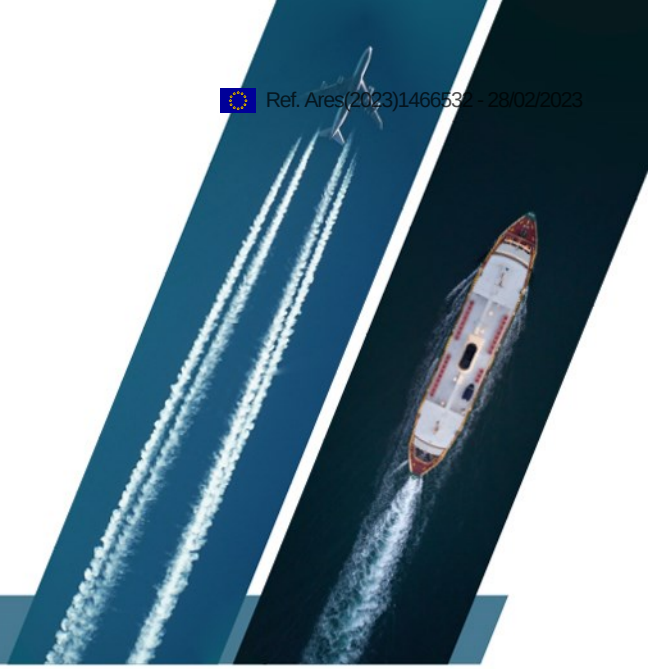
BioSFerA
Biofuel for biotravels



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Optimization of syngas fermentation process parameters for acetic acid production (1st stage)

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Abbreviations

CSTR	Continuous stirred tank reactor
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
H_2SO_4	Sulphuric acid
$OD_{600\ nm}$	Optical density at 600 nm
TAGs	Triacylglycerides
TSB	Tryptic soy broth
YTF	Yeast tryptone fructose

Contents

1 Executive Summary

Deliverable 3.4 “Optimization of syngas fermentation process parameters for acetate production (1st stage)” is a public document of the BioSFerA project, delivered in the context of WP3 “Biological production of lipids from syngas at lab scale”.

This Deliverable contains only the public version of the results achieved in task 3.3. The rest of the results are presented in much more detail in the confidential version of D3.4.

The main objective of WP3 is to develop a two-stage biological gas-to-liquid process for the conversion of CO_2/CO/H_2 produced by gasification into medium (C14) and long (C16-18) chain triacylglycerides (TAGs) that will be upgraded to biofuels.

The aims of this task 3.3 are:

- To optimise the operation conditions of the syngas fermentation process at lab scale: Acetate titer [30 g/L], productivity: [0.55 g/L/h], syngas conversion: [90%]

- To study the influence of the syngas composition in order to optimize the performance of the gas fermentation stage and optimize the operating conditions of the gas fermentation stage for a possible adaptation to the upstream syngas composition.

In this deliverable the main operating parameters for the culture of *Moorella thermoacetica* have been studied and optimized at lab scale using gas fermentation bioreactors (1 and 10 L). Acetate production was compared by testing different culture media, gas ratios and operation modes.

Results showed that the fed-batch fermentations on 1 L scale resulted in the production of more than 30 g/L, thereby reaching the BioSFerA target, with a productivity of 0.26 g/L/h.

Furthermore, a proof-of-concept for continuous fermentation with cell-recycle was established in a 10 L gas fermentation bioreactor. This allowed for the production of a sterile acetic acid stream containing around 30 g/L acetic acid, and reaching productivities up to 0.42 g/L/h.

Given the gained expertise, the gas fermentation process can be scaled up to TRL5 and coupled with the gasification process in BioSFerA WP4.

2 Introduction

Task 3.3 is focused on the evaluation at lab scale of the performance of the gas fermentation process for the production of acetate testing syngas as main gaseous substrate. The most suitable acetogenic bacterial strains selected in Task 3.2 during the serum bottles experiments were used for the experiments of this task. Within the strains and in order to optimize the fermentation parameters for the production of acetate, CARTIF and BBEPP tested in parallel the *Moorella thermoacetica* DSM 2955 strain using first CO/H_2 and then syngas ($CO_2/CO/H_2$) at different gas ratios.

-Syngas as gaseous substrate for microbial conversion

BioSFerA project focuses on gasifying biogenic residues and waste to produce syngas, which will be fermented to produce bio-based triacylglycerides. In this process, the biogenic residues are submitted to a thermochemical process to produce a syngas stream. Then, the syngas is converted to acetate (1st stage) and then the acetate is converted to TAGs (2nd stage). Depending on the composition of the C1 gases and particularly depending on the CO content of syngas, some bacteria can be more efficient than others to produce acetate. Since acetogenic bacteria can secrete a mixture of compounds (acetate, ethanol, lactate, butanediol, etc.) during syngas fermentation, metabolic engineering and synthetic biology are powerful tools to increase the acetate production and reduce the spectrum of unwanted byproducts. Therefore, the main product of gas fermentation, i.e. acetate, can be used as substrate for a second fermentation process.

-Role of acetogenic bacteria in syngas fermentation

In syngas fermentation, microorganisms use hydrogen (H_2) as an electron donor, carbon monoxide (CO) as a carbon source and electron donor, and carbon dioxide (CO_2) as a carbon source for producing chemicals such as acetate and ethanol. This collection of bacteria is commonly referred to as acetogenic bacteria, or “acetogens”. Acetogens play an integral role in the global carbon-assimilation cycle as they are extremely common in nature and can be found in soil sediments, intestinal tracts of mammals. Within the known 22 genera of acetogens, *Acetobacterium* and *Clostridium* accommodate the most known acetogenic species.

