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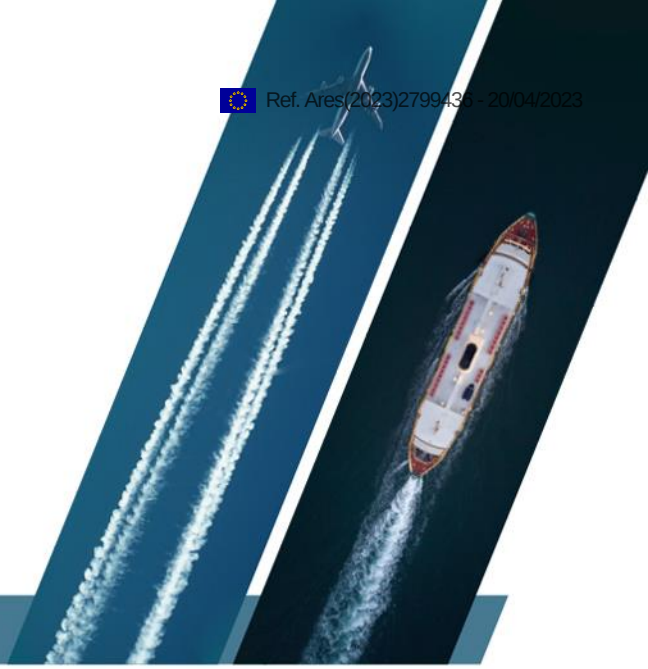
Biofuel for biotravels



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Deliverable 3.6

Lab scale downstream processing for TAGs recovery and purification using conventional and novel strategies

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Abbreviations

BBEPP	Bio Base Europe Pilot Plant
TAG	Triacylglycerol
GC	Gas chromatography
MS	Mass spectrometry
MeTHF	2-methyltetrahydrofuran
HPH	High-pressure homogenization
TMP	Transmembrane pressure

Contents

1 Executive Summary

This report describes the advances of BioSFerA task 3.6. Task 3.6 focuses on the exploration of different techniques for the extraction and purification of triacylglycerol (TAG), which was produced by fermentation and occurs intracellularly in the yeast *Y. lipolytica*, on lab scale. The fermentation process was developed within BioSFerA task 3.5, hence, the broth produced during those experiments was used as input stream for this work. This report aims to conclude all findings into an efficient and technically feasible method for TAG extraction. This will be used as a starting point for the scale-up of the process which will be performed in BioSFerA task 4.4.

All work was performed by BBEPP and EnviPark. A summary of their main experiments and insights is given below:

BBEPP focused on:

1. Development of a **TAG extraction method for analysis purposes**. A protocol was developed consisting of the following steps:
    - Efficient cell disruption by harsh conditions, namely acid hydrolysis with HCl at 80 °C
    - TAG extraction through the addition of butylacetate
    - TAG methylation with methanol at 80 °C
    - Recovery of the formed methyl esters in butylacetate
    - Quantification of the methyl esters by gas chromatography mass spectrometry (GC-MS)
2. Development of a **downstream processing (DSP) train** consisting of several unit operations to isolate pure TAGs suitable for hydrotreatment, which will be evaluated in BioSFerA workpackage 5. Therefore, various techniques were investigated for each step of the process:
    - Biomass concentration: centrifugation, microfiltration
    - Cell disruption: homogenization, sonication (focus on mechanical instead of enzymatic and chemical techniques envisioning a sustainable and economically feasible process)
    - TAG extraction with solvents: different solvents were screened with extraction under various conditions (temperature, pH, …), prevent emulsion formation by addition of salts and co-solvent.
    - Separation of the water and solvent phase: decantation, centrifugation

    First exploratory experiments were performed, followed by more in-depth experiments which focused on the bottlenecks of the process. These mainly included the cell disruption, as it was found that the TAG-containing yeast cells were very robust, and the solvent extraction. It was found important to select a solvent that was industrially feasible for scale-up, thereby considering safety (avoiding highly toxic solvents), environmental (preferably green solvents), and technical (focusing on solvents with low boiling point and good phase separation) aspects.

    Conclusively, BBEPP defined a suitable DSP train for the scale-up of TAG extraction which will be performed in BioSFerA task 4.4. The strategy consists of: (1) biomass concentration and washing by microfiltration and diafiltration, (2) cell disruption by homogenization (up to 70% cell disruption), (3) TAG extraction with ethyl-acetate using acetone as co-solvent to reduce the formation of emulsion (with at least 26% recovery), (4) Recover the solvent phase by decantation or centrifugation, (5) obtain pure TAG by evaporation of the solvent.

Envipark focused on:

1. Development of an alternative recovery strategy of TAGs using a steam explosion plant for physical extraction;
2. Identification of the best purification technique for the extracted TAGs using different separation method.
3. Evaluation of results by analysing the concentration of fatty acids, total amount and concentration of each TAG in the different fractions obtained

Some different tests on a steam explosion pilot plant have been performed in order to verify the yeasts fractionation and yield of lipids extraction to improve subsequent recovery and purification steps. In addition, some pilot tests with centrifuge and membrane purification process have been done in order to define the optimal process path for the industrial scale up and effectiveness of these technologies in this field of application.

Steam explosion tests were conducted by varying parameters such as the volume of *Yarrowia* used and operating pressure and temperature.

Micro and nanofiltration membrane systems were used for TAG separation tests on the product of steam explosion.

In subsequent tests, the use of centrifuge and filtration equipment before the steam explosion has also been evaluated.

All the samples have been analysed by Envipark using an analytical kit for total TAGs quantification.

In addition all the samples have been sent to an external laboratory able to quantify each fatty acid and TAGs as for total amount as well as for quantification of each triglyceride.

Results of internal analysis are reported and commented in this deliverable.

Some of the external laboratory results are in this report, others that are still in progress will be reported in Deliverable 4.5.

2 Introduction

One of the bottlenecks encountered during the processes of biofuels production from oleaginous yeast biomass is the lipid extraction costs. Cells disruptions alongside lipid extraction steps are critical for large-scale biofuel production in reference to cost adequacy.

The methods for extraction and purification of lipids from yeasts broth is to be identified and optimized from a technological and economic point of view. Scope of this task is the pre-treatment optimization and subsequent evaluation of the potential improvement in selected lipids production from the fermentation steps; this action will focus on lipids separation pretreatments potentials and pathways, by applying the technologies actually available and considering different process parameters and extraction procedures. In general, the yeast species have dense and rigid cell wall compared to the bacteria and are more similar to the algae cell wall, due to the presence of complex and long chain polymers such as chitin, mannan, glucan and glycoprotein which are very hard to disrupt. For this reason, strong mechanical forces are required for obtain the disruption of the cells wall of the oleaginous yeasts for the subsequent extraction of lipids. Concerning the actual knowledge about lipid extraction, the challenges that the project face, concern to bridge the gap between the preliminary results obtained, and the feasibility of this method for the industrial scale-up through the implementation of this sustainable extraction technique.

Several techniques are employed for the effective purification of the produced lipids. Indeed, as the oil forms intracellular for storage purposes, extraction is required to obtain TAGs. In this task various purification techniques have been evaluated and tested aiming the best of them to be chosen for upscaling in the following activities of WP4.

At first, BBEPP tested a lab scale downstream processing to extract, purify and analyse the total TAGs content from the fermentation broth. BBEPP evaluated an acid extraction followed by solvent separation and a centrifugation/microfiltration followed by sonication and homogenization followed by solvent separation techniques. At the same time, ENVPA investigated an alternative purification strategy based on steam explosion and centrifugation /membrane separation through a series of pilot scale tests in order to evaluate if these physical treatments could achieve better global results in terms of products yield. Beside this, some recovery and purification optimal pathways (centrifuge and filtration steps, singular or in combination) have been explored in order to enhance the yields of lipids recovery.

3 DSP for TAG analysis (BBEPP)

For TAG analysis purposes, the yeast cells were first disrupted under harsh conditions by hot acid hydrolysis with highly concentrated hydrochloric acid. For this, 100 µL sample of the broth fermentation was transferred into a glass vial and mixed with 200 µL of water and 300 µL of 12 M HCl. The mixture was then heated at 80°C for 2 hours. Afterward, the extracted TAG molecules were converted by transesterification with methanol. For this, the sample was cooled down to room temperature and mixed with 300 µL of methanol and 300 µL of internal standard solution dissolved in butylacetate. It was then centrifuged at 2000 rpm for 5 minutes to get phase separation. The upper layer was transferred to a new glass vial and 250 µL of 2.5% H_2SO_4 in methanol solution was added. The mixture was heated at 80°C for 2 hours and cooled down to room temperature. After cooling, 500 µL of 1 M NaCl and 250 µL of internal standard solution in butylacetate were added to the mixture, which was then allowed to stand for 15 minutes until phase separation appeared. Finally, 100 µL of the upper layer was transferred to a new glass vial for GC-MS analysis. An overview of the sample treatment is shown in Figure 1.

Figure 1: Step-by-step process for conversion of intracellular TAGs into its methyl-esters for analysis by GC-MS.

The prepared samples were analyzed with by gas chromatography (GC) (Trace GC 2000, ThermoQuest Italia S.p.A.) equipped with a robotic injector (PAL system, CTC Analytics AG), a capillary column (30 m x 0.25 mm x 0.25 µm, Rxi-5Sil MS, Restek), and a mass spectrometry (MS) detector (Trace DSQ) using electron ionization and selected ion monitoring as scanning mode. The injection volume was 1 µL. Helium was used as the mobile phase at a constant pressure of 70 kPa. The column temperature was increased with a gradient from 70 to 300°C. Quantification of the methyl-ester compounds was achieved with the use of an internal standard (undecanoate) and a calibration curve prepared by using a certified reference FAME standard mix (AOCS FAME Mix RM-6, Supelco, Merck). Xcalibur (ThermoFisher) software was used for data acquisition and processing. By following these steps, we were able to extract and esterify the TAG molecules to quantify them and determine the lipid profile by GC-MS analysis. This analysis was also performed within BioSFerA task 3.4. As such, the fatty acid profile of the TAG produced during all fermentation process was reported in BioSFerA deliverable 3.5.

4 DSP for TAG isolation (BBEPP)

4.1 Experimental plan and equipment

For isolation purposes, a range of DSP techniques for each step of the process was assessed thereby aiming to develop a sustainable and scalable method. This also included the combination of different techniques to increase the overall lipid recovery. A schematic overview of the considered routes is illustrated in Figure 2.

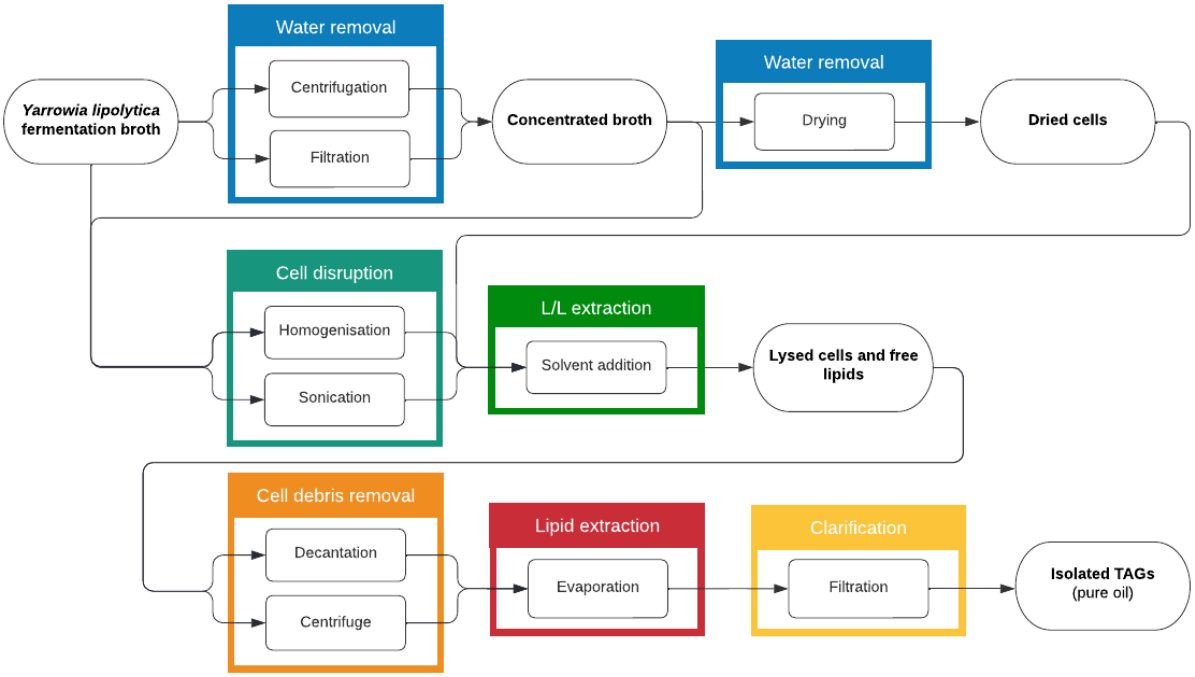


Figure 2: Overview of the different routes considered for TAG isolation.

