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AGRO LOUVAIN

Faculté des bioingénieurs

Fermentation of a complex organic feedstock to medium chain carboxylates by a natural microbial consortium : Investigation on the substrate influence and the process limitations

Author : Antoine de Halloy de Waulsort
Supervisor : Patrick Gerin
Readers : Jacques Mahillon, Patricia Luis Alconero
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Abbreviation list

4M-V: 4-methylvalerate

A2: ethanol

AML: acidogenic mixed liquor

BSG: brewer's spent grain

C2: acetate

C3: propionate

C4: n-butyrate

C5: n-valerate

C6: caproate

C7: heptanoate

C8: caprylate

cBSG: crude BSG

Cc: concentration

COD: chemical oxygen demand

COD_s: soluble COD

COD_{th}: theoretical COD

COF: complex organic feedstock

DM: dry matter

Equ: equation

EtOH: ethanol

Exp: experiment

Fig: Figure

FM: fresh matter

GC: gas chromatography

HRT: hydraulic retention time

MCCA(s): medium chain carboxylic acid(s)

Min: minute(s)

ML: mixed liquor

MMC: mixed microbial culture

NOF: no organic feedstock

pBSG: pressed BSG

p_{H2}: H_2 partial pressure; p_{CO2}: CO_2 partial pressure

RT: retention time

Rxx: bioreactor n°xx

SCCA(s): short chain carboxylic acid(s)

SRT: solid retention time

THF: tetrahydrofolate

UQ: ubiquinone

UQH_2: ubiquinol

VFA(s): Volatile fatty acid(s)

VM: volatile matter

1 Introduction

Our world is knowing an important demographic increase. The world population has more than tripled since 1950 (United Nations 2019). This demographic increase has been accompanied by an increase in the average level of life. Every year, more people live on Earth, and every year, a single person consumes more (consoGlobe 2021). This phenomenon results in a depletion of the natural resources, to start with fossil resources (Head 2011). Experts predict the depletion of the accessible fossil resources for 2050 (Head 2011). This will assuredly lead to an oil crisis. As a result, alternatives must be developed to replace oil.

Parallel to the demographic increase and to the rising average consumption, the volumes of organic waste have grown and still do (consoGlobe 2021). So far, the organic wastes usually serve as compost and fertilizer. Some coproducts of the food industry are also valorised as feed for livestock (Agler et al. 2011). Only a little part is processed to get some molecules of interest from it, or, in other words, is biorefined (Arslan et al. 2012; Steinbusch et al. 2008). The biorefining corresponds to "an integrated process that uses biomass as a feedstock for conversion into a range of differentiated products" (Law Insider). Among these differentiated products, two main product families stand out. These are gathered under the term of "platform". There is the sugar platform and the syngas platform (Arslan et al. 2012; Steinbusch et al. 2008).

The sugar platform utilizes enzymes which deconstruct biomass into sugars. The sugars produced correspond to intermediate molecules, which are then fermented to molecules of interest, such as fuels and chemicals (Fernández-Naveira et al. 2017).

The syngas platform consists in the gasification of organic matter via thermochemical techniques. The obtained gases are mostly CO and H_2. They are next converted into valuable chemicals like alcohols, fuels, etc (Fernández-Naveira et al. 2017).

To handle the upcoming oil crisis and, overall, the depletion of natural resources, there is a real opportunity to exploit more and better the growing volumes of organic waste. In this context, the recent development of a third biorefining platform - the carboxylate platform - showed encouraging prospects. However, it needs improvements (Weimer and Kohn 2016). This master's thesis investigates the potential of biorefining fermentable biomass into carboxylates through the use of bioreactors and examines the substrate influence and the limitations of the process.

2 State of the art

2.1 The carboxylate platform

2.1.1 History

Until now, the petrochemical industry produced enough carboxylates, so that it remained cheaper to extract fossil resources than bioprocessing organics into carboxylates (Arslan et al. 2012). Nevertheless, with the upcoming oil crisis, there is every day a greater interest to develop the biological procurement of carboxylates. That is why, in the last decade, a particular interest on carboxylates has grown (Weimer and Kohn 2016). Various researchers demonstrated the ability of microorganisms to ferment organic matter into carboxylates. The carboxylates are molecules which can efficiently be processed in many other types of molecules of great interest (alkanes, etc.). They are qualified as "platform molecules". Their bio-sourcing led to the definition of a new platform: the carboxylate platform (Weimer and Kohn 2016). The bioproduction of carboxylates is obtained via the "elongation" process (Spirito et al. 2014). Today, the difficulty resides in the efficiency of this process, and then in its economic profitability (De Groof et al. 2019).

2.1.2 Interests of elongation & medium chain carboxylates

Volatile fatty acids (VFAs), lactate and ethanol are the main intermediate products of the anaerobic digestion of organic matter. These are valuable metabolites, but their value is way lower than the value of a certain type of molecules: the medium chain carboxylic acids (MCCAs), or medium chain carboxylates.

The MCCAs correspond to a saturated carbon chain, with 6 to 10 C atoms, containing a carboxylic group. As the intermediate metabolites of the anaerobic digestion (VFAs, lactate and ethanol), MCCAs can also be obtained by anaerobic digestion. The intermediate metabolites, in addition to their lower value, have a lower energy content than the MCCAs. Above all, the intermediate metabolites are difficult to extract from the fermentation broth, unlike MCCAs (Spirito et al. 2014). The purpose of the elongation is then to convert those intermediate products into MCCAs, especially caproïc acid (C6). The MCCAs are poorly soluble in water (e.g., 10.19 g/l at 25°C for undissociated C6), so their recovery in an organic phase is relatively easy (Spirito et al. 2014). Besides, MCCAs have a larger range of applications. C6 is for example a valuable molecule in the pharmaceutical sector, in the agri-food sector and in the chemical industry (Cavalcante et al. 2017).

2.2 The anaerobic digestion to produce carboxylates

2.2.1 Microbial steps of the anaerobic digestion

Under anaerobiosis, many non-lignified organic feedstocks can be anaerobically digested by consortia of microorganisms, leading to the conversion of the organics into CO_2 and CH_4. Figure 1 shows the succession of the steps of this process.