Converting CO_2 into acetate relies on the Wood-Ljungdahl pathway (WL-pathway), which allows acetogens to conserve energy and produce one molecule of acetate for every two molecules of CO_2 assimilated. Like many bacteria, acetogens can utilize sugars as a primary growth and energy substrate but with the WL-pathway, it enables them to thrive in competitive, anaerobic environments. Within the context of industrial bioprocesses, acetogens are able to utilize mixtures of industrial waste syngas ($H_2/CO_2/CO$) into acetate or ethanol depending on the species.

An efficient transformation of C1 gases into acetate requires to funnel the carbon metabolism to the secretion of acetate as the main or unique product. This means that the production of undesired by-products such as acetone, lactate, ethanol, and other alcohols, must be reduced or eliminated by creating mutant strains with a reduced capacity to secrete these undesired compounds.

The technologies currently used to improve the producer strains are mainly based in conventional random mutagenesis and selection procedures that are grounded in typical trial and error

methodologies. Within BiosFerA, a more rational approach is implemented to construct the strains based on the use of new tools and technologies provided by systems and synthetic biology. This approach will be faster and more efficient since it allows us to construct complex mutants that will have an extremely low probability of being generated by random mutagenic approaches. Moreover, the proposed technologies can be combined when required with the random mutagenic approaches and with the more sophisticated Adaptative Laboratory Evolution for further improvement of the strains. Finally, the use of pressurized bioreactors at lab-scale is very infrequent since it requires special facilities but the potential of using these bioreactors will provide new insights in the fermentation of syngas.

-Main parameters influencing gas fermentation performance

Experiments were carried out in order to identify the best acetogenic bacteria in terms of productivity, yield and titer of acetate, which will be used to feed the subsequent fermentation process. Fully equipped pressurize fermenters were used. As the gas fermentation step is the bottleneck of the global process, a strategy for process optimization is necessary in order to improve both overall reaction rate and global yield of the process. The following key aspects should be considered:

- Optimization of syngas flow rates and pressure conditions. Solubility of H_2/CO/CO_2 are typically limiting fermentation kinetics. Increasing the pressure improves the gas solubility, potentially boosting the efficiency
- Optimization of the fermentation strategy. The optimal fermentation mode (batch, fed batch, continuous, or continuous with cell recycle) should be determined. Indeed, in line with experience from previous trials, setting up a continuous fermentation with cell recycle is likely to boost the productivity, as acetogens are known to be relatively slow growers.
- Implementation of a close loop gas system (recirculation of unconverted syngas to the liquid fermentation broth). A batch reaction system equipped with an internal recirculation system would increase significantly the overall yield and will prevent gas substrate losses.
- Optimization of the operation protocol (time, optimal ratios of H_2/CO/CO_2, etc.)

BBEPP and CARTIF studied every parameter involved in the fermentation process to satisfy the desired quality attributes. Some of these parameters were the substrate concentration and composition, impurities and inhibitors, removal of by-products or stability of microbial culture and fermentation product yield accordingly to process parameters (temperature, pH, etc.) and feedstock addition.

This strategy allowed the lab-batch operation mode to be studied with special emphasis in developing a cheap fermentation broth and highly efficient/yield process. The validation of syngas fermentability was performed progressively increasing fermenter volumes, from 1 to 10 L, and was carried out on clean gases, comparable to what would be used in FT-synthesis. One of the main advantages of BioSFerA technology compared to FT-synthesis is the tolerance of acetogenic bacteria towards contamination. Studies were performed to evaluate the maximum allowable concentration of a) tars and light hydrocarbons (Eg benzene, toluene, naphthalene, ethylene, acetylene, propylene, etc.), b) N-group impurities (NH_3, HCN, NOx), c) S-group impurities (H_2S, COS, SOx), d) halogen impurities (HCl, HF), and e) metals.

Apart from spiking these impurities, fermentation using scrubber water obtained from the actual gasification was performed as well with the majority of the actual contaminants present in the real

syngas. The obtained results will allow to reduce the purification of the syngas obtained by gasification of various biomass streams during the scale up in WP4.

-Mode of cultivation: serum bottles and bioreactors

Fermentations were carried out first on a small scale, in serum bottles, and then in a bioreactor. In serum bottles, variables such as agitation or pH were not controlled, so it was not possible to optimize the fermentations, although an initial study could be carried out in order to select the best culture medium. The bioreactor fermentations allowed greater control of the process, including pressure, pH and gas mixing. Likewise, the volume of the bioreactors used increased as the number of experiments increased.

3 Selection of culture media for the growth of acetogenic strains (CARTIF)

From CARTIF's side, the growth of the four initially selected anaerobic bacterial strains (*M. thermoacetica* DSM 2955, *M. thermoacetica* DSM 521, *C. ljundahllii* and *C. autoethanogenum*) was evaluated by means of growth assays using 100 mL serum bottles. All strains used were provided by CSIC as they have been developed in the framework of Task 3.2.

Different rich culture media, such as TSB or YTF, and other specific culture media for anaerobic microorganisms described in literature were tested. The composition of used culture media is described in the Annexes.

For the preparation of culture media in serum bottles under anaerobic conditions, the following protocol was carried out. All media ingredients were mixed in distilled water except those that are heat sensitive (vitamins), precipitating agents (sodium carbonate) and the reducing agent (cysteine-HCl). The pH was adjusted and then divided into serum bottles, which were tightly closed with butyl rubber septum and aluminium centre hole caps. Then, the bottles were bubbled with nitrogen gas at 1.5 bar for 30 minutes using sterile syringes and needles, in order to purge any trace of oxygen. Once the oxygen was purged, the bottles were autoclaved at 121 ºC for 15 minutes. After autoclaving, sterile and anoxic supplement solutions were injected into the medium using sterile needles and syringes.