As concerns the DSP of microbial lipids, wet or dry extraction is possible. While solvent extraction on dry cells is an effective method, dewatering methods (such as heating, spray drying, or freeze drying) have a high energy consumption, hence, wet extraction was chosen as the more industrially feasible route. Nevertheless, a concentration step might be interesting to remove part of the water thereby also decreasing the working volume. For this, centrifugation and microfiltration were investigated. As concerns cell disruption, two mechanical techniques, namely homogenization, and sonication, were evaluated. When the lipids are released from the cells, they can be extracted through the addition of a solvent. A range of solvents was screened for the most efficient extraction, including ethanol, isopropanol, methanol/chloroform, heptane, ethyl acetate, butyl acetate, amyl acetate, 2-methyl tetrahydrofuran, and dimethyl carbonate. Afterward, the solvent was evaporated to retain the TAG. Finally, also the need for a final clarification step by filtration was evaluated. Figure 3 presents some state-of-the-art DSP equipment used for TAGs purification at BBEPP.

Figure 3: Equipment used for DSP of TAG at BBEPP: ceramic filtration unit, high-pressure homogenizer, sonicator, rotavapor (from left to right).

4.2 Exploratory experiments

4.2.1 Starting material

For a first set of exploratory DSP experiments, fermentation broth from BioSFerA -T3.5-BBEPP-F09 (fermentation performed within BioSFerA T3.4 and reported in BioSFerA deliverable 3.5) was used. The broth consisted of acetate-grown Y. lipolytica yeast cells with a cell dry weight (CDW) concentration of 46 g/L and a TAG content of 13%.

4.2.2 Mechanical cell disruption

It was found that sonication was not effective in breaking the cells. Better results were obtained with high-pressure homogenization (HPH). After four cycles of HPH at 800 bar, a substantial amount of cells were damaged as seen from the microscopy pictures (Figure 4). The high shear stress applied during the HPH treatment leads to the disruption of the cells and the subsequent release of their content. However, *Y. lipolytica* shows some resistance to this pretreatment, as some of the cells are still intact at these conditions. It is known that oleaginous yeast cells possess a rigid cell wall consisting of high fractions of mannoproteins and chitin, which renders them more resistant to cell lysis.

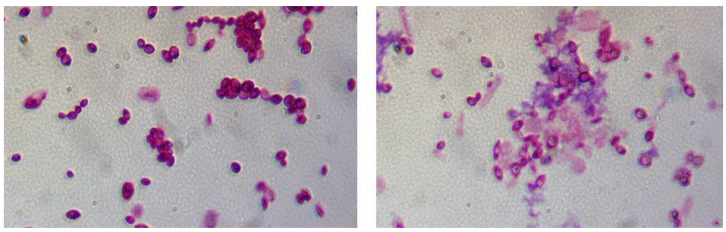


Figure 4: Microscopy pictures of the sample before and after HPHnop (800 bar, 4 cycles) at BBEPP.

The lipid recovery after HPH treatment was quantified by centrifuging the samples and analyzing the TAG concentration in the supernatant. The lipid recovery increased over the subsequent HPH cycles (Figure 5). After four cycles, approximately 15% of the lipids were out of the cells. As can be seen from the rising trend, more cycles could be beneficial. This was evaluated in follow-up experiments. Yet, in this context, economic and technical aspects should also be considered.

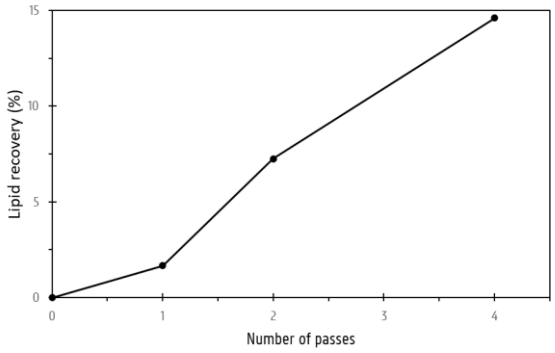


Figure 5: Lipid recovery from the samples after several cycles of homogenization at 800 bar.

4.2.3 Solvent screening for TAG extraction

After four cycles of HPH at 800 bar, the extraction of TAG using different solvents was evaluated. The selection of appropriate solvents for the screening was based on a literature search. Traditionally, hexane is the preferred solvent for lipid extraction (Imatoukene et al. 2020). It is however considered to be highly toxic and damaging to the environment. Heptane can be used as a less toxic counterpart of n-hexane. In the following extraction experiments, n-heptane and a chloroform/methanol mixture are used as a benchmark for lipid extraction since they are often used on lab-scale to quantify lipids in small samples. Alternatively, ester solvents such as ethyl acetate, butyl acetate, and amyl acetate were chosen because of their cost-effectiveness and safety. Moreover, these solvents are regarded as “green” alternatives. In addition, 2-methyltetrahydrofuran (MeTHF) was also considered as it can be derived from renewable resources and has shown to be a promising alternative to n-hexane (Breil et al. 2016).

For the screening, 2 mL of solvent was added to 2 mL of the sample. After mixing for 4 hours at 30 °C or 50 °C, the samples were left to settle for 24 hours and the volume of the separated solvent phase was estimated visually (Figure 6). In addition, the TAG content of this phase was measured by GC-MS analysis, hence, the recovery could be calculated (Figure 7).

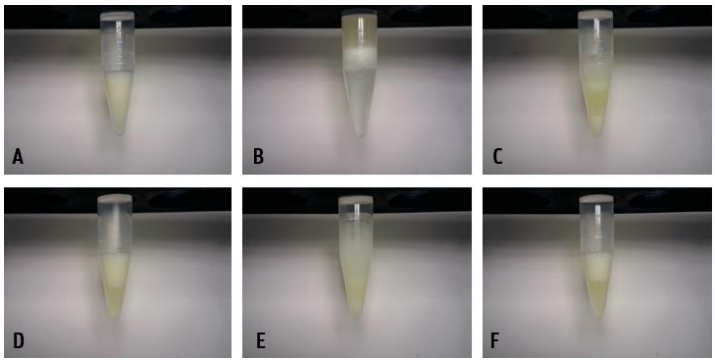


Figure 6: Samples after extraction with different solvents: n-heptane (A), chloroform/methanol (2:1) (B), MeTHF (C), butyl acetate (D), ethyl acetate (E), amyl acetate (F).

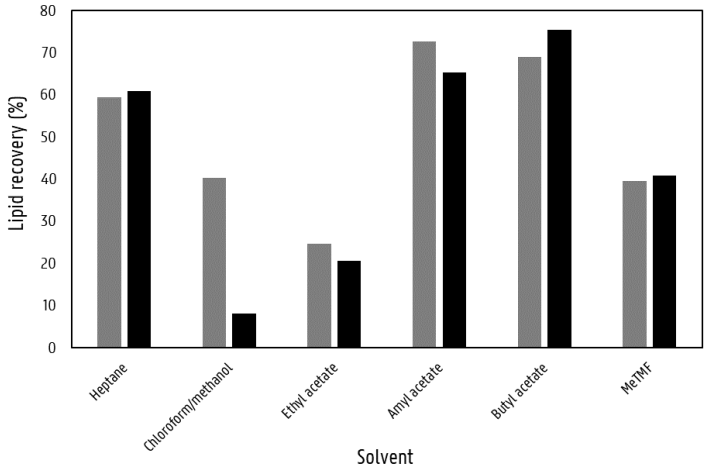


Figure 7: Lipid recovery from the samples after solvent extraction at 30 °C (grey) and 50 °C (black).

The ester solvents amyl acetate and butyl acetate show a remarkably higher lipid recovery than the benchmark conditions. Moreover, for most solvents, comparable lipid recoveries in both extraction conditions were found. Amyl acetate showed a higher lipid recovery of up to 73% at 30 °C, while butyl acetate shows the highest lipid recovery of up to 75% at 50 °C. In contrast, ethyl acetate has a notably lower recovery than the ester solvents. This could be attributed to the low solvent phase volume was observed, as there is no clear distinction between the solvent phase and the aqueous phase. It is important to note that this is contradictory to previous oil extractions performed on *Y. lipolytica*. Imatoukene et al. (2020) found that lipid recovery using ethyl acetate was comparable to other ester solvents. Although no conclusive decision can be made as these experiments were exploratory and show inconsistency in relation to data found in the literature, these results indicate that ester solvents appear to be a promising, green alternative to the conventional solvents.

Next to the recovery, also other factors should be considered to ensure a feasible industrial process. As such, given the nature of the complete TAG DSP process, the boiling point of the solvent is preferably low as it needs to be evaporated to retain TAG in the end. An overview of some important solvent properties to consider is given in Table 1.

Table 1: Overview of the properties of solvents considered for TAG extraction. With industrial feasibility defined by Joshi and Adhikari (2019) (3: Undesirable, 2: Usable, 1: Preferred at industrial scale), and following the hazards classification by the National Fire Protection Association (NFPA).

	Industrial feasibility	Health hazard	Flammability hazard	Instability hazard	Boiling point (°C)	Viscosity (mPa.s)	Density (kg/m³)	Water solubility (g/L)
Heptane	2	1	3	0	98	0.40	0.68	< 1
Chloroform	3	2	0	0	61	0.56	1.39	8.1
Methanol	1	1	3	0	65	0.55	0.79	Miscible
Ethyl acetate	1	1	3	0	77	0.42	0.90	8.3
Butyl acetate	1	2	3	0	126	1.00	0.88	6.8
Amyl acetate	-	1	3	0	142	0.69	0.89	3.0
MeTHF	2	1	3	1	80	0.60	0.85	41
Dimethylcarbonate	-	3	3	0	90	0.66	1.07	14
Isopropanol	1	1	3	0	83	1.96	0.79	Miscible

4.3 Development of a scalable DSP strategy

4.3.1 Starting material

For a next set of DSP experiments, fermentation broth from BioSFerA -T3.5-BBEPP-F12 (fermentation described in BioSFerA deliverable 3.5) was used. The broth consisted of glucose-grown *Y. lipolytica* yeast cells with a cell dry weight (CDW) concentration of 78.4 g/L and a TAG content of 29%. The complete broth (7.16 kg) containing 163 g TAG was used for TAG extraction.

4.3.2 Water removal

The inclusion of a water removal step before cell disruption was evaluated. This could be advantageous because: (1) the working volume and thus the operation time is reduced, and (2) this can increase the efficiency of the cell disruption process. Centrifugation and microfiltration were considered potential techniques for water removal. However, since oleaginous biomass has a similar or even lower density than water, centrifugation appeared not to be efficient to separate the cells since some of the cells were floating while another part settled (Figure 8)

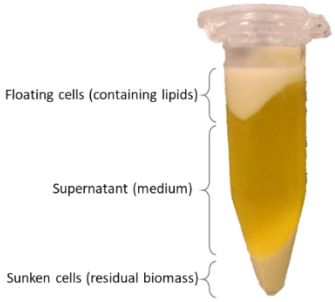


Figure 8: Centrifuged sample of *Y. lipolytica* broth showing lipid-containing floating cells.

Alternatively, by microfiltration with a ceramic 0.45 µm membrane, the broth could successfully be concentrated. To ensure efficient microfiltration, the following conditions were applied: 2.4 bar inlet pressure, 3.3 L/h recirculation flow, 1.9 bar transmembrane pressure (TMP), 0.8 bar differential pressure. Furthermore, also diafiltration was performed to wash the cells. As such, the biomass was concentrated resulting in 166 gCDW/L and 55 gTAG/L (Figure 9).

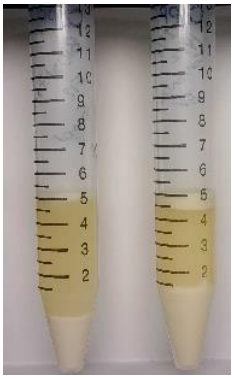


Figure 9: Centrifuged samples before (left) and after microfiltration (right)

4.3.3 High-pressure homogenization

The concentrated broth was completely homogenized at 800 bar and 8 cycles. After each cycle, a sample was taken and analyzed. The TAG concentration of the supernatant was as follows: 4.09 g/L (1x), 1.07 g/L (2x), 1.62 g/L (3x), 1.08 g/L (4x), 4.53 g/L (5x), 4.55 g/L (6x), 1.84 g/L (7x), and 1.53 g/L (8x). Remarkably, more emulsion was observed with an increasing number of homogenization passes (Figure 10). This could explain the unusual results in the TAG analysis of the supernatant, as centrifugation did not lead to good phase separation. However, since this is a crucial step in the process, the influence of the applied settings during HPH and the number of cycles was further investigated (Section 4.4).

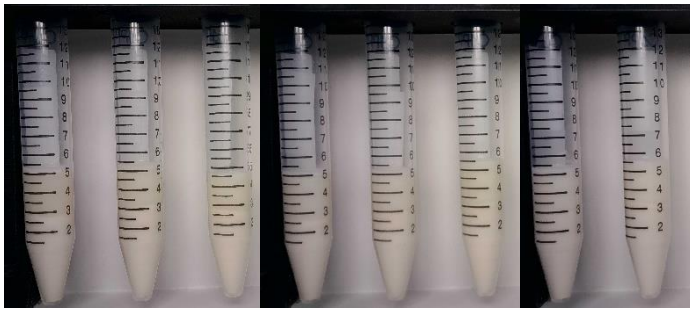
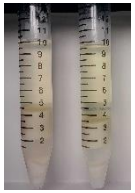


Figure 10: Samples after homogenization for 1 up to 8 cycles at 800 bar (from left to right).