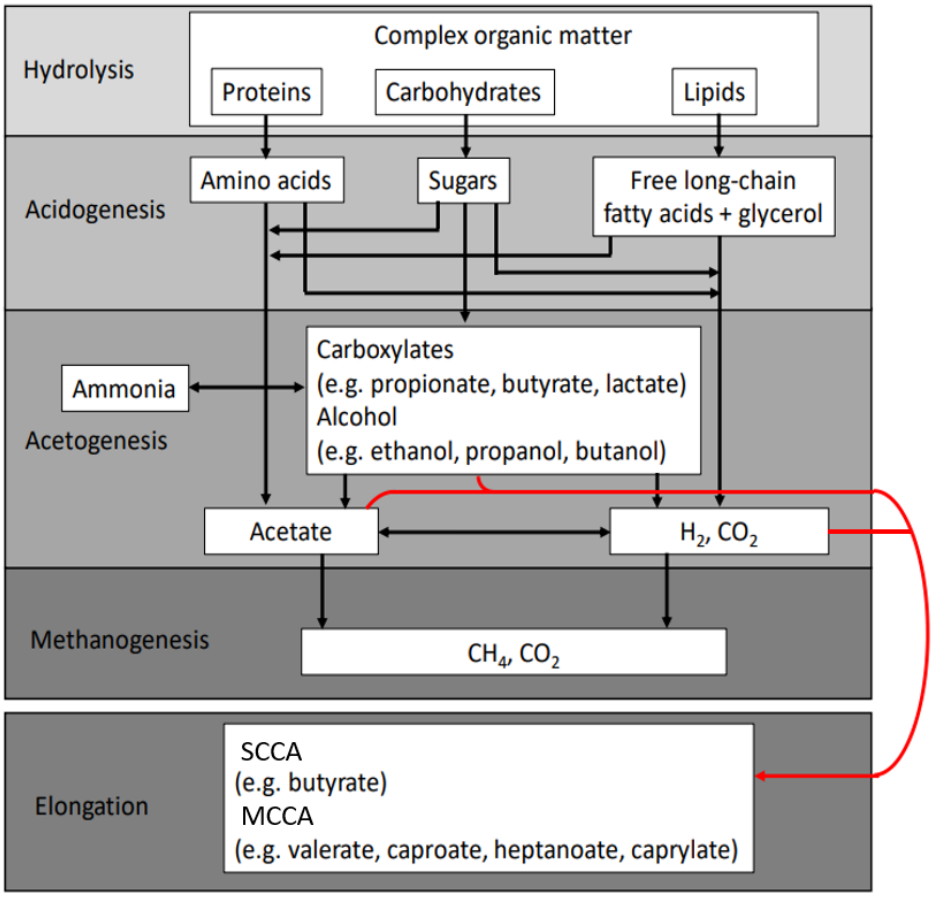

Figure 1: Connection of the microbial elongation of carboxylic acids with the anaerobic digestion process (Heymans et al. 2020).

The first step is the hydrolysis, in which polymers are decomposed and solubilized into their corresponding monomers and oligomers. Thereby, fats give place to fatty acids and glycerol, proteins give place to peptides and amino acids, etc (Arslan et al. 2013).

The second step is the acidogenesis. The products from step 1 are biologically converted to a mixture of short chain carboxylic acids (SCCAs), alcohols, CO_2 and H_2. The SCCAs are mostly acetate, propionate, lactate and n-butyrate, whereas the alcohols are mainly represented by ethanol (Agler et al. 2011). In this phase, inorganic compounds can be produced, such as ammonia, sulfides, and H_2 (Arslan et al. 2013).

The acidogenesis is in principle followed by the acetogenesis, in which the step 2 products are converted into exclusively acetate, H_2 and CO_2.

Finally, the methanogenesis consumes the products of acetogenesis and acidogenesis to produce only CH_4 and CO_2.

2.2.2 Methanogenesis, a competitive pathway

The elongation pathway is in competition with methanogenesis (Fig 1). This latter anaerobic conversion is thermodynamically and kinetically more favourable than the elongation pathway (Spirito et al. 2014). Methanogenesis operates then, in most of the conditions, preferentially to the elongation. Both can happen simultaneously, but the elongation is slowed down when it is simultaneous to methanogenesis (Agler et al. 2014).

The production of CH_4 is not bad since methane can be used in multiple applications. Nevertheless, it is a low value compound, and MCCAs offer better and larger possibilities than CH_4 (Angenent et al. 2016). So, having the objective to produce MCCAs, the methanogenesis needs to be inhibited.

Various techniques exist to inhibit methanogenesis.

- A simple method is to add in the fermenter a methanogenic inhibitor. The 2-bromoethylsulfonate has shown good results, but its elevated cost as well as its environmental impact limit its relevance (Reddy et al. 2018).

- Many studies reported that a heat pre-treatment of the inoculum could temporarily prevent the development of methanogen archaea (Agler et al. 2012; Mei et al. 2016), while not suppressing the elongating spore-forming bacteria (Weimer and Stevenson 2012). The disadvantage of this method is that the heat shock only has a short-term effect (Luo et al. 2010). Consequently, a periodic heat shock can be used (Agler et al. 2011).

- A common other technique is the control of the pH. Methanogenesis operates best in the pH range 6.0-8.3, while acidogenic and elongating bacteria bear greater acidity (Angelidaki and Sanders 2004). Several experiments were conducted maintaining the pH between 5.0 and 5.5 (Agler et al. 2014; Duber et al. 2018; Jankowska et al. 2018). This allowed to impede methanogenesis and did not prevent elongation. However, some researchers reported the occurrence of methanogenesis even working at pH 5.0 (Taconi et al. 2008).

- Finally, Spirito et al. (2014) reported that methanogenesis could be avoided by applying a high H_2 partial pressure.

2.2.3 The elongating microorganisms

A lot of studies about the impact of the inoculum on the elongation process have been driven. Several of them focused on *Clostridium kluyveri*. In the elongation experiments where a microbial analysis was conducted, this bacteria has indeed always been identified (Agler et al. 2012; Weimer and Stevenson 2012).

Yet, the majority of the studies worked on mixed microbial cultures (MMCs) (De Groof et al. 2019). These were used as natural microbial consortia, typically from sludge of wastewater treatment plant.

- MMCs present the advantage of being able to ferment very different complex substrates[1], because of the natural diversity of the consortium (Agler et al. 2012).

- This diversity as well allows the culture to be resilient and tolerate disturbances which could occur in the fermenter (rapid pH drop, presence of pollutants in the organic feedstock, etc.) (Agler et al. 2012).

- Besides, working with a MMC can enable to avoid sterilization. Sterilization is energy-consuming, time-consuming, and difficultly ensures a perfectly sterile environment (Grootscholten et al. 2014).

The drawback with MMCs is that they produce a mixture of metabolites, which must then be processed, extracted, or isolated. Then the final step is usually more complicated than with pure cultures, in which only a few metabolites are produced (Arslan et al. 2013).

2.3 The elongation process

2.3.1 Known elongation pathways

2.3.1.1 Homoacetogenesis

This pathway has been identified in many bacterial genera, including *Clostridium* (Spirito et al. 2014). It consists in a succession of sub-reactions involving cofactors such as coenzyme A and tetrahydrofolate (THF). The general reaction is:

Equ 1) $4\ H_2 + 2\ CO_2 \longleftrightarrow CH_3COO^- + H^+ + 2\ H_2O$

At 37°C with a pH of 6.82 and a H_2 partial pressure (p_{H2}) of 1 atm, it is largely favourable with a Gibbs free energy of -86.8 kJ/mol of acetate (Spirito et al. 2014).

[1] A complex substrate is a natural matrix composed of multiple molecules, taken as it is (De Groof et al. 2019).

2.3.1.2 Succinate formation

The formation of succinate from glycerol has been identified in genera less "classic" than *Clostridium*, like *Actinobacillus* (Spirito et al. 2014). Glycerol is a product from hydrolysis and acidogenesis since, in anaerobic digestion, it mostly originates from triglycerides and, in general, fatty organic feedstocks. Under standard conditions, the formation of succinate from glycerol is thermodynamically favourable. At 37°C and pH 6.82, the Gibbs free energy is worth -41 kJ/mol of succinate (Spirito et al. 2014). As for homoacetogenesis, the pathway is long and includes cofactors. The general reaction is summarized as (Zhang et al. 2010):

Equ 2) Glycerol + CO_2 + NAD^+ + UQH_2 → $succinate^{2-}$ + H_2O + NADH + 3 H^+ + UQ

Succinate is a 4-carbon carboxylate that can be later reduced into n-butyrate by *C. kluyveri* (Wolff et al. 1993), which can be elongated into n-caproate, as explained in the next paragraph.