For each growth assay, two consecutive precultures were performed for each experiment. The first pre-culture with a total volume of 10 mL (in 100 mL serum bottles) contained 1 mL of thawed cryo-stock and was cultivated for 24 h. The second preculture with a total volume of 50 mL (in 100 mL serum bottles) was inoculated in a 1:10 ratio.

Clostridium sp. cultures were grown at 37 °C and *Moorella* sp. cultures were grown at 60 °C in different lab´s incubators. No shaking was performed.

Two replicates of each growth test were performed for each type of anaerobic bacteria and growth tests were maintained up to 96 hours.

Liquid samples were taken from the fermentation bottles and acetate concentration analyzed by HPLC.

The growth of the microorganisms was determined by measuring the optical density at a wavelength of 600 nm ($OD_{600\ nm}$) using a UV-Vis spectroscope.

The following Figure 1 shows the results of the growth tests in rich media (YTF, TSB).

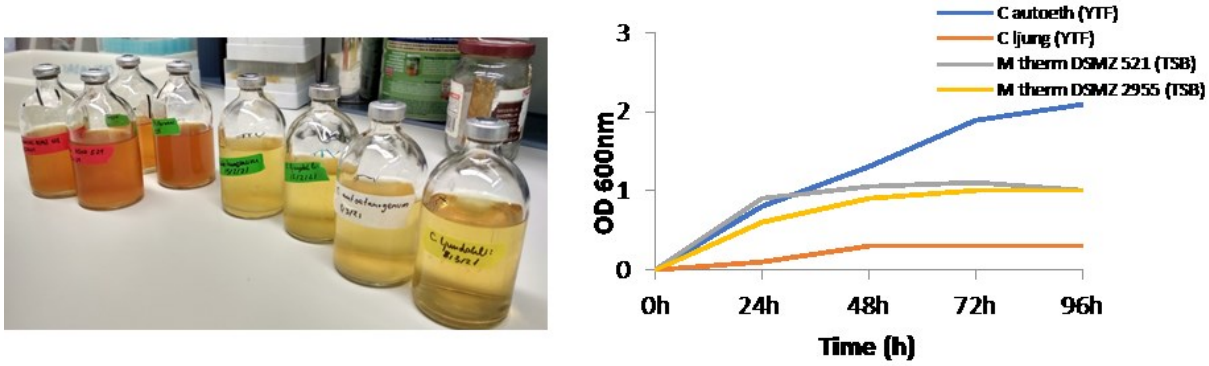

Figure 1: Growth of acetogenic strains on rich media (YTF, TSB) during 96 hours.

Results indicated that all four strains are able to grow on rich media for 96 hours. Specifically, *C. autoethanogenum* showed the highest growth rate, although both *Morella* strains also grew adequately.

In addition, the growth was also tested using a minimal media previously described by Fröstl et al. (1996). This media could be used in the bioreactor trials and subsequent scale-up activities at pilot plant due to their lower cost and absence of yeast extract.

The results of the 96-hour growth assays in 100 mL serum bottles showed that all three strains tested (*M. thermoacetica* DSM 2955, *C. ljundahlii* and *C. autoethanogenum*) are also able to grow on these minimal media and produce acetate (Figure 2).

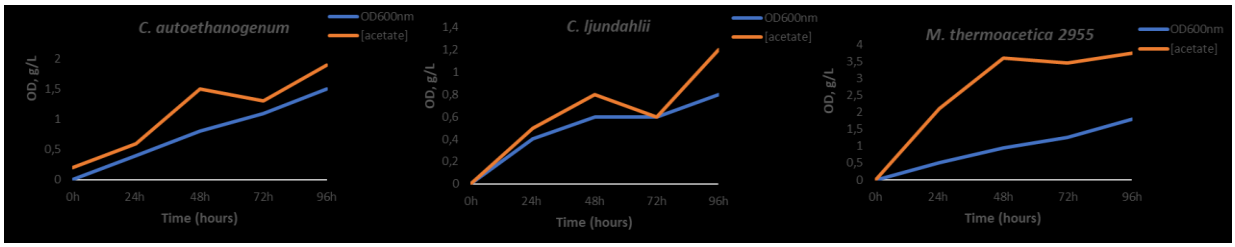

Figure 2: Growth, acetate production of acetogenic strains on minimal medium during 96 hours.

The best results in terms of OD_{600nm} (1.5) and acetate production (3.63 g/L) were achieved with *M. thermoacetica* DSM 2955, so it was decided to continue working with this strain to further optimise acetate production in the bioreactor.

4 Selection of an optimal fermentation medium for *M. thermoacetica* (BBEPP)

4.1 Anaerobic cultivation in serum bottles

For the production of acetic acid through gas fermentation, BBEPP focused efforts on *Moorella thermoacetica*. This acetogenic bacterium was acquired as a freeze-dried culture from the public culture collection of Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (DSMZ, Germany). Since *M. thermoacetica* is an anaerobic organism, a methodology was developed to perform anaerobic cultivations using closed serum bottles (Figure 3).

Figure 3: Serum bottles used for media screening experiments at BBEPP.

In this public report, BBEPP included the main outcome and conclusions of all performed serum bottle experiments.

4.2 Suitable media for *M. thermoacetica*

In order to identify a suitable medium to support growth and acetic acid production by *M. thermoacetica*, eight different liquid media were selected from literature. Both heterotrophic (on glucose with CO_2 in the headspace) and autotrophic (no glucose and with CO_2 and H_2 in the headspace) conditions were assessed for all media.

Under heterotrophic conditions, a maximal OD_{600} of 4.14 and acetate concentration of 7.9 g/L acetate were obtained. With the same medium, also the best results were obtained under autotrophic conditions, reaching an even higher concentration of 8.2 g/L acetate. This best-performing medium was used as the basis for all further experiments.