4.3.4 Solvent extraction

The homogenized broth was used for solvent extraction. Again, different solvents were evaluated. Focusing on a feasible scale-up, solvents with a boiling point below 100 °C and relatively low hazard were selected, namely ethyl acetate, ethanol, dimethyl carbonate (DMC), and isopropanol (IPA) (Table 1). The tests were performed on 5 mL of sample to which solvent was added in a 1:1 ratio. In addition, for some samples, salt was added at a concentration of 300 g/L to evaluate whether this could enhance the phase separation. The salt can make the water phase more polar resulting in a better separation from the hydrophobic solvent phase containing the oil. The extraction reactions were mixed for 2 hours at 30 °C. Afterwards, the samples were centrifuged and the TAG concentration in the solvent phase was determined. The results are presented in Table 2.

Table 2: Overview of the results of solvent extraction tests with different solvents and salts.

Solvent	**Ethyl acetate**										**Ethanol**										**DMC**		**IPA**	
Salt	**-**		**$(NH_4)_2SO_4$**		**$CaSO_4$**		**KH_2PO_4**		**$NaHCO_3$**		**-**		**$(NH_4)_2SO_4$**		**$CaSO_4$**		**KH_2PO_4**		**$NaHCO_3$**		**-**		**-**	
Solvent phase (mL)	7	7	2	2	3	3	3	2	4	4	1	1	4	4	8	8	6	7	7	7	2.5	2.5	8	8
TAG in solvent phase (g/L)	10.2	11.2	0.58	10.9	3.5	9.1	1.2	10.1	10.4	-	3.5	2.5	2.9	3.3	2.8	2.7	2.2	1.9	6.0	6.2	0.24	0.23	5.2	4.2
Recovery	26%	29%	0%	8%	4%	10%	1%	7%	15%	-	1%	1%	4%	5%	8%	8%	5%	5%	16%	16%	0%	0%	15%	12%
Picture after centrifugation																								

4.3.5 Separation

During solvent extraction, an emulsion is formed between the water phase of the broth, the amphiphilic TAG molecules which act as surface active agents (surfactants), and the solvent phase (Figure 11). Although this clearly indicates cells have successfully been disrupted and TAG has been released, the formation of an emulsion is undesired. In contrast, a clear separation between the water phase containing the hydrophilic cell debris and the solvent phase containing the TAG is preferred to easily separate the product. Several techniques to prevent the formation or to break the emulsion were investigated, such as changing the temperature and pH.

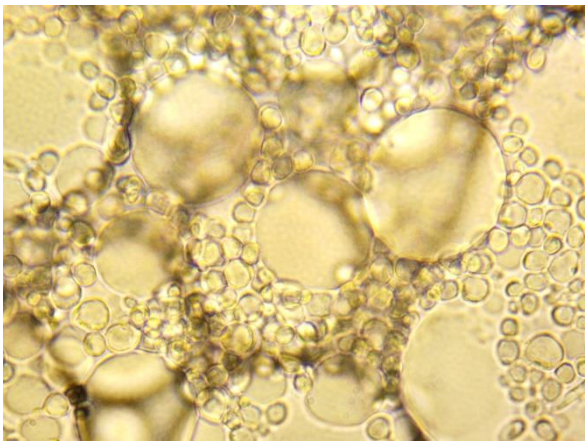


Figure 11: Microscopic picture (400x) of emulsion formed during extraction with ethyl acetate.

Therefore, additional solvent extraction tests were performed with 20 mL of homogenized sample and 20 mL of ethyl acetate. Emulsion formation decreased by performing solvent extraction at lower temperatures (cooling at 4°C). However, also less oil was extracted under these conditions. While higher temperatures (50 °C) during extraction showed more extraction and also more emulsion. Furthermore, it was found that the addition of acetone as a co-solvent improved phase separation. Nevertheless, so far, no ideal strategy could be defined to optimize TAG extraction and avoid emulsion formation. Centrifugal forces of centrifugation can aid to break the emulsion when it is formed.

4.4 Additional homogenization tests for improved cell disruption

With the aim to increase cell disruption efficiency by homogenization, BBEPP contacted the supplier of their HPH equipment to get some advice based on their expertise in this matter. GEA showed great interest in this challenging project and kindly proposed to perform some additional homogenization tests at their testing facility in Italy. Thanks to the good relationship between GEA and BBEPP, these tests were free of charge.

4.4.1 Starting material

For the additional homogenization tests, fermentation broth obtained from BioSFerA-T4.4-F01 (fermentation executed with BioSFerA task 3.4 and described in BioSFerA deliverable 4.5) was shipped to the testing facility of GEA in Italy. The broth consisted of glucose-grown *Y. lipolytica* yeast cells with a cell dry weight (CDW) concentration of 67 g/L and a TAG content of 46%.

4.4.2 Homogenization conditions

The trials have been performed using several types of GEA homogenizers (Figure 12). The effect of multiple passes and different operating pressures was evaluated.

Figure 12: Homogenizers used for DSP of TAG at GEA (for BBEPP): Panda Plus, X-stream, Panther (from left to right).

4.4.3 Analyses of homogenization samples

To verify the homogenization efficiency, samples have been analyzed by using different analysis techniques:

- Microscopy: Small amounts of sample were analyzed with an optical microscope. The microscope is the latest version of Nikon Eclipse H550L motorized series with digital camera full frame sensor. Equipped with a microscope, there is a statistical software package: NIS Elements Ar. Based on this data, the visual rupture of the cells was determined.
- DNA extraction quantification: The sample was centrifugated at 10000 RCF for 15 minutes and then the DNA dissolved in the supernatant was quantified by spectrophotometric analysis. For this, the sample is exposed to ultraviolet light at a wavelength of 260 nanometers (nm) and a photo-detector measures the light that passes through the sample. Some of the ultraviolet light will pass through and some will be absorbed by the DNA. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. By using the Lambert Beer law it is possible to relate the amount of light absorbed to the concentration in µg/ml of the absorbing molecule by multiplying the absorbance with factor 50.
- Centrifugation: Small quantities of the sample were centrifugated and then used to fill transparent containers to make comparisons of their visual properties. The measure, although simple in its implementation, gives an immediate and reliable response on the quantity of oil extracted.

4.4.4 Results

An overview of the tested conditions with the three different types of homogenizers and the obtained results are presented in Table 3. The microscopy pictures of the samples before and after homogenization are presented in Table 4.

Using the Panda Plus at 800 bar for 4 passes, showed similar results as were obtained by BBEPP at the same operating conditions, thereby indicating the reproducibility of the results at GEA and BBEPP. The best results, namely a visual cell rupture of 70%, were obtained with the Panther homogenizer at a pressure of 1200 bar and with 10 cycles. In all cases, there is a clear positive influence of increased cycles, which was also observed by BBEPP during the exploratory experiments.

Table 3: Overview of the results obtained during the additional homogenization tests performed by GEA for BBEPP. The visual rupture was calculated from the microscopy pictures.

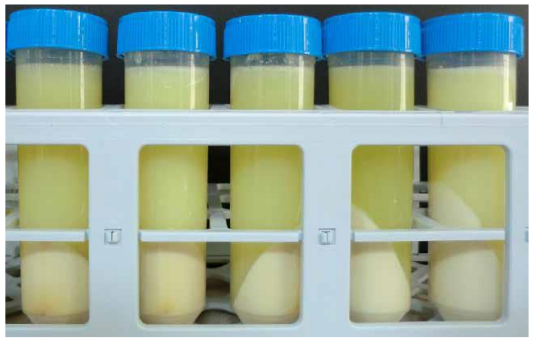
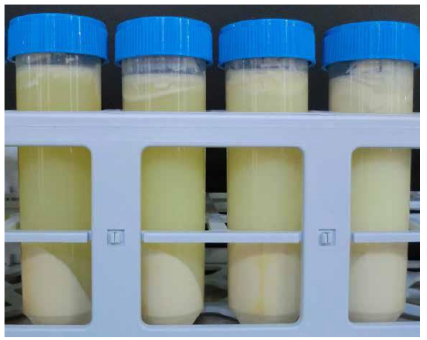
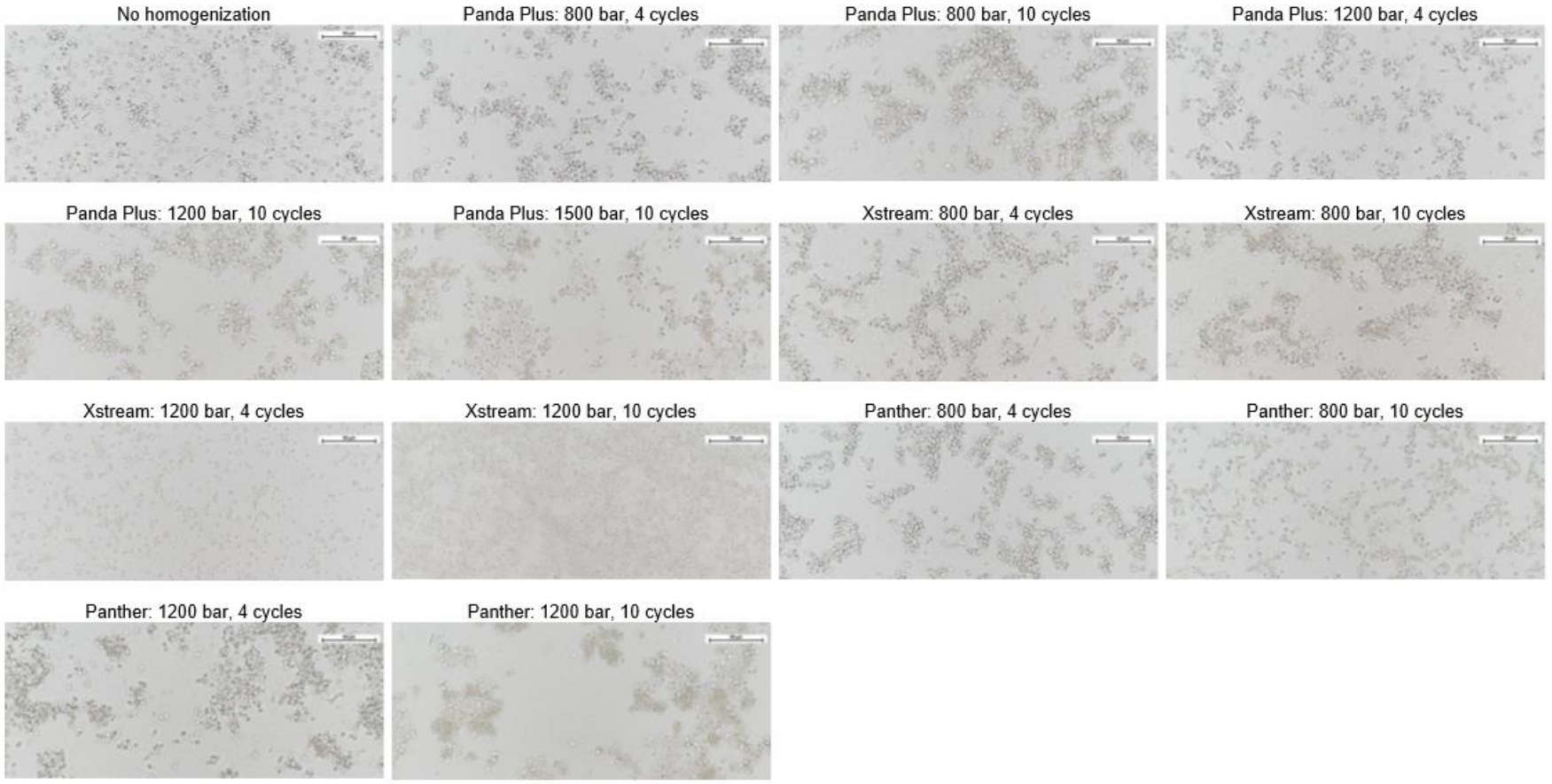
	None	Panda Plus					Xstream				Panther			
Pressure (bar)	**-**	**800**	**800**	**1200**	**1200**	**1500**	**800**	**800**	**1200**	**1200**	**800**	**800**	**1200**	**1200**
Cycles	**-**	**4**	**10**	**4**	**10**	**10**	**4**	**10**	**4**	**10**	**4**	**10**	**4**	**10**
Visual rupture	0	25%	40%	30%	50%	60%	30%	60%	20%	65%	30%	60%	40%	70%
DNA concentration	177	177	177	184	182	274	185	175	186	183	173	175	171	182
Picture after centrifugation														

Table 4: Microscopy pictures of the samples after homogenization under different conditions tested by GEA for BBEPP.



4.4.5 Summary of results

- TAG analysis was developed in which the TAG was extracted under harsh conditions (hot acid hydrolysis) and trans-esterified for quantification by GC-MS.
- Several DSP techniques for TAG isolation have been evaluated and an optimal was established consisting of the following process steps:
    1. Biomass concentration and water removal by ceramic filtration: to reduce the working volume and increase efficiency of other process steps.
    2. Cell disruption by HPH: higher pressure and increased cycles improve cell rupture. The highest rupture was obtained with 10 cycles of homogenization at 1200 bar in GEA's Panther homogenizer.
    3. Solvent extraction with ethyl acetate and acetone as co-solvent: ethyl acetate was identified as the optimal solvent based on industrial feasibility and TAG extraction efficiency. The addition of acetone as a co-solvent improved phase separation of the water and the solvent phase. Extraction at an increased temperature (50°C) resulted in the highest efficiency, however, also increased the formation of the emulsion.
    4. Removal of the water phase containing the cell debris: centrifugation was required to get phase separation and allow to decant 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.
- The main bottleneck of this process was the robustness of the cells resulting in difficult cell disruption. With a standard procedure of 1 homogenization cycle at 800 bar, less than 5% of the TAG was released. Therefore, more harsh conditions, thus higher pressure, and more cycles were required. However, this is energy-intensive and the scalability should be investigated in WP4.
- The formation of the emulsion during solvent extraction was indicated as another challenge inherently coupled with the amphiphilic nature of the TAG. Phase separation by decantation was found the be impossible, hence, centrifugation was included.

