concentrated homogenized broth is extracted into a solvent in the chemical reactor R5400. The same solvent, co-solvent, and ratios were used as for the 150 L scale DSP. The mixture was stirred at an increased temperature for improved extraction. Afterward, the temperature was decreased to allow for phase separation. Figure 25 shows a picture of a centrifuged sample at the end of the extraction.

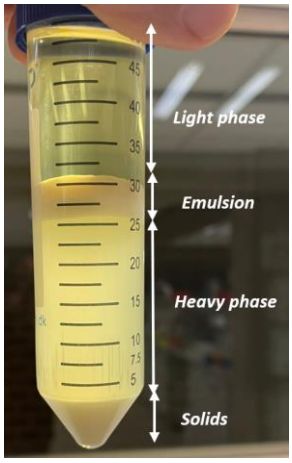


Figure 25: Centrifuged sample after solvent extraction (batch 1). Four different phases could be distinguished.

After extraction and settling, it was tried to separate the heavy phase from the light phase of batch 1 (Figure 26). 120 kg of heavy phase was collected in an IBC and a sample was analyzed which contained already 30% of the light phase. Since no proper phase separation occurred in the reactor, the phase separation was stopped, which resulted in the collection of 350 kg of heavy phase in total. A sample was taken from the R5400 and only contained 2.5 vol% of solids (= 90 L solids), which indicated that most of the solid cell debris was already collected in the plastic IBC. Since the complete mixture during solvent extraction contained approximately 9 vol% of solids, it could be concluded that most solids were in the IBC. The heavy water phase with a lot of cell debris was collected and

the volume remaining in the reactor was used for the next process step. For batch 2, 400 kg of heavy phase with solids was collected.

Figure 26: Set-up for separating heavy phase from the light phase.

At this point in the process, the remaining water-solvent mixture of batch 1 contained ± 55 kg TAG and batch 2 ± 50 kg TAG. In total, the collected heavy phases of both batches together contained ± 10 kg of total TAG. The heavy phases were discarded which means that this step had a yield of 90%. The mass balance of this process step is given in Table 5.

Table 5: Mass balance of solvent extraction of BioSFerA-T4.4-F03-DSP (step 3).

Type	Parameter	Unit	Actual value
BATCH 1			
Input	Homogenized broth	kg	1997
	Co-solvent	L	200
	Solvent	L	2000
Output	Solvent-water mixture	kg	3618
	Heavy phase with solids	kg	340
BATCH 2			
Input	Homogenized broth	kg	1933
	Co-solvent	L	200
	Solvent	L	2000
Output	Solvent-water mixture	kg	3494
	Heavy phase with solids	kg	400

6.6 Step 4: Oil separation by centrifugation

After the decantation of the heavy phase with solids from the water-solvent mixture, the water phase was separated from the solvent phase using the TSE20 centrifuge (Figure 27). Both batches were processed separately. During parameter optimization, the heavy phase and light phase were recirculated back to the feed tank. During production, the heavy phase and light phase were collected in separate IBC's. The mass balance of this process step is given in Table 6. The light phase of both batches contained together around 50 kg of free TAG, while the removed heavy phase and the solids contained also around 50 kg of free TAG. This shows that the extraction efficiency of the TAG into the solvent and subsequent separation had a yield of only 50%.

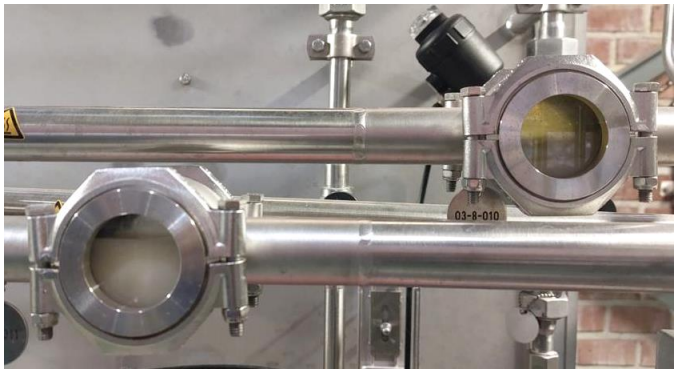


Figure 27: Sight glasses of TSE20 centrifuge with separation of the heavy phase (left) and light phase (right).

Table 6: Mass balance of oil separation of BioSFerA-T4.4-F03-DSP (step 4).