5 *M. thermoacetica* tolerance towards syngas contaminants (BBEPP)

Moreover, BBEPP explored the influence of syngas contaminants on the performance of *M. thermoacetica*. In the BioSFerA concept, these contaminants are present in the syngas obtained by biomass gasification and can be eliminated by a series of purification steps, as described by VTT in BioSFerA Deliverable 3.1.

To evaluate the tolerance of *M. thermoacetica* towards these contaminants serum bottle experiments were performed. The tolerance towards water soluble contaminants was assessed by using water samples provided by VTT. Interestingly, after some purification, the growth obtained on contaminated water peered with results obtained on pure water. These findings provide valuable insights for setting up a purification process, which is crucial when performing the coupled gasification and gas fermentation process at pilot scale within BioSFerA WP4.

6 Acetate production by *M. thermoacetica* in bioreactor (CARTIF)

After completion of fermentation trials in 100 mL serum bottles, work was also carried out on the setting up of a pressurized bioreactor that was be used in the anaerobic fermentations with *M. thermoacetica* DSM 2955. The bioreactor, which can be seen in Figure 4, is made of stainless steel, operates up to a pressure of 10 barg allowing greater solubility of the gases in the medium, allows selection of the desired flows of each of the gases (CO_2, CO, H_2, N_2), has the usual control systems (pH, stirring, temperature). It also has two ports for taking liquid and gas samples. It has a gas recirculation line, equipped with filters and a compressor, which would allow continuous recirculation of gases in order to operate in a batch system but with continuous bubbling, favoring the gas-liquid mass transfer.

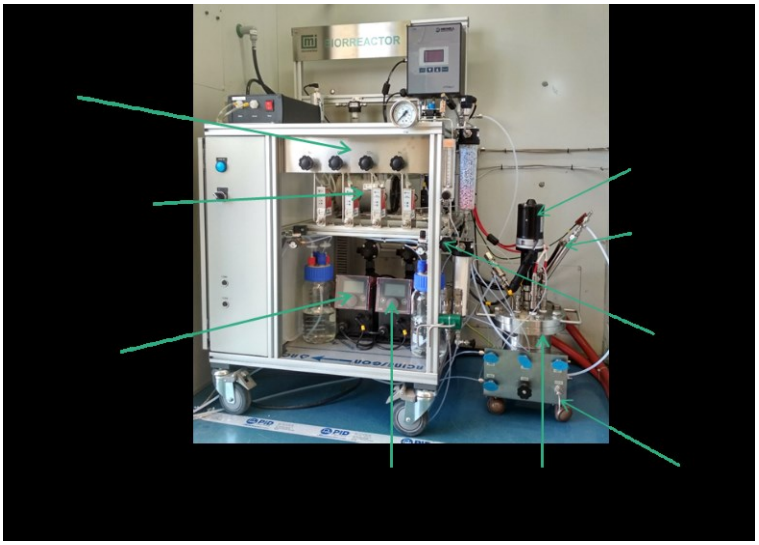

Figure 4: 1 L bioreactor for gas fermentation at CARTIF.

Once the bioreactor was fully operative, the first anaerobic fermentations were started with the wild-type strain *M. thermoacetica* DSM 2955 to study acetate production under controlled conditions of pH, temperature, agitation, pressure and gas feed.

For all fermentation experiments, the preinocula of *M. thermoacetica* were grown on *Moorella* medium (see Annexes).

Table 1 shows the fermentations performed in 1 L bioreactor at CARTIF. As for the experiments carried out, mainly the use of pressure has been made to favor the solubility of gases. All fermentations were carried out at 60 °C. Fermentation F1 took place at 5 barg, with a H_2:CO:CO_2:N_2 gas ratio of 2:1:1:1, pH 7 and no-cosubstrate addition. In the following experiments (F2, F3, F4) co-substrate in the form of 20 g/L glucose was always added to increase biomass growth. The pressure in these fermentations reached 5 barg, but it was give through a progressive incrase that allowed the microorganisms to get used to a high concentration of gases gradually. The pH in these fermentations was reduced to 6 as recommended by BBEPP. In these fermentations the effect of the co-substrate and the effect of different ratios of H_2:CO:CO_2:N_2 gases were studied, being F2 (2:1:1:1), F3 (3:1:1:1).

Table 1. Description of gas fermentation experiments.

	T (ºC)	P (barg)	pH	Glucose (g/L)	H_2-CO-CO_2-N_2	Acetate (g/L)	OD_{600nm}
F1	60	5	7.0	0	40%-20%-20%-20% (2:1:1:1)	3.3	2.5
F2	60	1.5-2.5-3.5-5.0	6.0	20	40%-20%-20%-20% (2:1:1:1)	13.2	4.5
F3	60	1.5-2.5-3.5-5.0	6.0	20	50%-17%-17%-17% (3:1:1:1)	6.8	3.93
F4	60	1.5-2.5-3.5-5.0	6.0	20	25%-25%-25%-25% (1:1:1:1)	7.78	3.4

In all fermentations the operation was carried out in batch mode and using Demler as culture medium.

Liquid samples were taken from the bioreactor and glucose, acetate analyzed by HPLC as described in the Annexes. The growth of the microorganisms was determined by spectrophotometry measuring the optical density at a wavelength of 600 nm ($OD_{600\ nm}$).

Figure 5 shows the evolution of acetate concentration and the biomass growth through $OD_{600\ nm}$. As can be seen, during the F1 fermentation there was no growth of microorganisms on the first day of fermentation, but in the following days a constant growth took place, showing a continuous consumption of gases. An $OD_{600\ nm}$ value of 2.36 was reached after 6 days of fermentation. This value is not high, although if the experiment had been continued longer it would probably have increased. In the case of F2, carried out with a gradual increase in pressure and with 20 g/L glucose as co-substrate, a higher concentration of *M. thermoacetica* was reached. The evolution in $OD_{600\ nm}$ was of an initial increase between days 1 and 3, followed by a decrease between days 3 and 9, and a marked final increase between days 9 and 14. This evolution may be due to an initial growth associated with glucose consumption followed by a period of adaptation to the new substrate (gas mixture) that ends with a considerable increase in the concentration of *M. thermoacetica*, reaching a maximum $OD_{600\ nm}$ value of 4.668 after 13 days of fermentation.