5 Recovery and purification tests: 1st part (ENVIPARK)

5.1 Introduction

ENVPA tested as alternative extraction strategy: the steam explosion process for the lipid extraction from wet digestate coming from the fermentation process developed by CARTIF and CSIC and finally scaled up by BBEPP. The concept of this treatment is the use of hydrolysis at high temperature and pressure for short duration, followed by sudden decompression. The process converts the thermal energy into mechanical energy and the shear forcing caused by the expansion of steam leads to the disruption of cell wall. Moreover, with autohydrolysis and explosive depressurization the particle size distribution, shapes and chemical composition of the biomass feedstock are altered. For these reasons, this pre-treatment has been employed as an effective process for the extraction and separation of active compounds such as microbial oil from biomass. The main parameter to be optimized in the

steam explosion is the severity factor that depends linearly on time and exponentially on the temperature inside the pressurization vessel.

Since in literature there aren't references that could be used for the identification of the steam explosion process parameters in relation to Yarrowia yeasts, Envipark identified the process parameters for the first tests based on its own past experience on lipids extraction from microalgae. The steam explosion process, in fact, has also been used for lipid extraction from microalgae. The application of this technique as a pretreatment offers two main advantages: a) the explosion caused by a sudden release of pressure disrupts the cellular structure of microalgae, making lipids more accessible and b) the technique is applicable directly on the wet biomass thus reducing the need for drying. Lorente et al. employed acid-catalyzed steam explosion to simultaneously extract lipids and sugar from microalgae as a pretreatment for biofuel production. High lipid recovery was obtained from wet microalgae pretreated with steam explosion. ENVIPA has already done experiments for the extraction of lipids from microalgae using the steam explosion technique directly on concentrated wet algal biomass with high yield in terms of lipids release (client company IPR).

The yeasts broth provided by the partners have been sampled and prepared. The first stage consists of the analytical characterization of samples in order to quantify the lipid content in the broth. After each steam explosion test the analytical characterization of the hydrolyzed samples provided the efficiency of the process. The chemical analysis before and after all the process steps allowed to improve the process in order to get useful information for the following step of scaling up in WP4.

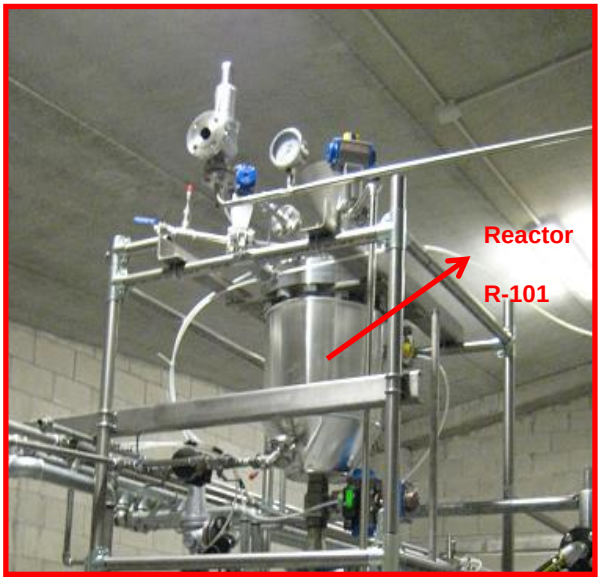
5.2 Steam explosion

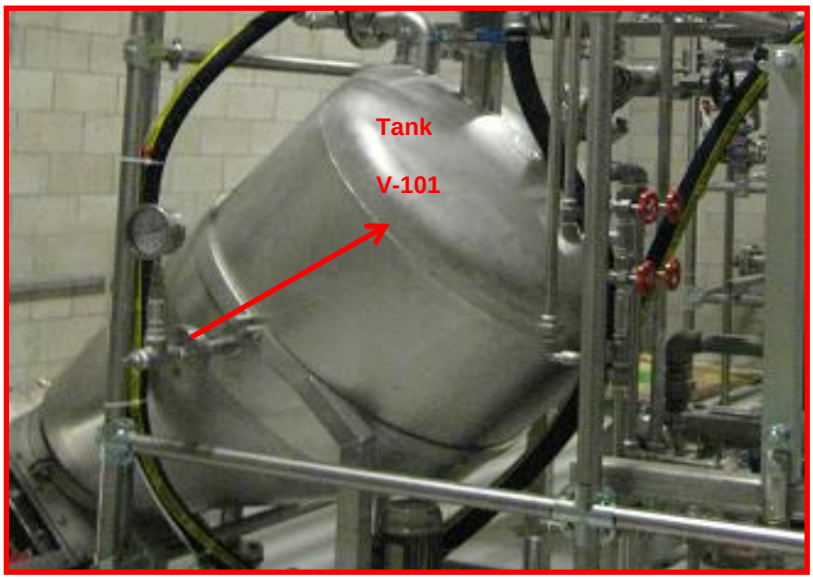
The steam explosion process is based on saturated or slightly superheated steam used to treat biomass for a predetermined time, followed by sudden expansion at atmospheric pressure. The sudden reduction in pressure causes the steam to expand, resulting in the deconstruction of the treated material.
The steam explosion plant at Environment Park consists of a batch reactor with a capacity of 22 liters. The reactor consists of a jacketed tank capable of operating internally at a maximum operating pressure of 26 bar with saturated steam and a temperature of about 227°C.
Once the reactor is brought up to process temperature, high-pressure steam is introduced, and an automatic control system maintains the process parameters. When the bottom valve of the reactor is opened, the material expands into the vessel below. The container has a volume of about 250 liters and is connected to a funnel in the atmosphere. Expansion occurs under near-isoenthalpic, critical pressure drop conditions, usually in a few seconds. Some of the condensates in the material vaporize, and the collecting vessel is under total pressure conditions equal to atmospheric pressure.

The plant control system releases the safety device when the pressure in the collecting vessel decreases and the internal temperature is such that a worker can operate. The safety device prevents the bottom butterfly valve from opening to collect the waste material.

The steam explosion reactor used for testing consists of two vessels, one for pressurizing the biomass (R101) and the other for subsequent expansion (V101) of the biomass at atmospheric pressure.

The characteristics of the two vessels are shown in Figure 13**: Steam explosion plant**





- V= 22 L
- Pmax= 26 bar
- Tmax=227 °C
- Pre-heating jacket V=10 L
- Temperature and pressure control system

- V= 300 L for the expansion
- Pmax= 1 bar
- Cooling jacket
- Hermetic butterfly valve for the recovery of the exploded biomass

Figure 13: Steam explosion plant

The steam explosion cycle can be represented as in Figure 14:

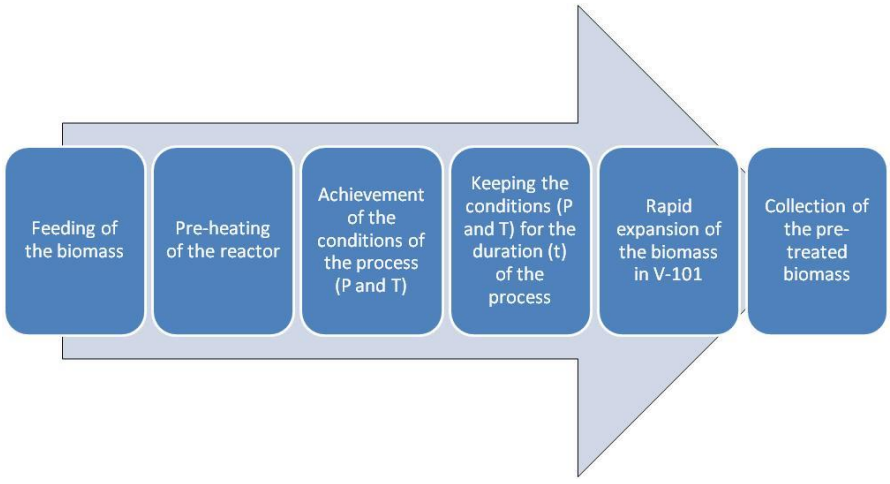


Figure 14: Steam explosion cycle

The main parameter to be optimized for the vapor explosion process is the severity factor, which depends linearly on time and exponentially on the temperature inside the pressurization vessel, as given in the following equation:

$$R_o = t \cdot e^{((T-100)/14{,}75)}$$

and linearly dependent on time t (minutes) and exponentially on temperature T (°C).

Between Oct. 1, 2021, and Sept. 30, 2022, Environment Park performed the first tests on the sample of *Yarrowia* Lypolitica broth provided by BBEPP. Process parameters are presented in Table 5.

Table 5: Process hypotheses

Steam Explosion Condition Hypotheses	**Test I**	**Test II**	**Test III**
Sample weight before steam (kg)	4	18	18
Process temperature (°C)	174	174	210
Process pressure (bar)	10	10	20
Process duration (min)	10	10	10
Average severity	**1528,7**	**17898,89**	**2227,90**

BBEPP sent Envipark a sample of *Yarrowia* lipolytica broth recovered from fermentations with the wild-type strain.

The first sample arrived consisted of 2 bottles, each containing about 2 L of fermentation broth (Figure 15).

Figure 15: *Yarrowia* broth first sample

Envipark conducted an initial test of the steam explosion pilot plant, determining the process parameters based on literature data and previous experience with biomass treatment.

The process was developed in four steps:

Phase I: biomass loading

After preheating the system to the temperature set for the test, the biomass was manually fed into the vapor-explosion reactor, trying to minimize material losses.

Phase II: pressurization

The reactor was brought from starting pressure (1 bar) to the desired conditions for the process in 0.933 minutes by opening a control valve to introduce steam.

Step III: Maintenance of process conditions

Once the desired conditions inside the reactor were reached, the automatic control software maintained temperature and pressure in the intervals shown in Table 6 for 10 minutes:

Table 6: Process conditions

	MIN	MAX	AVERAGE
Pressure (bar)	9.02	10	9.458
Temperature (° C)	172.2	176.5	174.2

Step IV: expansion, cooling and collection of exploded biomasses

Figure 16 containing the trend of the pressure and temperature parameters during the process and during the different phases described above monitored during the test.

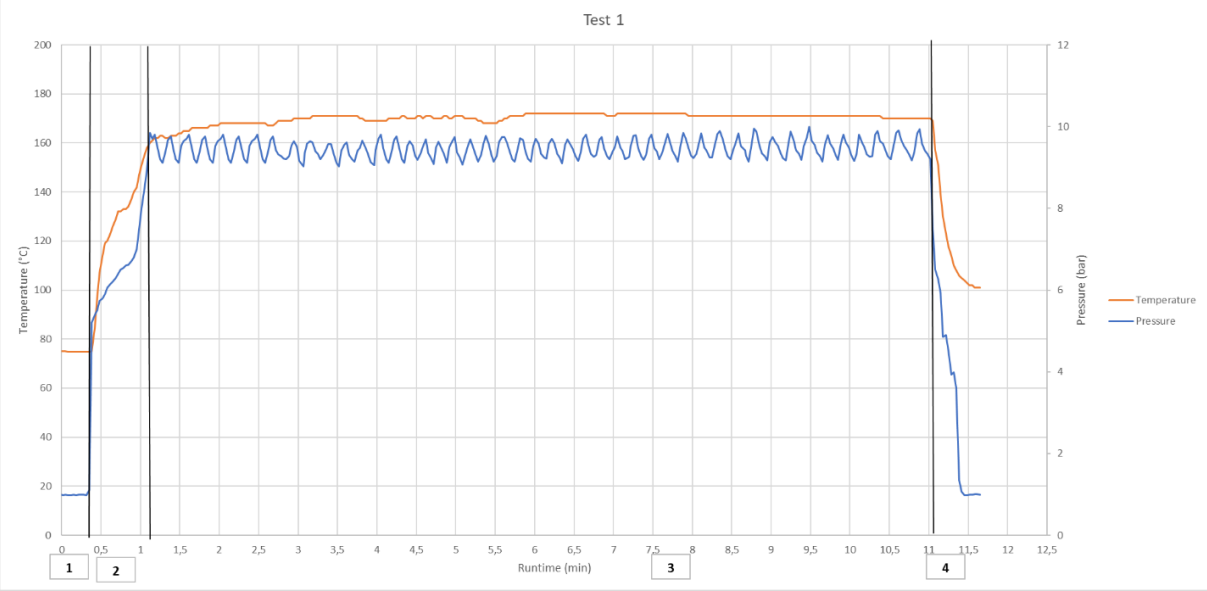


Figure 16: Trend of pressure and temperature during the process

The estimation of the severity factor reached during the process is reported in Table 7, computed according to both phase II (R1) and neglecting it (R2) and the average temperature of phase III (R3).