2.3.1.3 Reversed β-oxidation

The elongation by reversed β-oxidation is a major elongating pathway that has been identified in *C. kluyveri* (Agler et al. 2011). The pathway operates in 2 steps linked.

The 1st step corresponds to the generation of an acetyl-CoA, reducing equivalents and energy. The production of energy under the form of ATP makes the 1st step not thermodynamically feasible. It then needs to be coupled to a 2nd pathway thermodynamically feasible: the 2nd step (Spirito et al. 2014).

The 2nd step, cyclic, is the proper reversed β-oxidation. It consists in the condensation between the acetyl-CoA from step 1 and a carboxyl-CoA. The 2-C longer carboxyl-CoA produced is reduced by the reducing equivalents originating from step 1. Subsequently, the reduced 2-C longer carboxyl-CoA transfers its coenzyme A to a carboxylate and then forms the 2-C longer carboxylate. Eventually, the new carboxyl-CoA can start a new reversed β-oxidation cycle with another acetyl-CoA from step 1 (Spirito et al. 2014).

The following scheme (Fig 2) represents the overall pathway, in which the 1st step corresponds to an ethanol (EtOH) oxidation, while the 2nd step implicates an acetyl-CoA as carboxyl-CoA. The EtOH acts as an electron and C donor (step 1). The acetyl-CoA comes from an acetate molecule, which acts as electron and C acceptor (step 2).

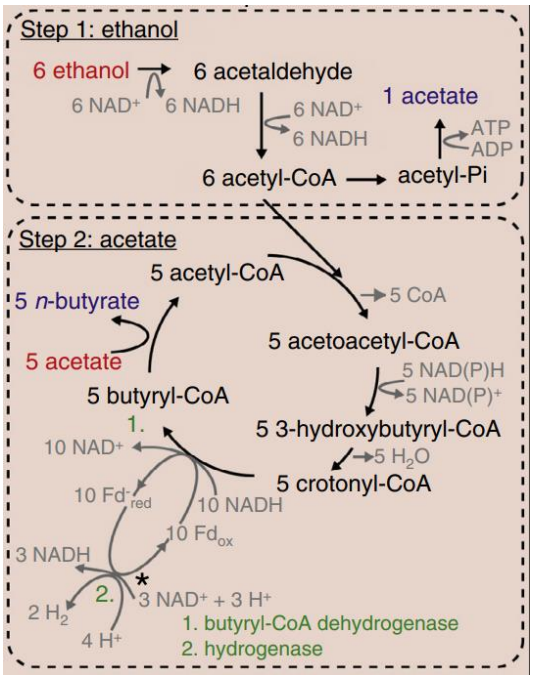

Figure 2: The mechanism of reversed β-oxidation from ethanol (Spirito et al., 2014)

For every EtOH oxidation into acetate (Equ 3), there are 5 reversed β-oxidations (Equ 4). In other words, for 1 oxidized mol of EtOH, 5 more mol of EtOH and 5 mol of acetate are transformed into 5 mol of n-butyrate. The two steps can be resumed with the simplified equations 3 and 4. The ΔG^0_r were obtained at 37°C with a pH of 6.82 (Spirito et al. 2014).

Equ 3) $CH_3CH_2OH + H_2O + ADP + P_i \rightarrow CH_3COO^- + H^+ + 2\ H_2 + ATP$

$\Delta G^0_r = +7.5$ kJ/mol EtOH

(*Equ 4)* $CH_3COO^- + CH_3CH_2OH \rightarrow$ n-butyrate$^-$ + H_2O) x5

($\Delta G^0_r = -38.3$ kJ/mol EtOH) x5 = -191.5 kJ/5mol EtOH

→ Total = -184.0 kJ/6mol EtOH

The 1st step, on its own, is not feasible, since its change in Gibbs free energy is positive. It needs the be linked to the 2nd step, that has a ΔG^0_r very negative. This results in a negative global ΔG^0_r, and then the whole pathway is thermodynamically favourable (Spirito et al. 2014). The global equation of the pathway is:

Equ 5) 6 EtOH + 4 acetate$^-$ + ADP + $P_i \rightarrow$ 5 n-butyrate$^-$ + H^+ + 2 H_2 + 4 H_2O + ATP

A few ATP is produced via step 1 (Equ 3), making this pathway relevant for bacteria (Seedorf et al. 2008). In brief, the two steps are linked, and the 1st step cannot operate without step 2. Step 1 provides acetyl-CoA's, reducing equivalents and ATP, while step 2 uses these acetyl-CoA's and reducing equivalents and ensures the overall thermodynamical favorability (Seedorf et al. 2008; Spirito et al. 2014).

The explanation of the pathway was made with EtOH as electron and C donor, and acetate as electron and C acceptor. It must be reminded that both steps can start with other metabolites (e.g., lactate for step 1) (Coma et al. 2016; Kucek et al. 2016).

2.3.1.4 Numerous elongating pathways

These three elongation pathways belong to the diversity of the elongation mechanisms which exist. Only a few were totally understood and written down with equations. Literature suggests that reversed β-oxidation is the most common (Angenent et al. 2016; De Groof et al. 2019; Spirito et al. 2014). It is important to conceive that, in a MMC, it is very complicated to know which mechanism(s) occur(s) for elongation. It is usually supposed that various different elongation pathways occur simultaneously or consecutively (Agler et al. 2011; De Groof et al. 2019; Spirito et al. 2014).

2.3.2 Limiting factors of the elongation process

The elongation and the production of MCCAs are quite rapidly limited (De Groof et al. 2019, 2019; Ge et al. 2015). To our knowledge, with batch fermentations, the highest C6 concentrations ever obtained reached up to 21.2 g/l or 46.9 g_{COD}/l (see Table I of Appendix for the conversion values), working with pure cultures of *C. kluyveri* on a synthetic substrate[2] (n°1 Table 1 section 2.5) (San-Valero et al. 2019). Literature suggests different factors which can limit the elongation process (Jarboe et al. 2013; Royce et al. 2013; Yin et al. 2017):

- Depletion of substrate. The degraded feedstock could lack of electron donor (e.g., ethanol), electron acceptor (e.g., acetate, n-butyrate) (De Groof et al. 2019) or essential compounds (e.g., micronutrients) (Fernández-Naveira et al. 2017; San-Valero et al. 2019), making the elongation pathway unfeasible.

- Thermodynamic inhibition by product accumulation. The product accumulation could cause a decrease in the ΔG of the elongation pathway, turning it into a thermodynamically unfavourable reaction (De Groof et al. 2019).

These two hypotheses explaining the stop of elongation have been contradicted by various researchers. For example, Weimer at al. (2015) observed an incomplete consumption of their substrate (both the electron donor and the electron acceptor) while the ΔG stayed favourable.

- A common explanation for the elongation stop is the metabolic inhibition by product accumulation. Indeed, the MCCAs, in their undissociated form, are toxic for microorganisms

[2] A synthetic substrate is a tailor-made mixture of molecules, or else a unique molecule, well defined. It is created to optimize the metabolic process(es) of interest (De Groof et al. 2019).

(Pratt et al. 2012; Weimer et al. 2015). Firstly, the undissociated MCCAs are able to diffuse through the cell membrane and dissociate intracellularly, thus releasing one proton. The bacteria then uses energy to export this proton to maintain its intracellular pH, instead of using this energy for the MCCA formation (Zhang et al. 2013). Moreover, the MCCAs cause metabolic trouble inside the cell (e.g., disturbance of the respiratory chain, inhibition of some essential metabolite production) (Jarboe et al. 2013). Besides, the undissociated MCCAs have antimicrobial properties impacting the bacteria from the outside of the cell. They can interact with the cell membrane and drive changes in its properties (e.g., in its fluidity, composition) (Jarboe et al. 2013).