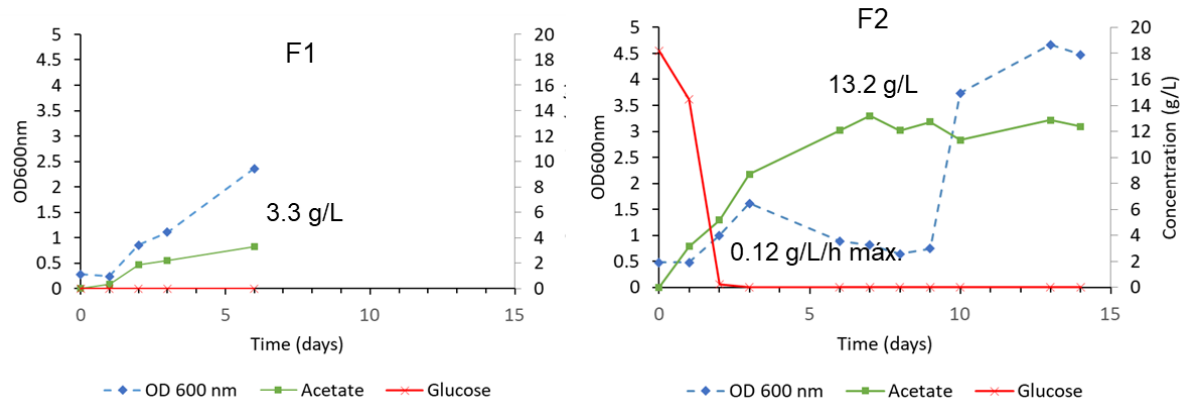

Figure 5: Results of anaerobic fermentations in bioreactor. F1) no glucose, F2) glucose.

Regarding the evolution in substrate consumption and product formation, it can be seen that in F1 acetate was produced up to a concentration of 3.3 g/L after 6 days of fermentation, which is a low production. The evolution in acetate production shows that it does not necessarily follow the same trend as the growth of microorganisms. In the case of the F2 fermentation with co-substrate, acetate production was more significant. This occurred from the beginning of the fermentation until day 7, reaching a maximum of 13.2 g/L. Glucose was totally consumed in the first two days of fermentation. After this initial period, acetate production continued to increase confirming the consumption of gases after glucose consumption. Again, the evolution in the acetate concentration did not seem to be related to the evolution in the concentration of microorganisms, as the biomass concentration experienced a decrease and a marked increased while acetate only experienced a gradual increase.

The maximum titer of acetate achieved was 13.2 g/L and the acetate productivity was 0.12 g/L/h.

A test was carried out with gas recirculation in order to keep them continuously bubbling, in batch mode and performing gas recharges when the pressure dropped below the desired value. The test was not valid due to the impossibility of sterilizing the compressor area, causing contamination problems even working at 60 °C. Since recirculation was not possible, the gases are not continuously recirculated but are in a head space, in contact with the liquid phase only though stirring. It is likely that this stirring does not provide sufficient mass transfer to ensure good solubility for a higher production of acetate.

The rest of fermentations (F3, F4) were carried out using different syngas ratios and glucose as co-substrate (20 g/L) at the beginning of the fermentation, but results obtained in terms of acetate concentration and biomass produced were lower than those of the previous fermentation F2.

During F3 a H_2:CO:CO_2:N_2 gas ratio of 3:1:1:1 was used and the pressure inside the bioreactor was gradually increased up to 5 barg during the course of the experiment. In this case a maximum acetate titer of 10.8 g/L was reached and the OD_{600nm} increased up to 3.93 after 11 days of fermentation. The maximum acetate productivity achieved was 0.098 g/L/h.

During F4 a H_2:CO:CO_2:N_2 gas ratio of 1:1:1:1 was used, maintaining the same operating conditions as in the previous fermentations. In this case, the maximum acetate titer achieved was 7.78 g/L, the OD_{600nm} reached 3.4 and the maximum acetate productivity was 0.032 g/L/h after 14 days of fermentation.

The following Figure 6 show the evolution of F3 and F4 in terms of biomass production and acetate, glucose concentration over time.

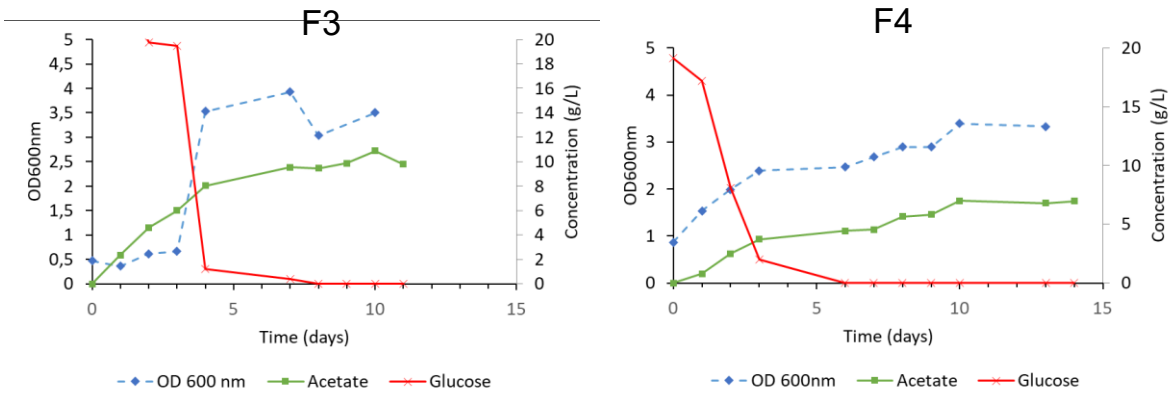

Figure 6: Time course of biomass (OD_{600nm}), acetate (g/L) and glucose (g/L) during F3 and F4.