Type	Parameter	Unit	Actual value
BATCH 1			
Input	Solvent-water mixture	L	3618
Output	Heavy phase	kg	1831
	Light phase	kg	1489
	Solids	kg	267
BATCH 2			
Input	Solvent-water mixture	L	2000
Output	Heavy phase	kg	1637
	Light phase	kg	1577
	Solids	kg	239

After processing both batches, a sample was obtained from the light phase. The light phase contained emulsion which settled down gravitationally very easily (Figure 28). Therefore, it was decided to pour the light phase of both batches together in reactor R5400 and subsequently decant the emulsion part into an IBC. The mass balance of this additional process step is given in Table 7. The decanted emulsion only contained 0.6 kg of total TAG.

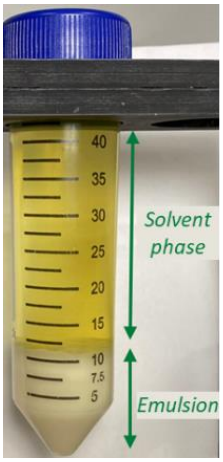


Figure 28: Sample of light phase after centrifugation.

Table 7: Mass balance of emulsion removal of BioSFerA-T4.4-F03-DSP (additional step).

Type	Parameter	Unit	Actual value
Input	Solvent-emulsion mixture	kg	3066
Output	Solvent mixture	kg	2674
	Emulsion	kg	239

6.7 Step 5: Oil recovery by solvent evaporation

To recover the oil, the solvent was evaporated in R5400 (Figure 29). When the volume in R5400 decreased below the stirrer, the mixture was transferred to R500 for further evaporation. The mass balance of this process step is given in Table 8. TAG analysis showed that the condensate was indeed free of oil, hence, as oil was totally recuperated in the concentrate.

Figure 29: Evaporation in R5400.

Table 8: Mass balance of solvent evaporation of BioSFerA-T4.4-F03-DSP (step 5).

Type	Parameter	Unit	Actual value
Evaporation in R5400			
Input	Solvent mixture	kg	2018
Output	Condensate	kg	2490*
	Concentrate	kg	184*
Evaporation in R500			
Input	Solvent mixture	kg	184*
Output	Condensate	kg	119*
	Concentrate	kg	65*

**Calculated values based on other measurements.*

6.8 Step 6: Oil clarification by filtration

Before filtration, the oil was heated to 43°C where total liquefaction occurs. After heating, a mixed sample was taken and spun down. This sample contained around 45% solids. The feed was recirculated over the chamber filter press until a clear filtrate was obtained. When everything was filtered, the chamber filter press was blow-dried. However, during blow drying, a breakthrough of solids was observed toward the filtrate. Pictures of the product during processing are shown in Figure 30.

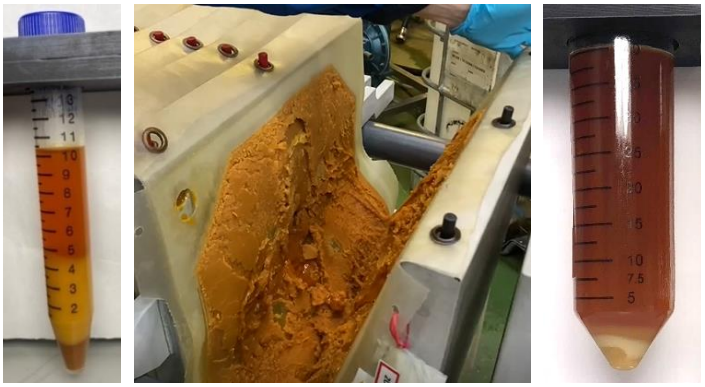


Figure 30: Pictures taken during filtration for oil clarification: centrifuged sample before filtration, cake retained on the chamber filter press, centrifuged sample after filtration which contained solids because of breakthrough (from left to right).

To remove the solids caused by the breakthrough, another filtration was performed over a bag filter. Yet, due to a decrease in the temperature of the product, the filters got clogged and some product was lost. The remaining product was therefore again collected in R500 and heated up to 55 °C in an oven and sieved manually (Figure 31). The mass balance of this process step is given in Table 9. Hence, while 37 kg of oil could potentially be recovered, only 22 kg of the product remained because of losses due to solidification.

Figure 31: Sieving of the filtrate after chamber filter press.

Table 9: Mass balance of filtration and sieving of BioSFerA-T4.4-F03-DSP (step 6).

Filtration over chamber filter press and sieves			
Type	**Parameter**	**Unit**	**Actual value**
Input	Concentrate after evaporation	kg	86
Output	Concentrate (solids)	kg	49
	Losses due to solidification	kg	15
	Concentrate (oil)	kg	22

7 Conclusions and future prospects

The TAG production process consisting of the fermentation of *Y. lipolytica* and DSP of TAG was successfully scaled from lab to pilot scale up to 15 m³ within BioSFerA task 4.4. Therefore, two fermentations at 150 L scale (BioSFerA-T4.4-F01 and BioSFerA-T4.4-F02) and one fermentation at 15 m³ scale (BioSFerA-T4.4-F03) were performed with DSP of F02 and F03. The main conclusions resulting from the scale-up of the fermentation and DSP process as well as suggestions for future developments are listed below.