Overall, above a certain concentration depending on the bacteria genera, the culture conditions and the carboxylic acid itself, the bacteria is inhibited and the elongation process stops (Jarboe et al. 2013; Royce et al. 2013). For example, the inhibitory concentration of protonated C6 on a MMC in yeast-fermentation beer is 1.92 g_{COD}/l (Angenent et al. 2016).

- The pH of the culture can also play a role on the limitation of elongation. Indeed, the majority of the environmental bacteria are neutrophilic. As a result, the MMC responsible of the elongation process is more efficient in neutral medium (Jankowska et al. 2018).

Furthermore, the pH determines the proportion between the dissociated and undissociated MCCAs. MCCAs have pKa's of 4.7-4.9 (e.g., 4.88 for C6) (Angenent et al. 2016). It means that the more acidic the pH, the more MCCAs are under their undissociated toxic form. A way to avoid the microorganism inhibition is then maintaining a neutral pH (Yin et al. 2017).

- On another note, Fernández-Naveira et al. (2017) were able to reactivate the elongation into MCCAs by replacing the fermentation broth. They suggested that some inhibitory metabolites (e.g., trace elements in the substrate, alcohols produced) were causing the stop of the MCCA production.

In summary, researchers have got very diverse results. No obvious trend explaining the stop of the elongation can be selected. It seems that this phenomenon principally depends on the characteristics proper of each experiment (the pH, the substrate, etc.). Some methods have been classically used to avoid this stop. Among them, we can mention the removal of the MCCAs, the regulation of the pH and the addition of substrate (Agler et al. 2011, 2012).

2.4 Influence of culture conditions on carboxylate elongation

2.4.1 pH

A neutral or nearly neutral pH seems to be the most effective pH to promote the elongation process. At pH 7.0, Many researchers reported their greatest C6 production yields obtained from a complex organic feedstock (COF) (i.e., a complex substrate) thanks to a MMC (Liang and Wan 2015; Nzeteu et al. 2018; San-Valero et al. 2019). However, a neutral medium entails dealing with methanogenesis since it mainly occurs within the pH range 6.0-8.3 (Angelidaki and Sanders 2004). So, the studies conducted at neutral pH most often employed a methanogenic inhibitor or a heat pre-treatment of the inoculum (see section 2.2.2) (Reddy et al. 2018; Weimer and Stevenson 2012). Without these measures, the pH takes a greater importance. Indeed, in addition to influencing the kinetics of elongation, it determines the risk of methanogenesis occurrence.

It is possible to work at a pH higher than 8.3 to avoid methanogenesis. However, this strategy has already shown its limitations since the overall elongation process is sensibly less thermodynamically favourable in basic conditions (Liang and Wan 2015).

In another way, a pH lower than 6.0 can be used. This strategy foremost makes sense since the hydrolysis enzymes operate the best in the pH range 5.0-7.0 (Jonke and Michal 2003). Concerning the acidogenesis, it is not disabled by the acidity since the acidogenic bacteria tolerate pH's lower than 6 (Angelidaki and Sanders 2004). Nevertheless, a pH too acidic is to avoid. Indeed, as explained in section 2.2.2, the MCCAs are bio-toxic in their undissociated form. Because they have pKa's of 4.7-4.9, it is then relevant to work at a pH higher than 5.0 (San-Valero et al. 2019).

Another point of attention about the pH is the pH stability. In a MMC, plenty of different microorganisms live together, and the population is different from one pH to another (Gildemyn et al. 2017). In pure cultures of *C. kluyveri* with only acetate (C2) and EtOH as substrates, San Valero et al. (2019) have got better MCCA productions when working at a constant pH of 6.8 (11.8 g_{C6}/l) than when re-adjusting the pH to 6.8 every day (8.4 g_{C6}/l) (the maximum pH drop being -1) (n°2-3 Table 1 section 2.5). Therefore, there is undoubtedly an interest to maintain the desired pH as well as possible. It is even more true when working with unbuffered media. As an example, Liang et al. (2015) reported for brewer's spent grain a drop from pH 6.5 down to 3.8 in only 24 hours.

In summary, when processing COF with a MMC, if no methanogenic inhibitor is used, the best compromise regarding the pH seems to be using a pH ranging from 5.5 to 6.0. This allows to prevent methanogenesis and to limit the product toxicity. At the same time, maintaining a constant pH offers more guarantees.

2.4.2 Substrate composition

The fermentable substrate used for the MCCA production can be of two types: synthetic (see section 2.3.2) or complex (see section 2.2.3). The best MCCA yields reported in literature were obtained from the fermentation of a synthetic substrate (De Groof et al. 2019; Grootscholten, Steinbusch, et al. 2013; San-Valero et al. 2019). It is relatively logic since all the substrate provided is fermentable, unlike any natural complex substrate. These synthetic substrates are useful to study the elongation process, but they are expensive and not relevant in the context of large-scale bio-production of MCCAs. Hence, the challenge is to perform the elongation from complex substrates.

Some studies investigated the effect of the complex substrate composition on the production of MCCAs. In batch with a MMC, Arslan et al. (2012) compared the efficiency of waste streams of a potato, a meat, and an oil processing industry. The potato waste stream was rich in carbohydrates, the meat waste stream was rich in proteins, and the oil waste stream was rich in lipids. The researchers reported total MCCA concentrations up to 4.9 g_{COD}/l, 2.0 g_{COD}/l and 0.1 g_{COD}/l, respectively (n°4-5-6 Table 1 section 2.5).

A lipid-rich substrate always gives low yields (Arslan et al. 2012; De Groof et al. 2019). This is explained by the fact that lipidic substrates are much less hydrolysable (De Groof et al. 2019). Hydrolysis is the rate-limiting step of the elongation process (Mata-Alvarez et al. 2000). For this reason, researchers conclude that the better the hydrolysis, the better the yield (Grootscholten et al. 2014). In order to favor the hydrolysis, a highly biodegradable matter should be selected (Arslan et al. 2012).

Furthermore, among the protein and the carbohydrate-rich substrates, the carbohydrate ones usually give better yields, especially if the carbohydrate substrate is starch-based (Arslan et al. 2013).

Lastly, literature suggests that higher the substrate concentration, higher the MCCA production (Arslan et al. 2012)

2.4.3 Supplementation with the limiting reagent

To promote the elongation, the technique of supplementing the fermentation broth with the limiting reagent makes sense. As developed in section 2.3.2, some researchers indicated that their elongation process ceased because of the substrate depletion (Fernández-Naveira et al. 2017; San-Valero et al. 2019).

Some reported, in fermentation of a COF by a MMC, an improvement in the MCCA production practicing supplementation. Grootscholten et al. (2013) obtained a 50% higher C6 concentration by supplementing the fermentation broth (in this case, the organic fraction of municipal solid waste at 169 g_{COD}/l) with 12.8 g_{COD}/l of EtOH, the electron and carbon donor. Yet, the total production of carboxylates was lower than without supplementation (Grootscholten, Kinsky dal Borgo, et al. 2013). The likely reason is that ethanol inhibits the growth of bunch of bacteria, including the ones implicated in the hydrolysis and acidogenesis steps (Grootscholten, Kinsky dal Borgo, et al. 2013; Lonkar et al. 2016). Lonkar et al. (2016) determined a maximal EtOH concentration of 97.4 g_{COD}/l, above which EtOH starts inhibiting the overall elongation process.