The results show that in order to increase the acetate concentration it is necessary to increase the biomass produced during fermentation by a much larger amount. This point is crucial because, even if the culture is stable, it is not able to increase acetate productivity.

7 Acetate production by *M. thermoacetica* in bioreactors (BBEPP)

Following the serum bottle experiments, BBEPP conducted a series gas fermentations in state-of-the-art bioreactors aiming to produce acetate. In this public report, BBEPP included the main outcome and conclusions of all performed fermentations.

7.1 Fed-batch gas fermentations at 1 L scale

For the first fermentations, four parallel 1 L continuously stirred bioreactors with a working volume of 800 mL were used (Figure 7). Each bioreactor is equipped with separate mass flow controllers for H_2, CO_2, CO and N_2, which allows continuous sparging of different inlet gas mixtures. In addition, they can be pressurized to stimulate the gas transfer towards the microbial culture. The reactors can be controlled separately for pH, temperature, stirring, gas flow, pressure and foaming. They also have the possibility of the addition of feed.

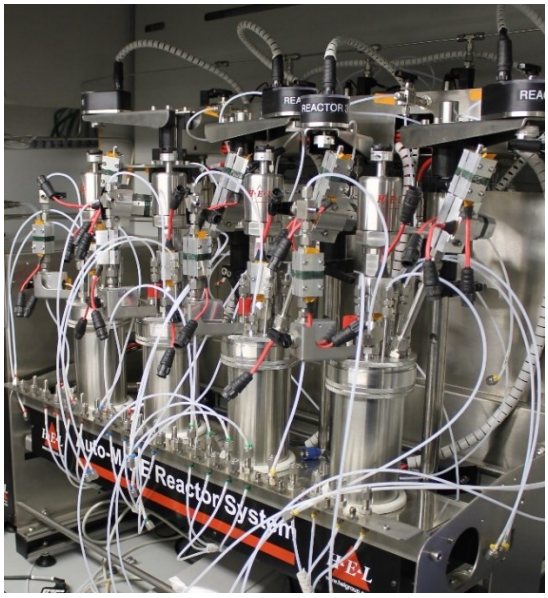

Figure 7: Four parallel 1 L bioreactors for gas fermentation at BBEPP.

Four fed-batch gas fermentations were successfully performed testing different gas mixes, pH, gas flows and pressures. The fermenters were inoculated with an active culture grown in serum bottles. All fermentations were carried out with continuous monitoring and control of key process parameters such as pH, temperature, stirring, gas flow, and pressure. Foaming was controlled by adding an antifoaming agent when necessary. Culture parameters such as optical density, metabolites, and off-gas were analyzed regularly to follow up on growth, acetate production, and gas consumption.

Each fermentation resulted in further optimization of the process at 1 L scale, with a final maximal productivity of 0.28 g/L/h and product concentration of 30 g/L acetate.

7.2 Continuous gas fermentations with cell-recycle at 10 L scale

For further development and optimization of the acetic acid production process, the gas fermentation process was scaled-up in a 10 L stainless steel CSTR with a 7 L working volume. The bioreactor allows for continuous sparging of CO_2, H_2, CO, and N_2, pressure control, pH control, addition of feed and antifoam. The reactor can work at elevated pressures to increase the gas transfer rate. An alternative fermentation mode, namely continuous fermentation with cell-recycle, was investigated. This allowed for continuous withdrawal of the produced acetic acid from the broth, while retaining the biomass in the bioreactor. As such, a higher biomass concentration could be reached, which subsequently led to a higher acetate productivity. In addition, this set-up led to the continuous production of a biomass-free acetic acid stream, which could directly be fed to the second-stage reactor (BioSFerA Task 3.4). The cell-recycle set-up was accomplished by installing an external membrane to retain the cells and three mobile high-pressure pumps for feed, filtrate, and recirculation as illustrated in Figure 8 and Figure 9.

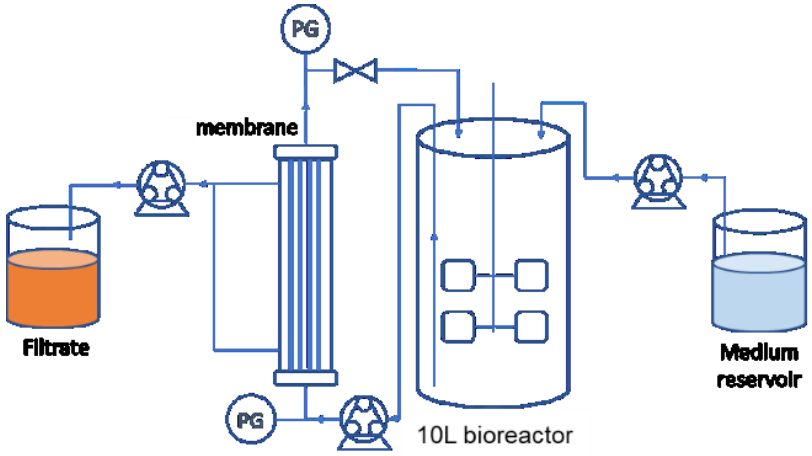

Figure 8: Schematic illustration of set-up for 10 L gas fermentation bioreactor with a ceramic membrane for a continuous process with cell-recycle