Conclusions from pilot-scale *Y. lipolytica* fermentations for the production of TAG from glucose:

- The process settings which resulted from the lab-scale process optimization experiments within BioSFerA T3.4 (described in BioSFerA deliverable 3.5) could be applied at a larger scale. In addition, thanks to advanced process control, TAG production could be improved.
- All performed fermentation runs at pilot scale were successfully executed. No contamination and no major process issues occurred. This shows the robustness of the process and the scale-up potential of the *Y. lipolytica* fermentation for TAG production.
- The highest production of TAG from glucose was obtained on 150 L scale in BioSFerA-T4.4-F01, namely 31 g/L TAG with a maximal productivity of 0.21 g/L/h.
- At 15 m³ scale, slower growth and lower production was observed. This could be attributed to changes in several parameters inherently related to scale-up, such as variations in inoculum development, hydrostatic pressure, mixing, cooling, aeration, foaming, etc.
- The 15 m³ fermentation run was performed to produce oil for hydrotreatment tests which will be performed in BioSFerA tasks 5.2-5.3.
- During the 15 m³ fermentation, the cells consumed slower and the feed was not fully added by the end of the fermentation. The final volume was 6.1 m³ instead of the target 10 m³ and the final concentration was 19 g/L instead of 30 g/L. Hence, a total of only116 kg TAG was produced, which was below the target of 300 kg.

Conclusions on pilot-scale DSP for extraction and purification of TAG:

- The DSP strategy developed in BioSFerA task 3.6 and described in deliverable 3.5, consisting of (1) biomass concentration by filtration, (2) cell disruption by homogenization, (3) solvent extraction, (4) phase separation by centrifugation, (5) oil recovery by evaporation, was successfully scaled. In addition, a filtration step was performed to purify the extracted oil.
- From the 150 L fermentation in which 2.3 kg TAG was produced, 0.68 kg TAG could be extracted and purified. The obtained recovery of 29% was in line with the results obtained on lab-scale (described in BioSFerA deliverable 3.6).
- From the 15 m³ fermentation in which 116 kg TAG was produced, 22 kg TAG could be extracted and purified. The lower recovery of 19% originates from solidification issues during the final filtration step. Because of a breakthrough at the end of the filtration process, additional handling was required and the temperature of the product decreased while processing. Since the product solidifies at room temperature, it got stuck in the equipment, hence the losses.

- Disregarding the losses due to solidification, 37 kg of oil could have been recovered from the 15 m³ run, which corresponds to a recovery of 32%. It can thus be concluded that the DSP protocol developed by BBEPP in BioSFerA task 3.6 and further optimized in task 4.4 was indeed designed in a fully scalable manner.

Future prospects for the scale-up of the TAG production part of the BioSFerA project:

- The production of TAG by *Y. lipolytica* from glucose was successfully scaled. However, it should be noted that the BioSFerA concept requires the use of acetic acid. As found in the lab-scale experiments of BioSFerA task 3.5, the *Y. lipolytica* strain needs to further improved to allow for high TAG production from acetic acid and make scale-up feasible.
- More advances in optimization of the strain and the fermentation process could potentially increase the TAG content of the cells. A higher TAG content will improve the final product concentration in the fermentation. In addition, this could also have a beneficial effect on the efficiency of the DSP train.
- A relatively low efficiency of the cell disruption was identified as the main bottleneck in the TAG extraction process. Hence, investigations into alternative cell disruption techniques are ongoing. Additional pilot-scale fermentations were performed to get more broth for additional cell disruption tests which are performed by ENVIPA and BBEPP.
- The produced TAG solidifies at room temperature. It was observed that this can have negative impacts on the overall purification processing. This should be taken into account in future work, and this effects can be avoided by increasing the operating temperature, keeping thus TAG away from solidification.

Next steps in other parts of BioSFerA project from this work:

- The oil produced from the 15 m³ run was shipped to CERTH for hydrotreatment tests which will be performed as a part of BioSFerA workpackage 5. Preliminary analysis of the microbial oil obtained from 150 L run F02 performed by KPRT showed that the quality meets the requirements.
- The insights obtained during these pilot-scale runs serve as input for the establishment of a full chain process model and cost engineering which are being developed within BioSFerA tasks 6.2 and 6.3, respectively . More specifically, the operational aspects such as the required process steps and the process data are shared.
- The process data will also be valuable for BioSFerA workpackage 7 to perform the techno-economic, market, environmental, social, and safety assessments.