2.4.4 One or two phase system

Recently, several studies investigated the possibility to work with a two-stage system (Grootscholten et al. 2014; Steinbusch et al. 2011). The first bioreactor is a batch, in which the optimal conditions are set to promote hydrolysis and acidogenesis (e.g., the pH, the inoculum). Its purpose is to accumulate the products of acidogenesis, or i.e., the VFAs, the lactate, the ethanol, the H_2 and the CO_2 (see sections 2.1.2 & 2.2.1). The pre-fermented leachate is then transferred into a second bioreactor. This one provides the optimal conditions to catalyse the elongation of the output metabolites of the 1st bioreactor. The major objective of this 2nd stage is to put the leachate in contact with a pre-selected elongating inoculum (Grootscholten et al. 2014). Several advantages of this two-stage system were reported by Grootscholten et al. (2014):

- The separation first offers the advantage of providing the best conditions for the two stages. Indeed, in a classic one-stage fermenter, the best conditions for phase 1 and phase 2 cannot be satisfied, and a compromise must be chosen.

- Secondly, this system avoids the problems of the MCCA toxicity on hydrolytic and acidogenic bacteria.

- Thirdly, if a supplementation technique is wished, it can be applied only in the wanted stage and does not negatively affect the other stage (e.g., the ethanol effect on hydrolysis and acidogenesis).

- Finally, the MCCAs tend to adsorb on solid particles and are then more difficult to extract. If a COF is used in the 1st stage, the 2nd stage reactor gets much fewer solid particles than the 1st since only the leachate is transferred into it. Hence, the problem of adsorption is considerably reduced.

However, alongside the advantages, drawbacks are also encountered working in two phases. The major problem is the greater complexity of the operations generated by this separation. The leachate from the 1st bioreactor must be collected in anaerobiosis and reinjected into the 2nd bioreactor (Grootscholten et al. 2014). This greater complexity means greater operating time, greater costs, greater disturbance on the microbes and as well greater risks of error (De Groof et al. 2019).

The few existing results found in literature suggest that a two-stage system offers better yields than a one-stage (Grootscholten et al. 2014; Nzeteu et al. 2018). Grootscholten et al. (2014) obtained a global MCCA production rate of 1.9 g_{MCCA}/l*d in their two-stage continuous system, which is 30% higher than what they had obtained in a single stage system (n°8-9 Table 1 section 2.5).

As a result, in MMC with a COF, it seems that the elongation can be independent from the acidogenesis. Nevertheless, the knowledge is still limited, and more studies would be worth.

2.4.5 Gas supply

The gas or gas mixture introduced in the bioreactor was shown to play a major role on the elongation (Arslan et al. 2013; Grootscholten, Steinbusch, et al. 2013). In fact, after dissolving into the fermentation broth, the gas can enter the metabolism of the bacteria.

Arslan et al. (2013) reported a double effect of the headspace gas. Firstly, the headspace gas influences the hydrolysis, the rate limiting step of the elongation process (see section 2.4.2). It then exerts an effect on the final MCCA concentration. Secondly, Arslan et al. (2013) noticed that the headspace gas influences the acidogenesis step. This was materialized by net bias in the type of the final fermentation products formed.

The conditions H_2, CO_2, and the mixture H_2+CO_2 were tested. They all gave better yields in terms of MCCAs than the controls, in which N_2 was used (Arslan et al. 2013).

2.4.5.1 Supply of CO_2:

In several experiments, it was revealed that a CO_2 addition promotes the elongation process (Grootscholten, Steinbusch, et al. 2013). Interestingly, Arslan et al. (2013) realized that the elongation could occur at high rates while the CO_2 consumption was low. They concluded that

CO_2 could affect the elongation even without entering any pathway. Besides, in line with the previous results of Kim et al. (2011), they noticed that a CO_2 partial pressure (p_{CO2}) higher than 0.7 bar starts to inhibit the acetogenesis from n-butyrate (Equ 6), a non-desired pathway.

Equ 6) $n\text{-butyrate}^- + 2\ H_2O \rightarrow 2\ acetate^- + H^+ + 2\ H_2$ $\Delta G^0_r = +47.55$ kJ/mol n-butyrate

It was hypothesized by Kim et al. (2011) and Arslan et al. (2013) that CO_2 damages the cell membrane, causing functioning problems, and that this phenomenon appears first with acetogens (Kim et al. 2011).

An upper limit for the p_{CO2} is as well to consider in order to promote the elongation. Effectively, Roghair et al. (2018) revealed that, with high p_{CO2} (2.5 bar), the oxidation of EtOH into C2 (Equ 7) is stimulated and becomes excessive. It means that a part of the EtOH oxidation is not coupled to a reversed β-oxidation (see section 2.3.1.3), penalizing the elongation (Roghair et al. 2018).

Equ 7) $CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\ H_2$ $\Delta G^0_r = +7.22$ kJ/mol EtOH

Overall, a p_{CO2} between 0.7 and 2.0 bar seems recommendable.

2.4.5.2 Supply of H_2:

The H_2 partial pressure (p_{H2}) greatly influences the dominating microbial pathway. It was shown that a p_{H2} inferior to 0.3 bar favours processes which are competitive with elongation, like hydrogenotrophic methanogenesis (Equ 8) (Agler et al. 2014; Ni et al. 2011), which competes with homoacetogenesis (Equ 1) for H_2 and CO_2.

Equ 8) $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ $\Delta G^0_r = -125.84$ kJ/mol acetate

Besides, homoacetogenesis becomes reversible at p_{H2} lower than 0.5 bar (Equ 9) (González-Cabaleiro et al. 2013).

Equ 9) $CH_3COO^- + H^+ + 2\ H_2O \longleftrightarrow 4\ H_2 + 2\ CO_2$ $\Delta G^0_r = +86.8$ kJ/mol C2

Similarly, at p_{H2} lower than 0.1 bar, the oxidative conditions promote the excessive EtOH oxidation into C2 (Equ 7). The reversed β-oxidation then loses its feasibility (Angenent et al. 2016; Grootscholten et al. 2014).

According to literature, it appears high p_{H2} are efficient to promote the elongation process (Angenent et al. 2016; González-Cabaleiro et al. 2013; Ni et al. 2011). Equally, bioreactors supplemented with H_2 most often showed better MCCA productions than bioreactors with no H_2 (flushed with N_2 for example) (Nzeteu et al. 2018; Steinbusch et al. 2011). Arslan et al. (2013) used H_2 at 2 bar and they reported an increase in carboxylate concentration greater than the COD consumption as H_2 from the headspace. According to them, this greater increase is due to a better hydrolysis, caused by the high p_{H2}. Interestingly, in a previous study, they

also revealed that high p_{H2} allow to get longer carboxylates (Arslan et al. 2012). For Steinbusch et al. (2011), high concentrations of H_2 make energy efficient the use of VFAs as electron acceptors by microorganisms, leading to better MCCA productions.

Nevertheless, an upper limit is to be considered for the p_{H2}. Indeed, above 1.5 bar, the p_{H2} starts inhibiting the growth of *C. kluyveri* (Steinbusch et al. 2011). In addition, carboxylates start being reduced into their corresponding alcohols (Equ 10) (Grootscholten et al. 2014; Steinbusch et al. 2008).