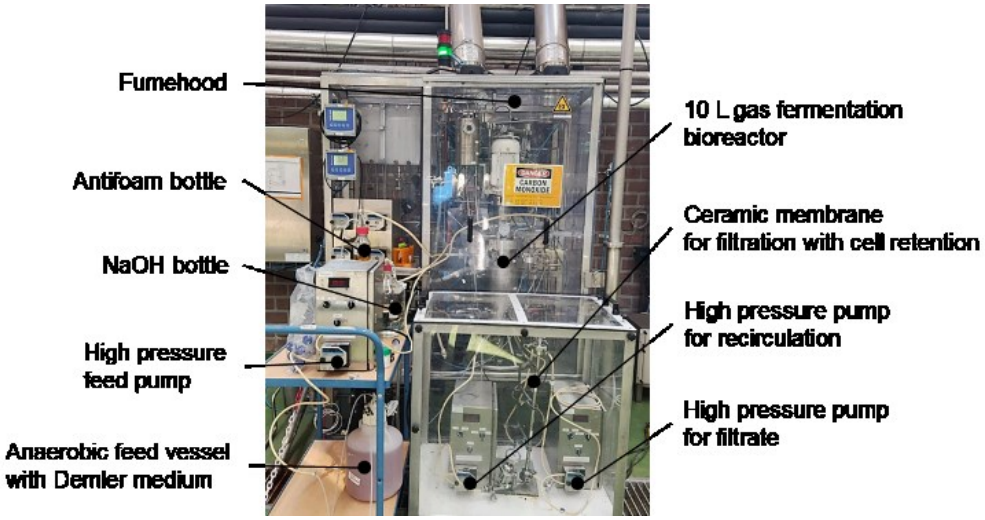

Figure 9: Actual set-up at BBEPP of the 10 L gas fermentation bioreactor with a ceramic membrane for a continuous process with cell-recycle

Four continuous gas fermentations were performed. Similar to the fed-batch fermentations, the culture was grown in serum bottles before inoculation into the bioreactor. All fermentations were carried out with continuous monitoring and control of key process parameters such as pH, temperature, stirring, gas flow, and pressure. When necessary, an antifoaming agent was used to control foaming. Optical density, metabolites, and off-gas were analyzed regularly to follow up on growth, acetate production, and gas consumption. The gas inlet stream consisted of a syn gas mixture, for which the ratios were determined based on the gas provided after biomass gasification. More specifically, these ratios were measured by VTT and described in BioSFerA Deliverable 3.1.

The fermentations indeed showed an increase in biomass concentration through the set-up of a continuous fermentation with cell-recycle. The target acetate concentration of 30 g/L was achieved, while the productivity could be improved up to 0.42 g/L/h.

8 Conclusions

Conclusions BBEPP:

- The commercially available wild-type strain *M. thermoacetica* DSM2955 showed sufficient performance for acetate production. Hence, although no optimized strain was obtained by CSIC, as described in Deliverable 3.2, fermentation development and optimization could take place.
- Media screening experiments in serum bottles by BBEPP identified an optimal medium for growth and acetate production by *M. thermoacetica*.
- Experiments assessing the tolerance of *M. thermoacetica* towards contaminants present in the VTT syngas stream (described in BioSFerA Deliverable 3.1), showed that certain gas cleaning steps are required to allow growth and acetate production.

- Fed-batch fermentations on 1 L scale performed resulted in the production of more than 30 g/L, thereby reaching the BioSFerA target, with a productivity of 0.26 g/L/h.
- A proof-of-concept for continuous fermentation with cell-recycle was established. This allowed for the production of a sterile acetic acid stream containing around 30 g/L acetic acid. This stream was used by BBEPP as a feedstock for the TAG production fermentation (described in BioSFerA Deliverable 3.5).
- The scale-up from 1 L to 10 L was performed successfully reaching productivities up to 0.42 g/L/h and also the BioSFerA target concentration of 30 g/L was achieved.
- Fermentations on syngas, for which the composition was identical to the gas obtained by VTT, showed a similar increase in biomass concentration and productivity as other tested conditions.

Conclusions CARTIF:

- Glucose as a co-substrate in the early stages of the anaerobic fermentation increases biomass and acetate production.
- 13.2 g/L of acetate were produced by *M. thermoacetica* using the syngas ratio of 40% H_2, 20% CO, 20% CO_2, 20% N_2 (2:1:1:1).

9 Future actions

In order to improve the results obtained under WP3 some future actions are proposed by CARTIF. The next trials to be carried out are described more specifically below:

- Test the performance of a clostridial strain (*C. autoethanogenum*) in terms of acetate production using the lab scale gas fermenter to compare yields with *M. thermoacetica*.

From BBEPP´s side:

- Given the gained expertise, the gas fermentation process can be scaled up to TRL5 and coupled with the gasification process in BioSFerA WP4.
- The continuous fermentation with cell-recycle strategy will be further optimized in BioSFerA WP4 to increase the biomass concentration and aim to reach the BioSFerA target productivity of 0.55 g/L/h.

10 Annexes

Annex 1. Media compositions

YTF medium

Routine medium growth for *Clostridium ljungdahlii* and *Clostridium autoethanogenum.*

Composition:

- Yeast extract, 10 g/L
- Tryptone, 16 g/L
- Fructose, 5 g/L
- Sodium chloride, 4 g/L
- Cysteine HCl, 0.75 g/L

The pH was set to 6 using 1 M HCl and the final volume was adjusted using autoclaved deionized water.

TSB medium

Tryptic Soy Broth (TSB) is a nutritious medium that supports the growth of a wide variety of microorganisms, especially common aerobic and facultatively anaerobic bacteria.

Composition:

- Casein peptone (pancreatic), 17 g/L
- Dipotassium hydrogen phosphate, 2.5 g/L
- Glucose, 2.5 g/L
- Sodium chloride, 5 g/L
- Soya peptone (papain digest.), 3 g/L

The final pH (at 25°C) was set at 7.3±0.2 and the final volume was adjusted using autoclaved deionized water.