$$R = \int_{t1}^{t2} \exp\left(\frac{T(t) - 100}{14.75}\right) dt \qquad (1)$$

$$R = t \cdot \exp\left(\frac{T - 100}{14.75}\right) \quad (2)$$

Table 7: Severity factors

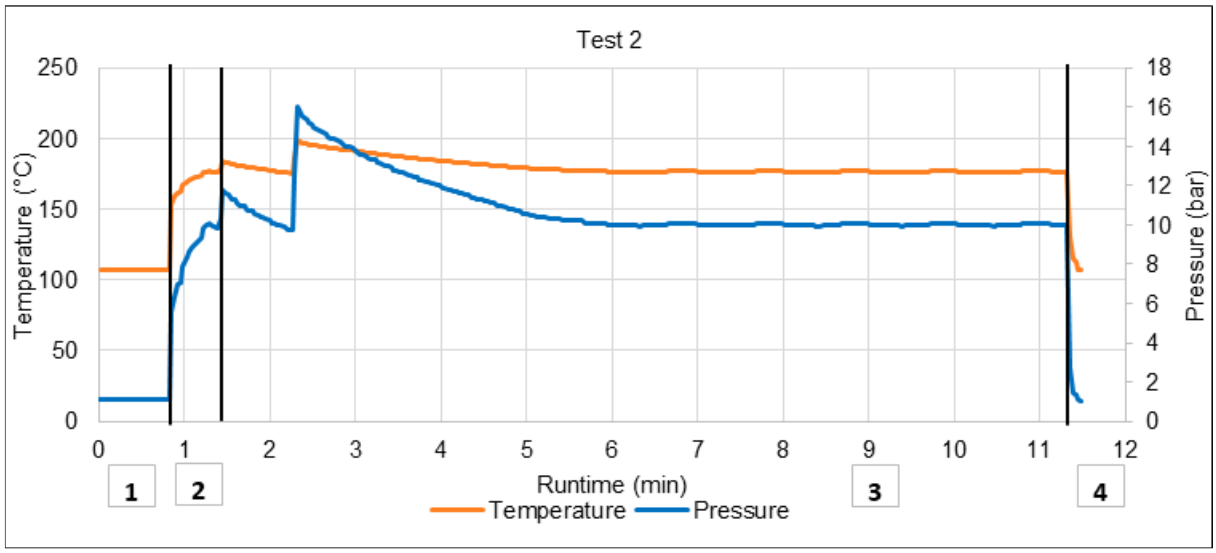
	Minutes	Equation
R_1 (step II+step III)	1566,25	1
R_2 (step III)	1490,6	1
R_3 (step III) average temperature	1528,7	2

The comparison between the severities generated with the two different calculation methods based respectively on the integral value and the average value (comparison R3 and R2) shows a modest difference confirming the correct maintenance of the process parameters set during phase III.

Envipark performed two more SE tests, using 18 kg for each test, which allowed to test the effects of the process with the reactor nearly filled, eliminating the problem of excessive dilution.

The conditions of 10 bar for 10 minutes were tested again to compare the results of the first test performed with only 4 l of broth. Moreover, a second test was done to evaluate more extreme conditions, i.e., 20 bar for 10 minutes, to see the influence of pressure on the breaking capacity of the yeast cells and whether or not this provides the release of fatty acids and TAG. The pressure and temperature trend of the two tests is shown in Figure 17: Temperature and pressure trend of two tests: a) 10 bar for 10 min and b) 20 bar for 10 min The operating conditions and severity factors are shown in Table 8 and in Table 9.

a)



b)

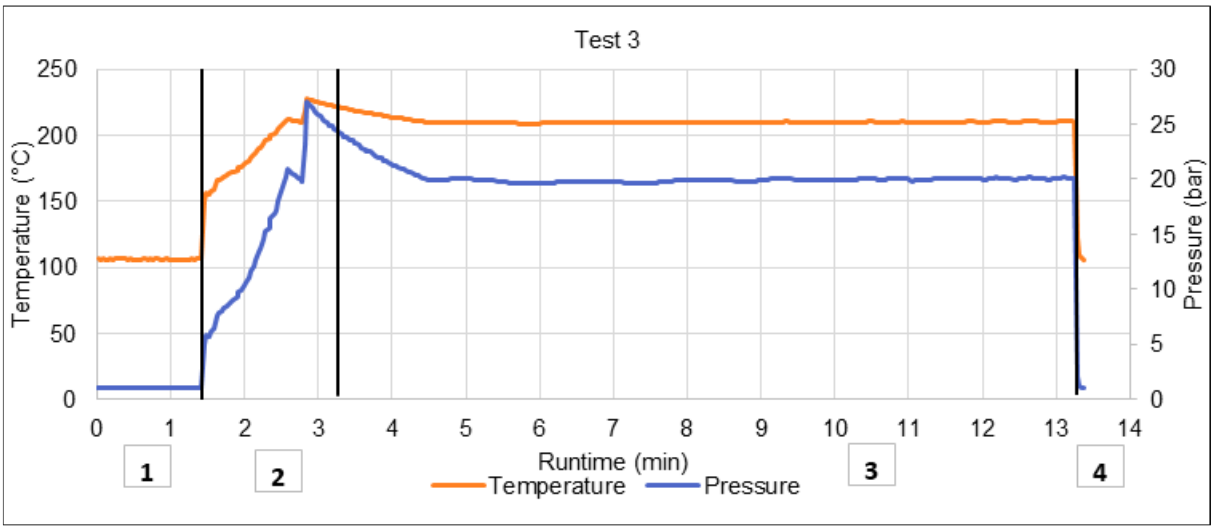


Figure 17: Temperature and pressure trend of two tests: a) 10 bar for 10 min and b) 20 bar for 10 min

Table 8: Process conditions

Test 2	**MIN**	**MAX**	**AVERAGE**
Pressure (bar)	9.73	16	10.8
Temperature (° C)	175.4	198.3	179.4
Test 3	**MIN**	**MAX**	**AVERAGE**
Pressure (bar)	19.6	24.4	20.2
Temperature (° C)	209	221.4	210.5

Table 9: Severity factors

Test 2	**Severity (min)**	**Time (min)**
Step II + Step III	21090,08	1,77
Step III	18197,24	10
Step III at average temperature	17898,89	-
Test 3	**Severity (min)**	**Time (min)**
Step II + Step III	2472,18	0,47
Step III	2415,36	10
Step III at average temperature	2227,90	-

Also in these tests, the comparison between the severities generated with the two different calculation methods based respectively on the integral value and the average value (comparison R3 and R2) shows a modest difference confirming the correct maintenance of the process parameters set during phase III.

Figure 18 and Figure 19 show the appearance of the hydrolysate after steam explosion.

Figure 18: Hydrolysate after steam explosion II

Figure 19: Hydrolysate after steam explosion III

5.3 Membrane filtration pilot plant

The first steam explosion product has been processed in a small pilot plant equipped with different filtration membranes (Figure 20):
1 – spiral wound PVFD (poly vinylidene fluoride) Microfiltration membrane 0,2 µ
2- spiral wound polyamide composite nanofiltration membrane cut off 300 Da

Figure 20: Filtration plant and samples

Samples (Figure 21) were shipped to a specialized analytical laboratory for the quantification of C16-C18 fatty acids. The results coming from micro- and nanofiltration operations are shown in Table 10.

Figure 21: Samples post filtration

Table 10: Results of micro- and nanofiltration on first steam explosion product

Acids	Feed (mg/l)	MF permeate (mg/l)	MF retentate (mg/l)	NF permeate (mg/l)	NF retentate (mg/l)
Myristic Acid	0,1	0,023	1,1	0,010	0,3
Palmitic Acid	6,8	1,156	95,1	1,404	36,7
Stearic Acid	3,4	0,264	20,7	0,335	9,7
Oleic Acid	10,4	0,041	159,9	0,054	36,9
Linoleic Acid	13,4	0,163	230,7	0,211	63,4
Linolenic Acid	0,2	0,002	10,8	0,002	1,5
Arachic Acid	0,3	0,015	1,6	0,014	1,2

As shown above, oleic and linoleic acids are the ones present in the highest amounts, and their concentration increases after microfiltration treatment, in which they are concentrated in the retentate, as well as most of the other acids, while the same cannot be said of the permeate or retentate of nanofiltration. Therefore, membrane microfiltration step is capable for fatty acid separation.

Therefore, hydrolysates from steam explosion II and steam explosion III operations were purified using a microfiltration plant. Both permeate and concentrate samples were sent to a certified laboratory for quantitative analysis on the presence of TAGs, particularly trilinolein, trilinolenin, trimyristin, triolein, tristearin, and the mixed TAGs composed of the fatty acid mix. Some samples of untreated *Yarrowia*, hydrolysate, and concentrate were centrifuged, phases separated, and sent to the laboratory for analysis. After centrifuging the concentrated product post-microfiltration step, three defined phases were identified: precipitate, upper supernatant phase, and lower supernatant phase. The three phases can be distinguished in Figure 22.

Analysis results are shown in

and Table 12.

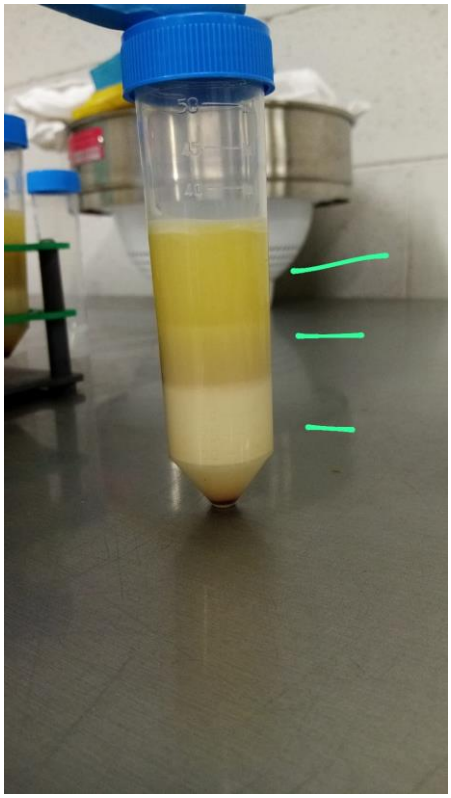


Figure 22: Three visible phases after centrifugation

Table 11: TAG results. Green boxes: increased concentration; yellow boxes: similar concentration; red boxes: decreased concentration.
LLL=trilinolein; LnLnLn=trilinolenin; MMM=trimyristin; OOO=triolein; SSS=tristearin

	mg/kg					
Sample Name	**LLL**	**LnLnLn**	**MMM**	**OOO**	**SSS**	**Triglycerides**
Yarrowia	2,12	-	-	16,5	14	43,8
Precipitated *Yarrowia*	3,81	-	-	7,94	10,4	34,6
Supernatant *Yarrowia*	-	-	-	-	0,8	2,03
Yarrowia Steam Explosion 2	4,34	-	-	16,4	14	44,6
Precipitate Concentrate 2 *Yarrowia*	5,05	-	-	3,88	6,53	24,3
Supernatant upper phase Concentrate 2 *Yarrowia*	1,63	-	-	9,8	7,64	21,5
Supernatant lower phase Concentrate 2 *Yarrowia*	5,34	0,55	-	12,6	9,2	30,8
Yarrowia Steam Explosion 3	2,52	-	-	11,1	9,79	27,7
Yarrowia Steam Explosion 3 Concentrate	4,09	-	-	7,57	8,33	27,8
Precipitated Concentrated 3 *Yarrowia*	3,53	-	-	3,77	6,29	22,1
Supernatant Concentrate 3 *Yarrowia*	1,07	-	3,66	9,32	7,67	24,7

The results show that after the steam explosion at 10 bar (179°C), most TAGs concentration remains about the same as in untreated *Yarrowia* (yellow boxes), while the total amounts of TAGs concentration decreases (red boxes).

The situation worsens after the steam explosion at 20 bar (210°C).

That may be due by:

- dilution caused by the addition of water after the steam explosion (an unlikely hypothesis since the concentration does not increase after purification steps such as centrifugation or filtration);
- triglycerides degradation: the carboxyl chains deteriorate in a T range between 160 and 350 °C (Vecchio et al.).

For this reason, the following steam explosion tests have been conducted at lower pressures (and thus temperatures).

Furthermore, since the permeate and some supernatant samples were free of TAG or with lower amount of TAG, the compounds of interest are concentrated in the solid phase. Therefore, in the next phase the *Yarrowia* was treated with centrifuge and membrane microfiltration equipment before the steam explosion to remove excess water in the fermentation broth.