Equ 10) $n\text{-butyrate}^- + H^+ + 2\ H_2 \rightarrow n\text{-butanol} + H_2O$ $\Delta G^0_r = -3.58$ kJ/mol n-butyrate$^-$

Overall, maintaining a p_{H2} superior to 0.1 bar and below 1.5 bar seems recommendable.

2.4.5.3 Supply of both H_2 & CO_2:

As for the supply of CO_2 or H_2 separately, a lower limit of 0.1 bar for the p_{H2} and an upper limit of 2.0 for the p_{CO2} are desirable to avoid an excessive EtOH oxidation (Equ 7) (Angenent et al. 2016; Roghair et al. 2018). More precisely, Weimer et al. (2016) calculated the best theoretical H_2/CO_2 ratio at the thermodynamic level: 1 bar of H_2 and 0.3 bar of CO_2.

A few researchers studied the possibility to produce MCCAs with a MMC from only CO_2 and H_2. The idea is to form C2 by homoacetogenesis, reduce this C2 into EtOH, and then have the combined EtOH oxidation - reversed β-oxidation. With the mixture H_2/CO_2 60/40 v/v at an absolute pressure of 1.4 atm, Zhang et al. (2013) were able to produce 1.4 g_{MCCA}/l or 3.20 g_{COD_MCCAs}/l, containing 70% of C6 (i.e., 2.17 g_{COD_C6}/l) (n°7 Table 1 section 2.5). Spirito et al. (2014) attributed this low productivity to the low kinetics of the C2 into EtOH reduction (Equ 11).

Equ 11) $CH_3COO^- + H^+ + 2\ H_2 \rightarrow CH_3CH_2OH + H_2O$ $\Delta G^0_r = -7{,}22$ kJ/mol C2

In summary, working with MMCs on COFs, the headspace composition plays an important role into the elongation process, whether the gases are fermented or not. The mixture H_2+CO_2 seems to be a relevant strategy to enhance the production of MCCAs.

2.5 Overview of the medium chain carboxylate yields in literature

Table 1 gathers the MCCA and C6 yields - associated to their experimental conditions - mentioned along the State of the art.

Table 1: Overview of the values of MCCA and C6 yields associated to their experimental conditions from literature.

Id n°	Operating mode	Inoculum	Organic substrate (non- gaseous)	pH	Headspace gas	MCCAs	C6***	Reference
1	batch	C. kluyveri	synthetic: C2, C4, EtOH	6.8	N_2 flushed	NA**	21.2 g/l = 46.9 g_{COD}/l	San Valero et al. 2019
2	batch	C. kluyveri	synthetic: C2, EtOH	6.8 constant	N_2 flushed	NA	11.8 g/l = 26.1 g_{COD}/l	San Valero et al. 2019
3	batch	C. kluyveri	synthetic: C2, EtOH	6.8	N_2 flushed	NA	8.4 g/l = 18.6 g_{COD}/l	San Valero et al. 2019
4	batch	MMC	complex: potato waste stream	5.0	N_2 flushed	4.9 g_{COD}/l	NA	Arslan et al. 2012
5	batch	MMC	complex: meat waste stream	5.0	N_2 flushed	2.0 g_{COD}/l	NA	Arslan et al. 2012
6	batch	MMC	complex: oil waste stream	5.0	N_2 flushed	0.1 gCOD/l	NA	Arslan et al. 2012
7	batch	MMC	none	6.0	H_2/CO_2 60/40, 1.4 atm	1.4 g/l = 3.2 g_{COD}/l	0.98 g/l = 2.17 g_{COD}/l	Zhang et al. 2013
8	continuous 1 phase, HRT 11h	MMC	complex: OFMSW*	6.5-7.0	N_2 flushed	1.43 g/l*d	NA	Grootscholten et al., 2014
9	continuous 2 phases, HRT 11h	MMC	complex: OFMSW	6.5-7.0	N_2 flushed	1.9 g/l*d	NA	Grootscholten et al., 2014

* OFMSW = organic fraction of municipal solid waste

** NA = not available

*** The conversion ratio to COD is calculated in section A of the appendix.

2.6 Current knowledge in our laboratory

During the past few years, the Bioengineering and Biorefining Team of UCLouvain investigated the potential of fermenting COF into MCCAs, by the action of a MMC. The COF currently studied is brewer's spent grain (BSG). BSG is a by-product from the brewery industry. It consists in the insoluble and undegraded part of the barley malt, obtained at the end of the wort production (Mussatto 2014). It is rich in hemicellulose, cellulose, and proteins (see section 5.1.2).

The Team investigated the effect of the gas supplied to the bioreactors on the elongation. Bioreactors in batch mode were supplied with either N_2, CO_2, H_2 at 1.3 bar, H_2 at 1.6 bar, or the mixture H_2/CO_2 77/23 v/v at 1.3 bar. As presented in Figure 3, the accumulation of MCCAs was observed under all conditions, and it stopped around day 7-9. Interestingly, after day 11, a 2nd phase of MCCA production started, only under the condition H_2+CO_2. This 2nd MCCA accumulation was accompanied by CO_2 and H_2 consumptions (Figure 4). Unfortunately, the experiment was stopped when the MCCA production was still going on, so more investigations on this subject were planned.

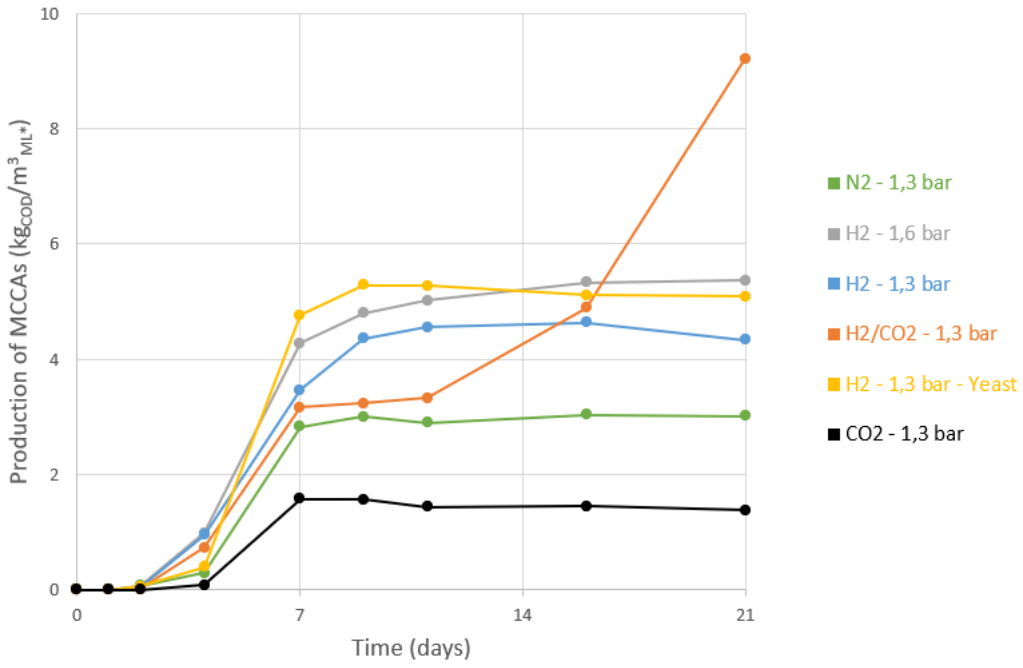

*Figure 3: Evolution of the MCCA production over time for different headspace gas conditions. * ML = mixed liquor, which corresponds to the fermentation broth.*