Fröstl medium

Fröstl medium is a minimal medium used for the growh of *Moorella thermoacetica* described by Fröstl et al. (doi: 10.1128/jb.178.15.4597-4603.1996).

Composition:

- Sodium hydrogen carbonate, 7.5 g/L
- Potassium dihydrogen phosphate, 5 g/L
- Sodium chloride, 0.4 g/L
- Ammonium chloride, 0.16 g/L
- Magnesium chloride hexahydrate, 0.05 g/L
- Calcium chloride dihydrate, 0.01 g/L
- Nicotinic acid, 0.002 g/L

- Cyanocobalamin, 0.25 mg/L
- p-aminobenzoic acid, 0.25 mg/L
- Calcium D-pantothenate, 0.25 mg/L
- Thiamine HCl, 0.25 mg/L
- Riboflavin, 0.25 mg/L
- Lipoic acid, 0.25 mg/L
- Folic acid, 0.1 mg/L
- Biotin, 0.1 mg/L
- Pyridoxal HCl, 0.1 mg/L
- Sodium nitrilotriacetate, 7.5 mg/L
- Manganese(II) sulfate monohydrate, 2.5 mg/L
- Iron(II) sulfate heptahydrate, 0.5 mg/L
- Cobalt(II) nitrate hexahydrate, 0.5 mg/L
- Zinc chloride, 0.5 mg/L
- Nickel(II) chloride hexahydrate, 0.25 mg/L
- Selenious acid, 0.25 mg/L
- Copper(II) sulfate pentahydrate, 0.05 mg/L
- Aluminium potassium sulfate dodecahydrate, 0.05 mg/L
- Boric acid, 0.05 mg/L
- Sodium molybdate dihydrate, 0.05 mg/L
- Sodium tungstate dihydrate, 0.05 mg/L
- Sodium sulfide nonahydrate, 500 mg/L

The final pH (at 25°C) of the medium was adjusted at 6.8 and the final volume was adjusted using autoclaved deionized water.

Demler medium

Demler medium is a nutritious medium that supports the growth of different anaerobic microorganisms (*Acetobacterium*, *Moorella*). The ingredients of this medium can be classified into base nutrients, trace elements, vitamins, sodium hydrogen carbonate and cysteine HCl H_2O. These five groups of ingredients were prepared separately.

Composition:

- Ammonium chloride, 1 g/L
- Potassium dihydrogen, 0.33 g/L
- Potassium hydrogen phosphate, 0.45 g/L
- Magnesium sulfate heptahydrate, 0.16 g/L
- Yeast extract, 2 g/L
- Sodium hydrogen carbonate, 10 g/L
- Cysteine hydrochloride monohydrate, 0.5 g/L
- Trace elements *
- Vitamins **
- Fructose 10g/L

*: nitriloacetic acid 30 mg/mL, $MnSO_4 \cdot H_2O$ 10 mg/mL, NaCl 20 mg/mL, FeSO4·7H2O 2 mg/mL, CoSO4·7H2O 3.6 mg/mL, CaCl2·2H2O 2 mg/mL, ZnSO4·7H2O 3.6 mg/mL, CuSO4·5H2O 0.2 mg/mL, KAl(SO4)2·12H2O 0.4 mg/mL, H3BO3 0.2 mg/mL, Na2MoO4·2H2O 0.2 mg/mL, NiCl2·6H2O 0.5 mg/mL, Na2SeO3·5H2O 0.006 mg/mL.

**: biotin 0.00004 mg/L, folic acid 0.00004 mg/L, pyridoxine-HCl 0.2 mg/L, thiamine-HCl·2H2O 0.1 mg/L, riboflavin 0.1 mg/L, nicotinic acid 0.1 mg/L, D-Ca-pantothenote 0.1 mg/L, cyanocobalamine 0.000002 mg/L, p-aminobenzoic acid 0.1 mg/L, and lipoic acid 0.1 mg/L.

***Moorella* medium**

The medium is composed of six different solutions, as follows:

Solution A	843 mL
Solution B	50 mL
Solution C	50 mL
Solution D	40 mL
Solution E	10 mL
Solution F	10 mL

Solution A:

- Magnesium sulfate heptahydrate, 0.1 g
- Ammonium sulphate, 0.5 g
- Ammonium ferrous sulfate hexahydrate, 0.04 g
- Sodium molybdate dihydrate (0.1% w/v), 2.4 mL
- Disodium selenite hydrate (0.1% w/v), 0.15 mL
- Tryptone, 5 g
- Yeast extract, 5 g
- Sodium resaruzin (0.1% w/v), 0.5 mL
- Distilled water, 840 mL

Solution B:

- Potassium dihydrogen, 4.5 g
- Potassium hydrogen phosphate, 7 g
- Distilled water, 50 mL

Solution C:

- Glucose, 18 g
- Distilled water, 50 mL

Solution D:

- Sodium carbonate, 2 g
- Distilled water, 40 mL

Solution E:

- L-Cysteine hydrochloride monohydrate, 0.3 g
- Distilled water, 10 mL

Solution F:

- Sodium sulfide nonahydrate, 0.3 g
- Distilled water, 10 mL

Solution A was sparged with 100% CO_2 gas for 30 - 45 min to make it anoxic, then distributed under same gas atmosphere into serum bottles and autoclaved. Solutions B, C, E and F were autoclaved separately, under 100% N_2 gas and solution D under 80% N_2 and 20% CO_2 gas atmosphere. Appropriate amounts of solutions B to F were added to the sterile solution A in the sequence as indicated in order to complete the medium. The pH of the complete medium was adjusted to 6.9.

Annex 2. Analytical methods

HPLC analysis

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