Table 12: Mixed TAG results. Green boxes: increased concentration; yellow boxes: similar concentration; red boxes: decreased concentration. L=linolein; Ln=linolenin; M=myristin; O=olein; S=stearin

	mg/kg												
Sample Name	**LLnL/OLnLn**	**LPL/OPLn**	**MLnM**	**OLL/OLnO/SLLn**	**OLLn/SLnLn**	**OLO/LSL/OSLn**	**OLP/PSLn**	**OOO**	**OPO/PLS**	**OSO/LSS**	**PLLn**	**PLnLn**	**SLnS/OLS**
Yarrowia	1,56	0,75		1,61	1,99	1,29	0,64	16,5		0,53	0,98	0,97	0,86
Precipitated *Yarrowia*	1,47	1,2		1,08	1,29	1,43		7,94	1,13	1,46	0,53	0,84	2,03
Supernatant *Yarrowia*				0,62		0,6							
Yarrowia Steam Explosion 2	1,24	0,65		1,14	1,4	1,13	0,78	16,4	0,53	0,58	0,65	0,71	0,93
Precipitate Concentrate 2 *Yarrowia*	0,87	1,09		0,79	0,75	1,29		3,88	1,01	1,23			1,78
Supernatant upper phase Concentrate 2 *Yarrowia*				0,93	0,84	0,71		9,8					
Supernatant lower phase Concentrate 2 *Yarrowia*	0,71			0,77	1,02	0,57		12,6					
Yarrowia Steam Explosion 3	0,79			0,64	0,87	0,72	0,57	11,1					0,75
Yarrowia Steam Explosion 3 Concentrate	0,7	0,75		0,66	0,7	0,87	1,18	7,57	0,71	0,93			1,33
Precipitated Concentrated 3 *Yarrowia*	0,54	1,17		0,67	0,62	1,37		3,77	1,03	1,31			1,78
Supernatant Concentrate 3 *Yarrowia*			0,69	0,87	0,73	0,69		9,32					

6 Steam explosion tests: 2nd part (ENVIPARK)

The diagram of the tests carried out in the second period of activity (February – March) is shown in

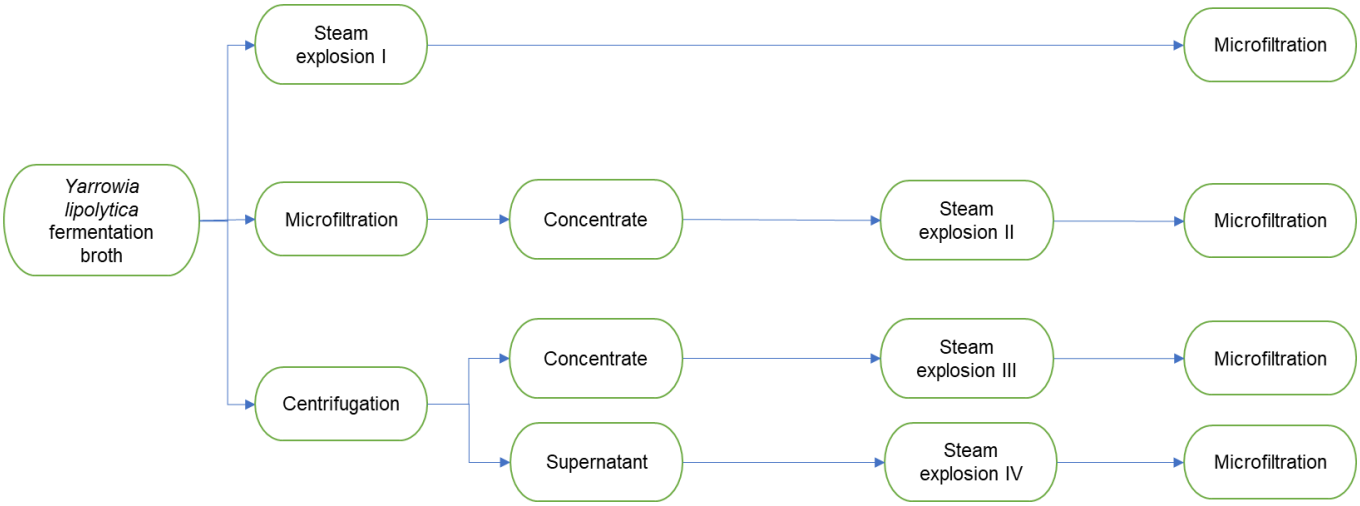


Figure 23.

Figure 23: Summary of performed tests

The final microfiltration operations were not performed on the entire volume of hydrolysate produced from each steam explosion but only on a portion. Two liters of hydrolysate obtained from each steam explosion test were subjected to microfiltration separately, yielding 1.5 l of permeate and 0.4 l of concentrate. The samples were sent to the laboratory for quantitative analysis of fatty acids and TAGs. Based on the results obtained, filtration of the entire volume of hydrolysate that gave the best results will be carried out in order to provide results useful for the scaling up in WP4. The concentrate was very fouling and very adherent to the membrane. The remaining 0.1 l from matter balance was lost in the membrane washes between filtrations.

6.1 Steam explosion + Microfiltration

The test conditions of the first steam explosion are shown in Table 13. Permeate and concentrate samples from microfiltration after steam explosion are shown in Figure 24.

Table 13: Steam explosion I

Steam Explosion Conditions	Test I
Biomass volume (l)	20
Biomass weight (kg)	20,9
Process temperature (°C)	150,2
Process pressure (bar)	5
Process duration (min)	10
Average severity (min)	**300,6585**
Biomass weight after steam (kg)	24,7
Volume after steam (l)	23
pH before steam	2,82
pH after steam	2,84

Figure 24: Permeate and concentrate samples after microfiltration steps

6.2 Microfiltration + Steam explosion + Microfiltration

30 L of fermentation broth was microfiltered in the small-scale plant (Figure 25), yielding 4 L of concentrate (Figure 26) that was subsequently subjected to steam explosion with the process condition explained in Table 14. Both a sample of concentrate and a sample of permeate were taken for analysis. The pressure and temperature trend of the test is shown in Figure 27.

Figure 25: Membrane small scale plant

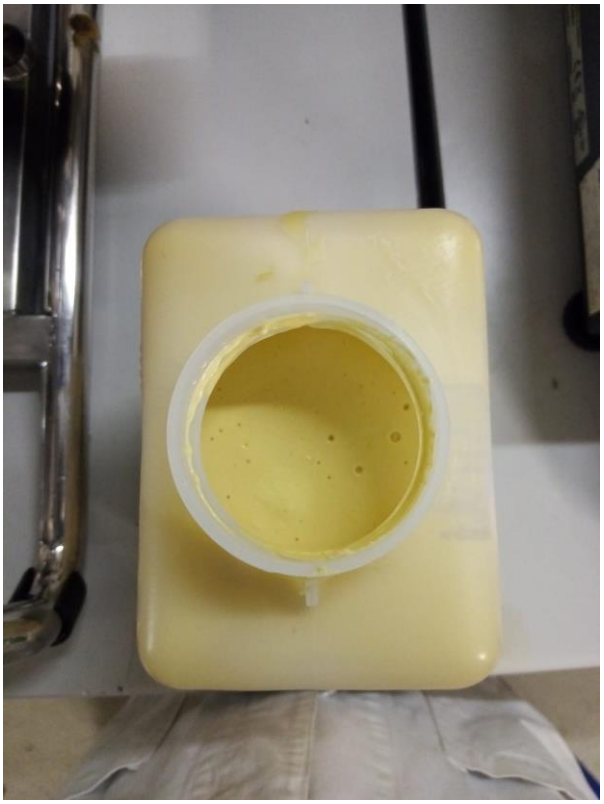


Figure 26: Microfiltration concentrate

Table 14: Steam explosion II

Steam Explosion Conditions	Test II
Biomass volume (l)	4
Biomass weight (kg)	4,41
Process temperature (°C)	150,2
Process pressure (bar)	5
Process duration (min)	10
Average severity (min)	**300,6585**
Biomass weight after steam (kg)	27,9
Volume after steam (l)	28
pH before steam	3,05
pH after steam	3,4

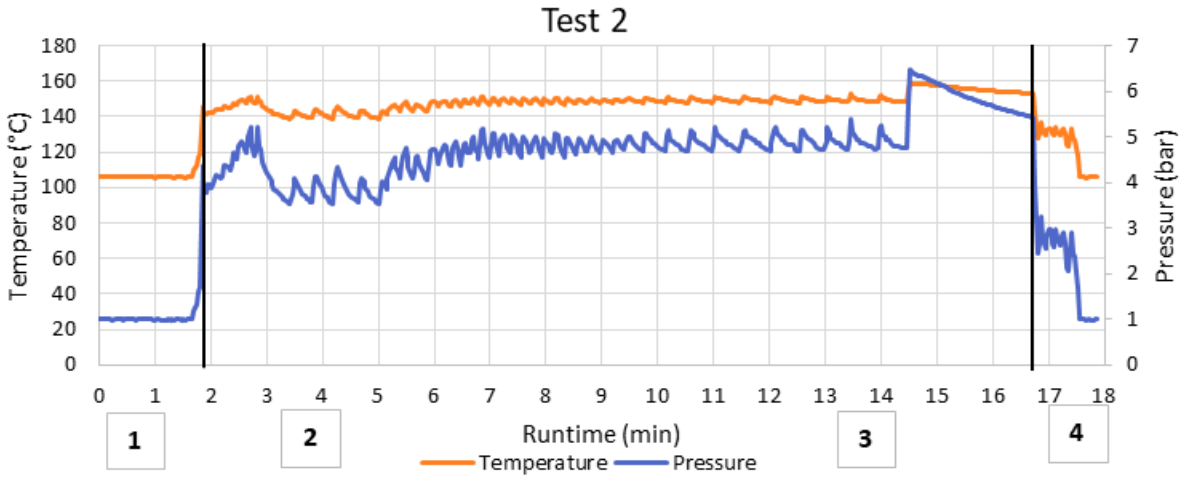


Figure 27: Temperature and pressure trend of steam explosion II

6.3 Centrifugation + Steam explosion + Microfiltration

20 L of *Yarrowia* fermentation broth was processed in a three-phase centrifuge (CLARA 20 – Alfalaval – Figure 28). 5 L of concentrate and 15 L of supernatant were obtained. One sample of centrifugate and one of the supernatant were sent for analysis as usual.

Figure 28: Clara 20

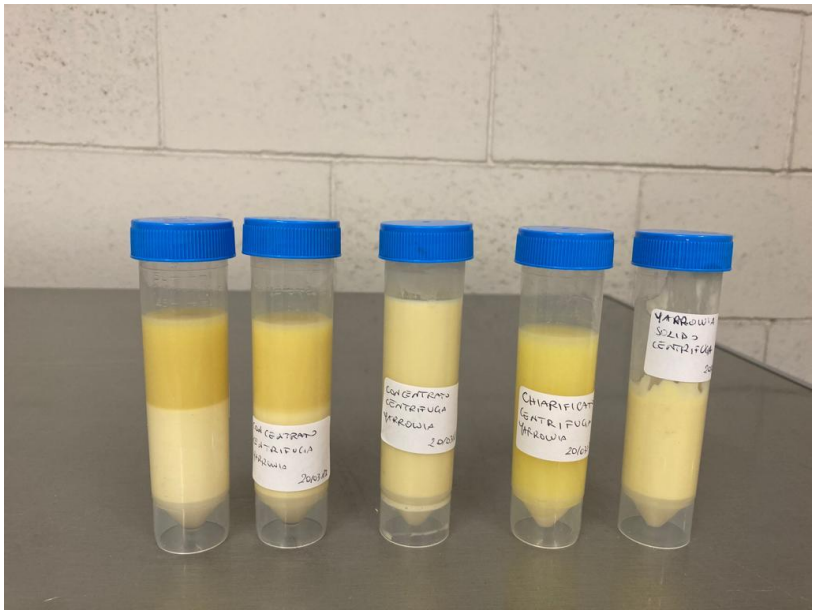


Figure 29: samples taken after centrifugation

6.3.1 Concentrate

5 L of concentrate was subjected to steam explosion with the process condition explained in Table 15. The pressure and temperature trend of the test is shown in Figure 27.

Table 15: Steam explosion III: centrifugate concentrate *Yarrowia*

Steam Explosion Conditions	Test III
Biomass volume (l)	5
Biomass weight (kg)	5,66
Process temperature (°C)	150,2
Process pressure (bar)	5
Process duration (min)	10
Average severity (min)	**300,6585**
Biomass weight after steam (kg)	27,5
Volume after steam (l)	27
pH before steam	3,04
pH after steam	3,34

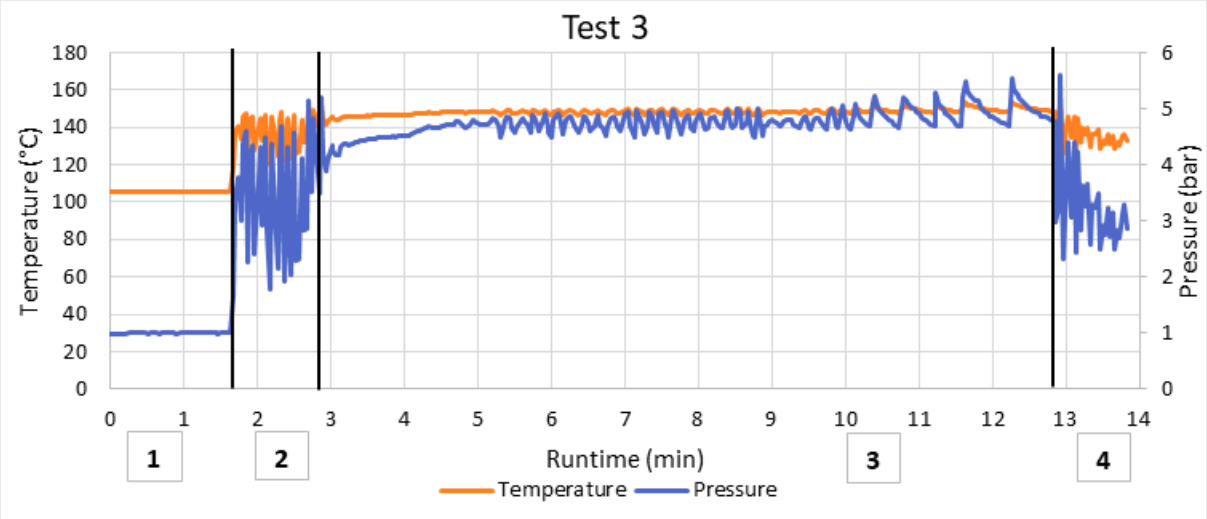


Figure 30: Temperature and pressure trend of steam explosion III

6.3.2 Supernatant

15 L of concentrate was subjected to steam explosion with the process condition explained in Table 16. The pressure and temperature trend of the test is shown in Figure 31.