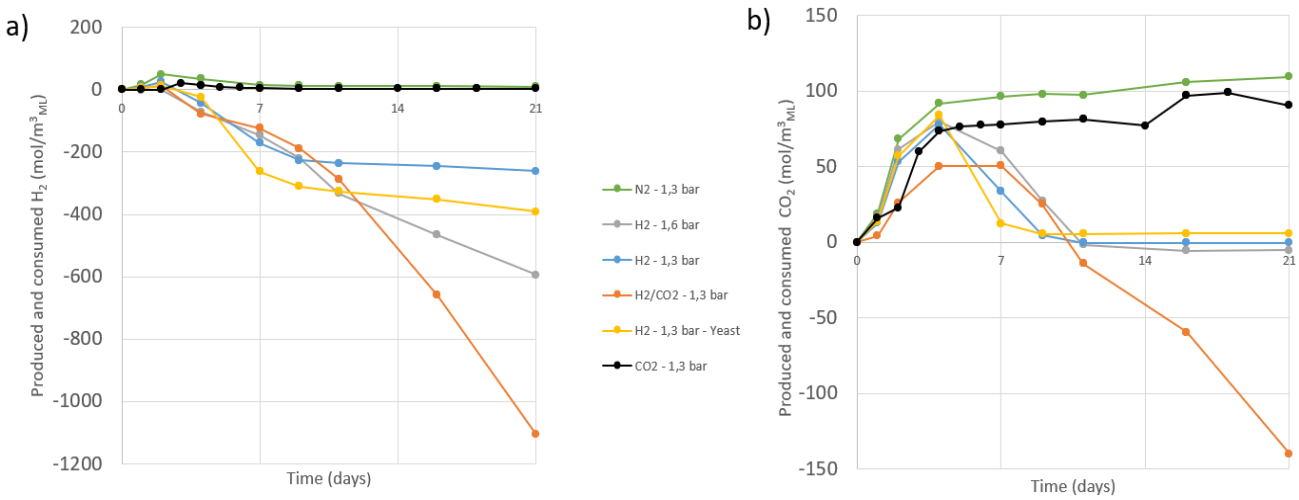

Figure 4: Evolution of the consumption and production over time of a) H_2 ; b) CO_2, for different headspace gas conditions.

3 Aims of the master's thesis

This master's thesis is integrated within this several-year project of MCCA bio-production from BSG. It is part of a larger project aiming at converting complex organic substrates (biowastes, agro-food residues) to MCCAs, using MMC to perform the steps of substrate hydrolysis, acidogenic fermentation to SCCAs, and elongation of SCCAs to MCCAs. In this context, the master's thesis aims at answering three main questions:

1) Is the 2nd phase of MCCA production reproducible?

2) What is the influence of the COF used on the MCCA production?

3) When does the MCCA production stop and what causes this stop? Is it possible to avoid this stop by passing to a semi-continuous system?

4 Experimental Approach

To answer the three questions of this master's thesis, the strategy was:

- In a 1st experiment, to reobtain the 2nd phase of MCCA production and get insight about the COF influence. For that, it was decided to keep working in batch mode with the mixture H_2/CO_2 77/23 v/v at 1.3 bar, in this experiment as well as in the next ones.

- In a 2nd experiment, to investigate the interruption of the 2nd MCCA production phase, in order to know when it occurs and get a first idea of why.

- In a 3rd experiment, to understand better the cause of the MCCA production stop and to see if this stop can be avoided by passing to a semi-continuous feeding mode.

5 Material and methods

5.1 Substrates and culture medium

5.1.1 Culture medium additives

A mineral medium was prepared and put in the reactors from the beginning of each experiment in order to provide the nutrients necessary for the microorganisms.

Table 2: Composition of the mineral medium.

Substance	Concentration (g/l)	Concentration (mol/l)
$NH_4H_2PO_4$	216	1.88
$MgCl_2.6H_2O$	7.6	$3.74*10^{-2}$
$CaCl_2.2H_2O$	1.6	$1.09*10^{-2}$
KCl	0.9	$1.21*10^{-2}$
H_3BO_3	0.032	$5.18*10^{-4}$
$FeCl_2.4H_2O$	0.158	$7.95*10^{-4}$
$ZnSO_4$	0.0110	$6.81*10^{-5}$
$MnCl_2$	0.0041	$3.26*10^{-5}$
$CoCl_2$	0.0200	$1.54*10^{-4}$
$CuCl_2$	0.0009	$6.69*10^{-6}$
$NiCl_2$	0.0020	$1.54*10^{-5}$
Na_2MoO_4	0.0032	$1.55*10^{-5}$
$Na_2Se_2O_3$	0.0013	$5.16*10^{-6}$

Table 3: Characterization of the mineral medium.

COD	0.000 g_{COD}/g_{FM} 0.000 g_{COD}/g_{DM}
Dry $Matter_{105°C}$	0.083 g_{DM}/g_{FM}
Volatile $Solid_{550°C}$	0.05 g_{VM}/g_{FM} 0.58 g_{VM}/g_{DM}

Different stock solutions were prepared to adjust the pH and supply EtOH when needed (Table 4).

Table 4: Characterization of the prepared stock solutions.

	KOH 2M	EtOH	H_3PO_4 1M
Targeted cc (g/l)	112.21	9.63	98
COD (g_{COD}/g_{FM})	0	0.01627	0
COD (g_{COD}/g_{DM})	0	1.69	0
DM (g_{DM}/g_{FM})	0.123	0.000	0.103
VS (g_{VM}/g_{FM})	0.036	0.000	0.091
VS (g_{VM}/g_{DM})	0.294	0.00	0.891

5.1.2 Brewer's spent grain

The BSG came from the brewery Bertinchamps of Gembloux, in Belgium. It was picked up fresh, then stored frozen at -20°C. The BSG used came from two different brewer batches. Table 5 describes the mean BSG composition in terms of components.

Table 5: Composition of the BSG (Godin, 2019).

Lignin	4.8 $\%_{DM}$
Hemicellulose	33.4 $\%_{DM}$
Cellulose	17.9 $\%_{DM}$
Starch	7.0 $\%_{DM}$
Soluble sugars	0.8 $\%_{DM}$
Proteins	25.1 $\%_{DM}$
Minerals	4.1 $\%_{DM}$
Other	7.0 $\%_{DM}$

The BSG from the 2nd brewer batch was used crude or pressed, whereas the BSG from the 1st brewer batch was only used crude. The pressed BSG (pBSG) was obtained by pressing the crude BSG (cBSG) at 3 bar on a sieve with a hydraulic press (Speidel 20 L) until the end of the press liquid outflow. Table 6 characterizes the BSGs used for each experiment, in terms of COD, water content and volatile solids.