Table 16: Steam explosion IV: centrifugate supernatant *Yarrowia*

Steam Explosion Conditions	Test IV
Biomass volume (l)	15
Biomass weight (kg)	15,2
Process temperature (°C)	150,2
Process pressure (bar)	5
Process duration (min)	10
Average severity (min)	**300,6585**
Biomass weight after steam (kg)	29
Volume after steam (l)	28
pH before steam	2,9
pH after steam	2,97

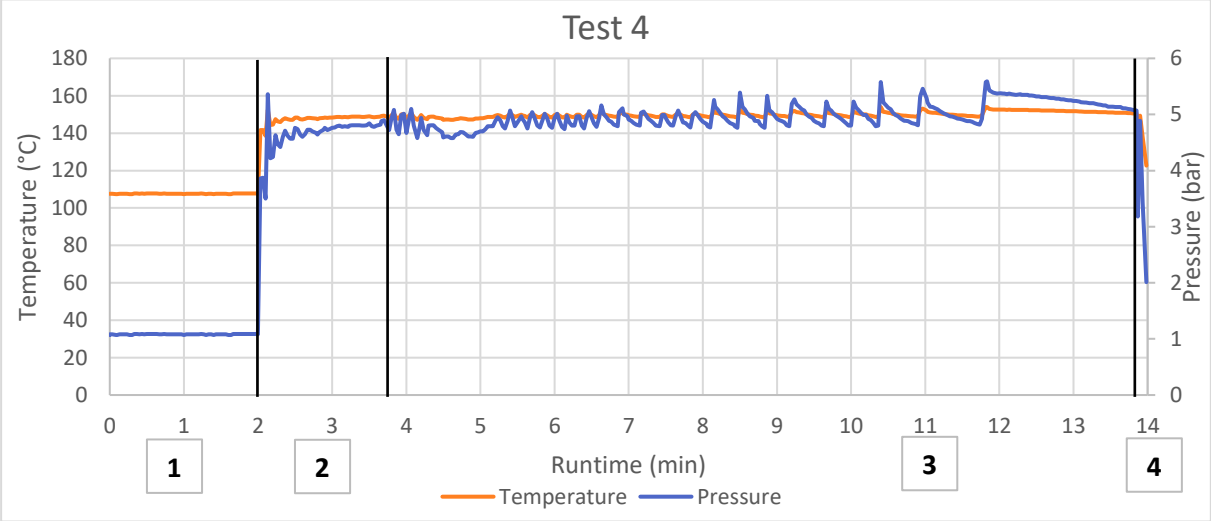


Figure 31: Temperature and pressure trend of steam explosion IV

6.4 Analytical results

Envipark analyzed the TAGs total amount using aa Analytical KIT Chronlab Triglycerides-LQ GPO-POD Liquid.

Analytical results, obtained by spectrophotometric kit, show the total amount of TAG present in the test sample. The working principle of the kit, provided by Chronolab, is as follows:

- sample triglycerides incubated with lipoproteinlipase (LPL), liberate glycerol and free fatty acids.

$$Triglycerides + H_2O \xrightarrow{LPL} Glycerol + free\ fatty\ acids$$

- Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase (GK) and ATP.

$$Glycerol + ATP \xrightarrow{Glycerol\ kinase} G3P + ADP$$

- Glycerol-3- phosphate (G3P) is then converted by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H2O2).

$$G3P + O_2 \xrightarrow{GPO} DAP + H_2O_2$$

- In the last reaction, hydrogen peroxide (H2O2) reacts with 4-aminophenazone (4- AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye.

$$H_2O_2 + 4 - AP + p - Chlorophenol \xrightarrow{POD} Quinone + H_2O$$

The intensity of the color formed is proportional to the triglycerides concentration in the sample.

As can be seen from the reaction formulas, the kit analyzes the amount of triglycerides present in the sample without extraction.

The concentration of TAGs and their amount expressed in grams in each sample analyzed can be seen in Figure 32. The sample signature is shown in Table 17.

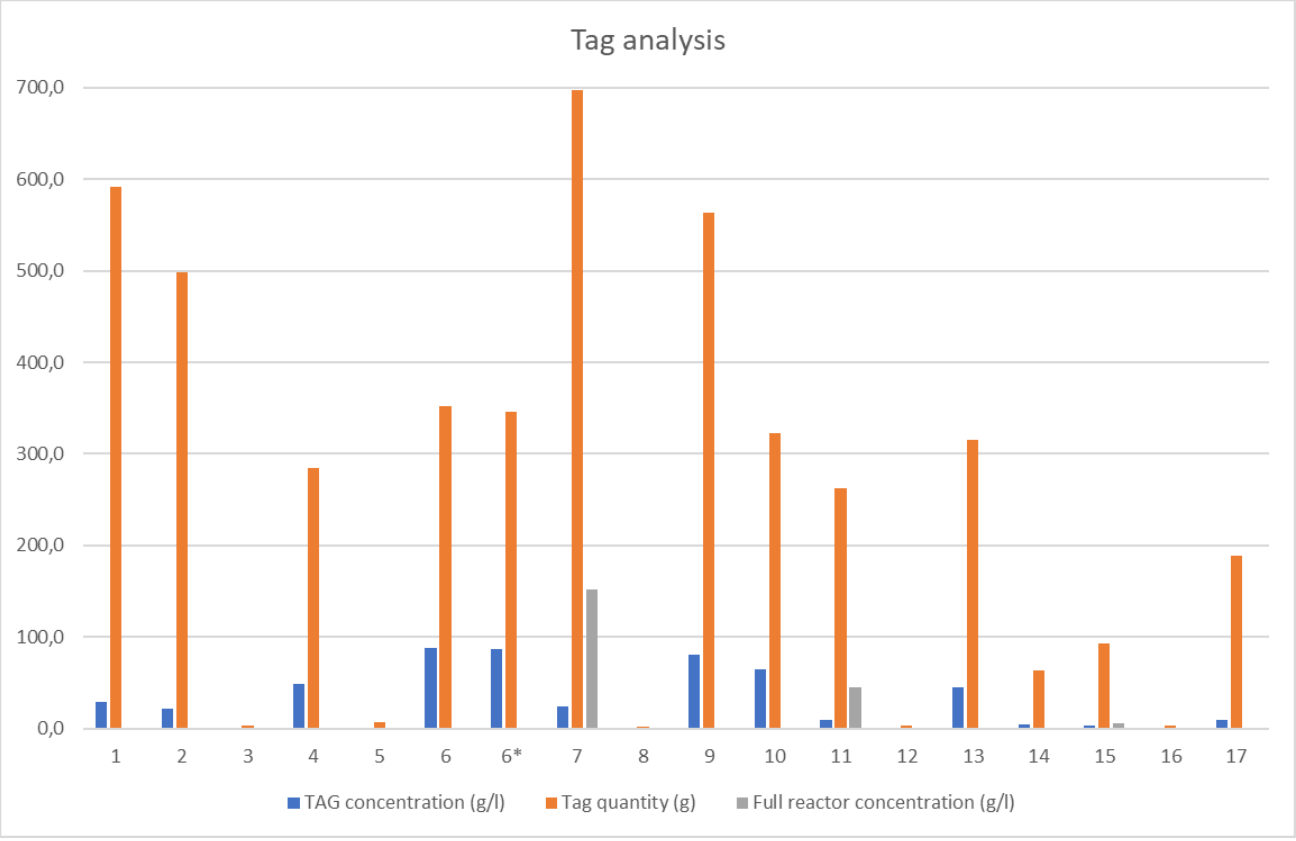


Figure 32: Tag analysis

Table 17: Sample signature

N°	Sample name
1	Yarrowia
2	Yarrowia steam explosion I 1:10
3	Yarrowia permeate microfiltration post steam explosion I
4	Yarrowia concentrate microfiltration post steam explosion I 1:10
5	Yarrowia permeate microfiltration
6	Yarrowia concentrate microfiltration 1:25
6*	Yarrowia concentrate microfiltration 1:50
7	Yarrowia steam explosion II 1:10
8	Yarrowia permeate microfiltration post steam explosion II
9	Yarrowia concentrate microfiltration post steam explosion II 1:10
10	Centrifuge concentrate Yarrowia 1:10
11	Yarrowia steam explosion III
12	Yarrowia permeate microfiltration post steam explosion III
13	Yarrowia concentrate microfiltration post steam explosion III 1:10
14	Centrifuge supernatant Yarrowia
15	Yarrowia steam explosion IV
16	Yarrowia permeate microfiltration post steam explosion IV
17	Yarrowia concentrate microfiltration post steam explosion IV 1:10

The graph shows that the highest concentration of TAG is obtained with steam explosion II, that is, treating the fermentation edge of concentrated Yarrowia with a microfiltration membrane. However, the Tag concentration obtained with this test appears actually be very low. It is important to note that steam explosions II, III, and IV were performed with the reactor not completely full. Assuming filling the reactor in the case of steam explosion II (green column): a higher TAG concentration is obtained than in all other tests, confirming that the reaction conditions of steam explosion II are the best.

Results of fatty acid analysis by an external laboratory are shown in Table 18.

As for the TAGs analysis with quantification of each triglyceride the work is still in progress and will be reported in deliverable 4.5.

Fatty acid analysis helps to define which are the predominant chains of TAGs present. The acids present in the highest amounts are oleic (C18), stearic(C18), palmitic (C16) and arachic (C20).

All have very good concentration yields by the microfiltration process with very high separation rates of up to 99%.

Those with the worst yields are lauric acid (C12) and myristic (C14), which, moreover, are also those present in the lowest amounts.

The only data that appears inconsistent (and for which we are requesting verification from the analytical laboratory that provided us with the results) are permeate and concentrate values after steam explosion II.

Table 18: Results of fatty acid analysis performed by an external laboratory

	Fatty acids concentration (mg/l)								
Sample name	**Methyl laurate**	**Methyl myristate**	**Methyl palmitate**	**Methyl stearate**	**Methyl oleate**	**Methyl linoleate**	**Methyl linolenate**	**Methyl arachidate**	**Total amount**
1 – Yarrowia	3,3	26,4	246,0	409,8	1046,7	200,0	1,7	312,1	2246,0
2 - Yarrowia permeate microfiltration	0,4	7,8	15,1	23,8	19,7	0,8	0,8	10,6	79,1
3 - Yarrowia concentrate microfiltration	3,0	129,0	1381,5	2925,9	5513,9	1031,1	6,2	2584,9	13575,5
4 - Centrifuge supernatant Yarrowia	1,9	18,0	114,5	197,4	518,9	80,2	1,2	202,6	1134,7
5 - Centrifuge concentrate Yarrowia	6,1	22,2	597,2	1206,5	2075,4	492,9	3,1	1195,4	5598,8
6 - Yarrowia steam explosion I	20,7	10,9	201,0	416,1	865,6	147,9	0,9	369,1	2032,2
7 - Yarrowia permeate microfiltration post steam explosion I	7,6	4,8	12,0	26,1	14,3	0,4	0,5	16,6	82,3
8 - Yarrowia concentrate microfiltration post steam explosion I	6,4	12,2	437,9	935,0	1524,2	133,3	1,7	663,6	3714,3
9 - Yarrowia steam explosion II	1,2	9,8	27,1	41,0	112,6	19,1	0,5	30,3	241,4
10 - Yarrowia permeate microfiltration post steam explosion II	3,9	7,9	270,0	575,6	1025,9	161,0	0,9	1080,5	3125,7
11 - Yarrowia concentrate microfiltration post steam explosion II	0,2	4,0	6,9	11,0	13,0	0,7	0,1	8,7	44,7
12 - Yarrowia steam explosion III	4,2	1,4	8,0	8,6	22,4	1,5	0,2	49,1	95,4
13 - Yarrowia permeate microfiltration post steam explosion III	0,6	3,4	11,2	7,5	12,2	0,9	0,2	5,1	41,1
14 - Yarrowia concentrate microfiltration post steam explosion III	0,3	5,9	27,6	31,9	66,2	6,6	0,2	17,6	156,3
15 - Yarrowia steam explosion IV	14,0	3,2	35,9	53,2	151,8	12,8	0,3	38,4	309,6

16 - Yarrowia permeate microfiltration post steam explosion IV	0,8	9,4	7,9	9,5	11,2	0,8	0,2	7,5	47,3
17 - Yarrowia concentrate microfiltration post steam explosion IV	1,8	6,7	96,8	231,8	466,1	37,2	0,8	159,9	1001,1

Full rector concentration (mg/l)									
Sample name	Methyl laurate	Methyl myristate	Methyl palmitate	Methyl stearate	Methyl oleate	Methyl linoleate	Methyl linolenate	Methyl arachidate	Total amount
6 - Yarrowia steam explosion I	20,7	10,9	201,0	416,1	865,6	147,9	0,9	369,1	2032,2
9 - Yarrowia steam explosion II	7,59	59,5	165	249	685	116	2,96	184	1469,7
12 - Yarrowia steam explosion III	19,8	6,71	37,6	40,6	105	7,11	0,804	230	448,1
15 - Yarrowia steam explosion IV	22,7	5,23	58,2	86,4	246	20,8	0,563	62,4	502,6

Table 19 – TAGs concentration scaling up the results in the case of full reactor process

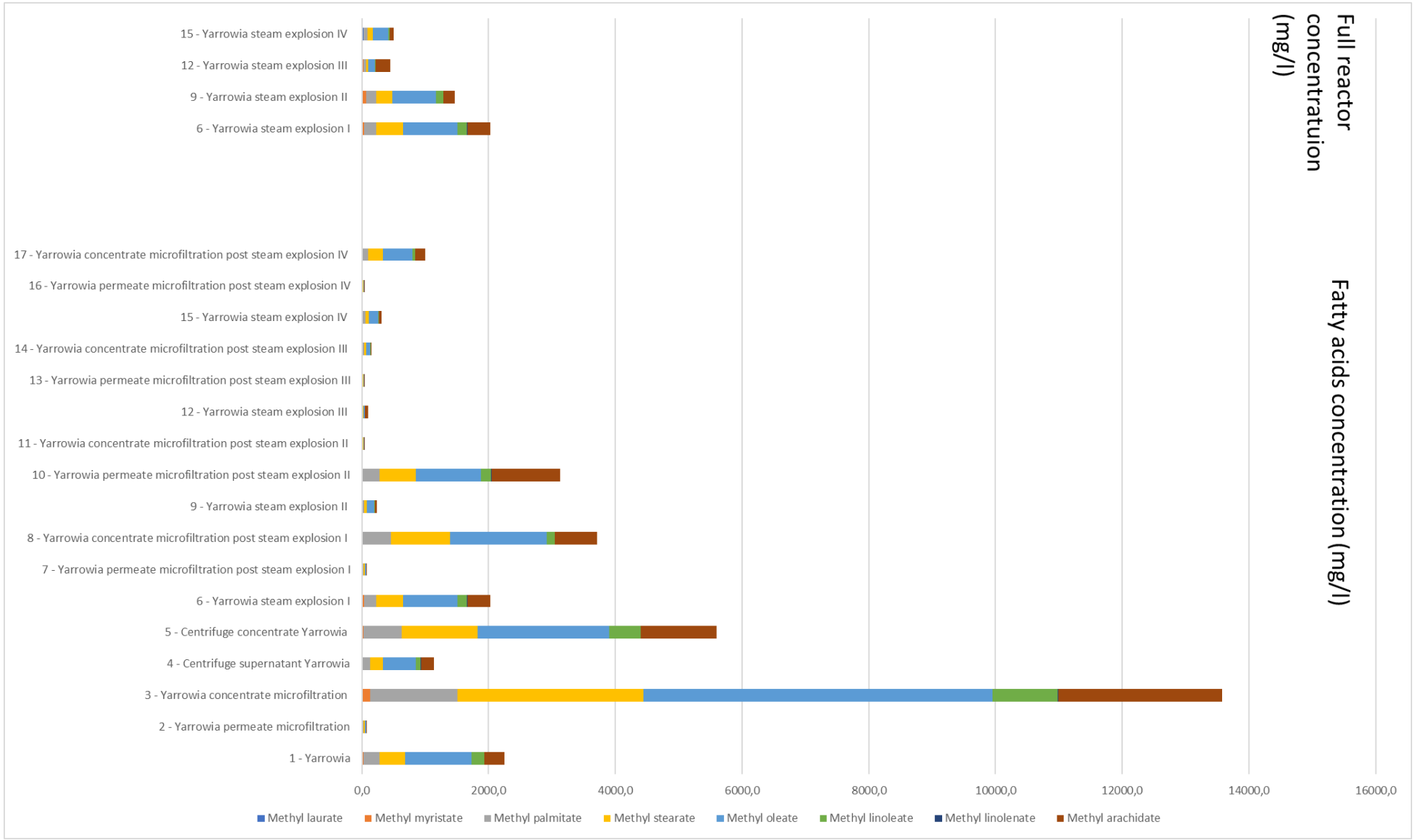


Figure 33 – Single Fatty acids quantities and concentration

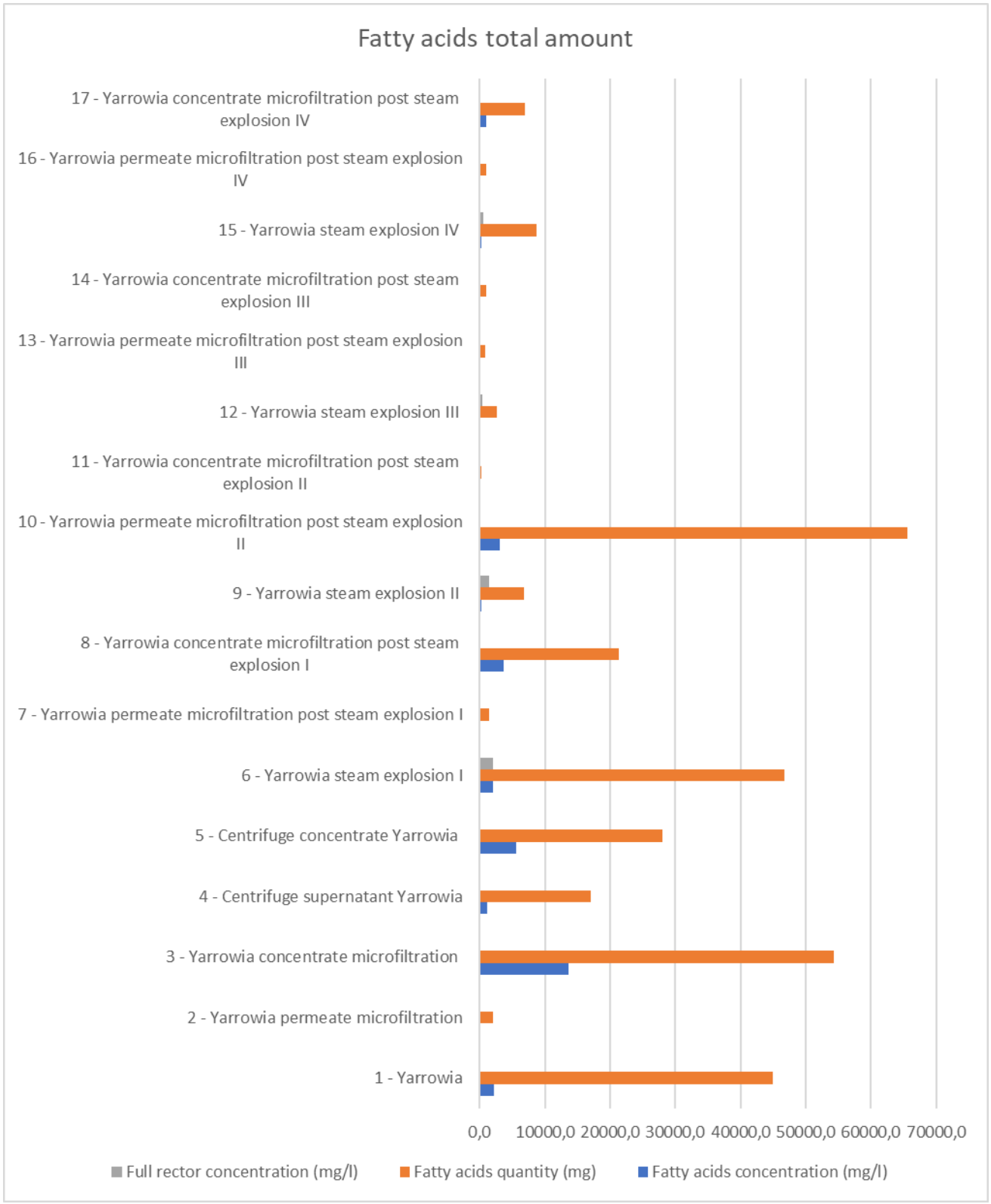


Figure 34 – Fatty acids total amount

6.5 Summary of results

- Steam explosion process, if used for TAGs extraction from yeasts cells, needs to be performed at low pressure and temperature (about 5 bar and 150 °C) in order to avoid the fatty acids separation from glycerol and the Tags disruptions
- The best sequence identified for the extraction and purification process is the Microfiltration of the yeasts followed by the steam explosion of the concentrated stream at 5 bar, 150 °C for 10 min (150 g/L total TAGs extracted)
- The steam explosion reactor needs to be fully filled in order to avoid TAGs dilution resulting in more expensive subsequent phases of concentration required

7 General conclusions

- It is convenient to carry out a broth concentration step prior to the cell disruption process to reduce the working volume and increase the efficiency of the subsequent process steps

- The best method to concentrate broth was found to be microfiltration because centrifugation could not separate the supernatant part of from the yeast biomass.

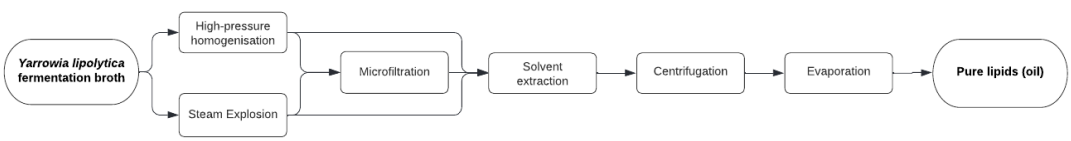
- Yeast cell rupture was found to be one of the most challenging steps in the process. On the one hand, BBEPP concluded that mechanical cell rupture could only be achieved under harsh conditions, namely multiple cycles of high-pressure homogenisation. On the other hand, ENVPA investigated the use of steam explosion which could be more efficient at high severities. However, the latter presents the problem of triglycerides degradation: the carboxyl chains deteriorate in a T range between 160 and 350 °C and thus a high concentration of fatty acids but not TAGs was found in the hydrolysates.

- The comparison between the results of the two partners BBEPP and ENVPA was not straightforward because ENVPA assesses the process performance by evaluating the final TAGs concentration in the hydrolysates (the highest concentration of TAGs obtained is 150g/L) and BBEPP provided the percentage of cellular breakdown. ENVPA can't evaluate with its own instrumentation the cellular breakdown. Therefore, samples obtained with SE will be provided BBEPP for TAG analysis in the upcoming months. The outcome will be reported in the following BioSFerA progress report.

- The maximum concentration of TAGs obtained by steam explosion is, however, the result of a theoretical calculation that takes into account the results obtained rescaled quantitatively by envisioning a full reactor

- The solvent extraction step is one that will need to be further investigated by both partners because of the difficulties associated with the formation of emulsions.

- Based on the insights gained in BioSFerA Task 3.5, an optimal DSP process scalable for the BioSFerA value chain should was defined, consisting of the following process steps:



- The choice between using the HPH or SE process for the cell-breaking stage for future scale up of the DSP process will have to be made following some technical and economic considerations. Before solvent extraction, a microfiltration step can be included to remove the cell debris.

8 Future prospects BioSFerA D3.6 (EnviPark & BBEPP)

- A higher TAG content in the cells could improve the recovery of the DSP process. Within BioSFerA Task 3.5, more lab scale fermentations are being performed with further improved *Y. lipolytica* strains from BioSFerA Task 3.2. cells could potentially reach a higher content of TAG (> 50%). If so, the influence of this higher content on the DSP train could also be investigated. This will then be reported in the following BioSFerA progress report.

- The cell disruption step needs to be further investigated to establish an efficient and industrially feasible process for the extraction of TAGs. In this respect, also a comparison between the proposed processes from the point of view of cost and energy consumption per kg TAG should be considered. Cell disruption by HPH is a widely performed at a commercial scale and investment costs can thus easily be found. The energy consumption of this method can be estimated around 0.037 kWh/kgTAG (Drévillon et al. 2019). In contrast, cell disruption by SE is not a common method but its investment cost estimation could be done following dedicated design according to the chosen process parameters. As a preliminary estimation for SE process, we can consider as hypothetical data (calculated with different biomass and process conditions) 0.017 $kWh_{el}/kg_{Biomass}$ and $0.44KWh_{ter}/kg_{Biomass}$ (ENVPA calculation made in previous activities). Obviously, all this information should be reconsidered according to the reference context (for example: in a hypothetical biorefinery that already has thermal waste capable of producing steam at 5 bar, the energy consumption for steam production would be much lower). A detailed investigation of the investment costs and the energy consumptions can be part of BioSFerA Task 6.3 (Process layout and cost engineering) and BioSFerA Task 7.1 (Techno-economic assessment).

- If more Yarrowia broth will be available, ENVPA could repeat the microfiltration test followed by steam explosion with a full reactor in order to validate the theoretically extrapolated data on the best concentrations obtainable with a full reactor process

- ENVPA is awaiting analytical data on the quantification of individual triglycerides. Once these results will be available, results obtained with analytical kit and reported in this deliverable will be validate. This will allow to make further considerations on the quality of the TAGs obtained with SE process. Also these data will be reported in the next progress report.

- On the basis of what has already been obtained from BBEPP, solvent extraction tests will be carried out on the samples with the highest amount of TAGs obtained in ENVPA to evaluate the subsequent separation of the oil. This will provide WP4 partners and partners dealing with hydrocracking and economic evaluations of the whole process some more data to work with.

9 References

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