Table 6: Characterization of the BSGs.

	cBSG from batch 2 (Exp 1)	cBSG from batch 1 (Exp 2)	pBSG from batch 2 (Exp 1 & 3)
COD	0.293 g_{COD}/g_{FM} 1.896 g_{COD}/g_{DM}	0.351 g_{COD}/g_{FM} 1.548 g_{COD}/g_{DM}	0.545 g_{COD}/g_{FM} 1.477 g_{COD}/g_{DM}
Dry Matter$_{105°C}$	0.155 g_{DM}/g_{FM}	0.227 g_{DM}/g_{FM}	0.364 g_{DM}/g_{FM}
Volatile Solid$_{550°C}$	0.149 g_{VM}/g_{FM} 0.963 g_{VM}/g_{DM}	0.217 g_{VM}/g_{FM} 0.958 g_{VM}/g_{DM}	0.350 g_{VM}/g_{FM} 0.962 g_{VM}/g_{DM}

5.1.3 Acidogenic mixed liquor of 4 days

The acidogenic mixed liquors (AMLs) used in the experiments were recovered after a 4-day fermentation at 35°C of 150 g_{COD_ML}/l_{ML}, under N_2 at 1.3 bar, in a 5 L bioreactor (see section 5.2.1). The pH was adjusted to 6.8. Table 7 characterizes the 4 days old AMLs used and Table 8 describes their composition.

Table 7: Characterization of the 4-day AMLs.

	AML 1 (Exp 1)	AML 2 (Exp 2)	AML 3 (Exp 3)
BSG of origin	Batch 2 pressed BSG	Batch 1 crude BSG	Batch 2 pressed BSG
COD	0.01905 g_{COD}/g_{FM} 1.102 g_{COD}/g_{DM}	0.02010 g_{COD}/g_{FM} 0.57 g_{COD}/g_{DM}	0.02035 g_{COD}/g_{FM} 1.41 g_{COD}/g_{DM}
Dry Matter$_{105°C}$	0.017 g_{DM}/g_{FM}	0.018 g_{DM}/g_{FM}	0.014 g_{DM}/g_{FM}
Volatile Solid$_{550°C}$	0.005 g_{VM}/g_{FM} 0.301 g_{VM}/g_{DM}	0.007 g_{VM}/g_{FM} 0.397 g_{VM}/g_{DM}	0.005 g_{VM}/g_{FM} 0.328 g_{VM}/g_{DM}

Table 8: Composition of the 4-day AMLs (g_{COD}/l).

Compound	AML 1	AML 2	AML 3
Ethanol	0.745	0.9164	0.741
2-butanol	0.068	0.0769	0.019
Propanol	0.046	0.0643	0.064
Butanol	0.008	0.0025	0.003
Pentanol	0	0	0
Hexanol	0	0	0
Heptanol	0	0	0
Acetic acid	3.341	3.2539	3.194
Propionic acid	5.122	4.961	4.935
Iso-butyric acid	0.033	0.0323	0.026
Butyric acid	2.434	2.2634	2.399
Iso-valeric acid	0.091	0.085	0.057
Valeric acid	3.463	3.3691	3.140
Hexanoïc acid	0.191	0.2139	0.260
Heptanoïc acid	0.046	0.0753	0.139
Octanoïc acid	0	0	0
Nonanoïc acid	0	0	0.003
Decanoïc acid	0	0	0
Phenylacetic acid	0.082	0.0985	0.077

5.1.4 Inoculum

Activated sludge from the wastewater treatment plant in Mont-Saint-Guibert was used as inoculum for the fermentation broth. It was homogenized before being collected and transferred into the reactors.

Table 9: Characterization of the inocula.

	Exp 1	Exp 2	Exp 3
COD	0.01020 g_{COD}/g_{FM} 1.18 g_{COD}/g_{DM}	0.00564 g_{COD}/g_{FM} 0.822 g_{COD}/g_{DM}	0.01020 g_{COD}/g_{FM} 0.893 g_{COD}/g_{FM}
Dry matter$_{105°C}$	0.008 g_{DM}/g_{FM}	0.007 g_{DM}/g_{FM}	0.011 g_{DM}/g_{FM}
Volatile Solid$_{550°C}$	0.004 g_{VM}/g_{FM} 0.436 g_{VM}/g_{DM}	0.003 g_{VM}/g_{FM} 0.471 g_{VM}/g_{DM}	0.005 g_{VM}/g_{FM} 0.456 g_{VM}/g_{DM}

5.2 Fermentation

5.2.1 Bioreactor design

Each bioreactor consists in a 2 L Scott glass bottle. Each bottle is closed by a stainless-steel headpiece with a 2-hole nozzle. The headpiece is pressed on a rubber seal by a pierced GL 45 screw cap. On the outside of the bottle, the nozzle of the headpiece is connected to two flexible PVC tubes that are sealed with a two-way Luer lock valve for the gas sampling tube and a Mohr pinchcock for the liquid sampling tube. On the reactor side of the cap, a flexible PVC tube is sealed on the liquid sampling nozzle; this tube is long enough to reach the top of the mixed liquor and is used as a liquid sampling tube. The sealing of the PVC tubes over the nozzles is done using a short piece of silicone tube. The silicone tube is then fitted inside the PVC tube and its compression ensures the tube's gas tightness.

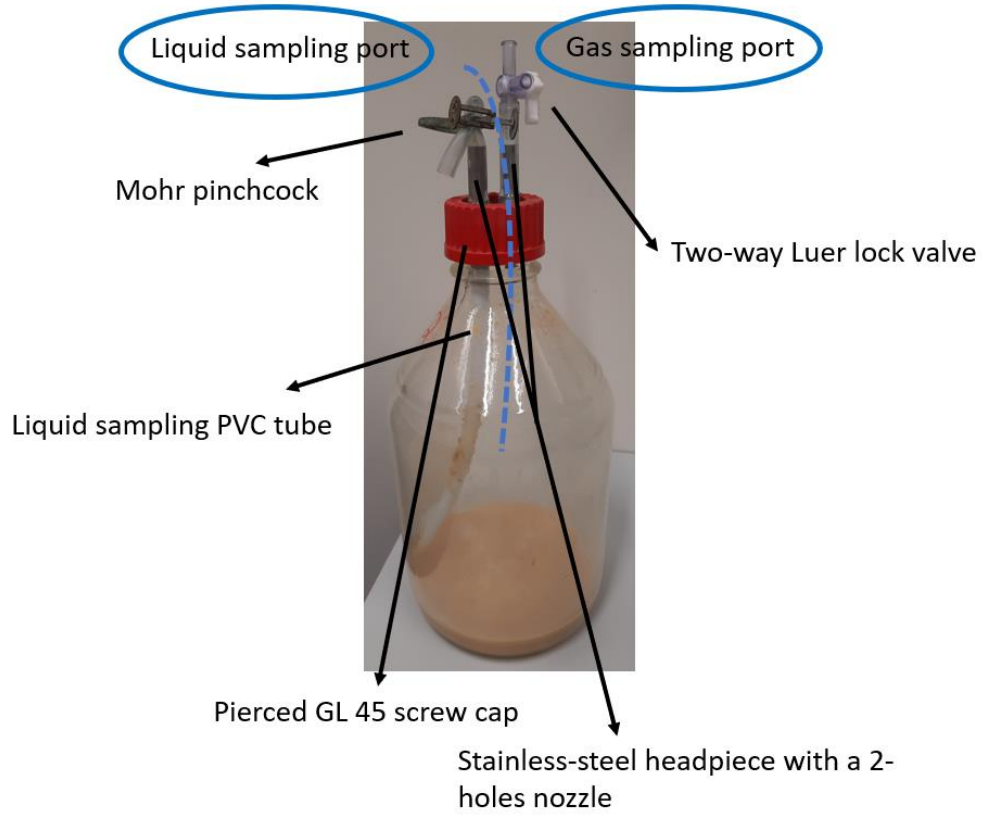

Figure 5: Bioreactor.

5.2.2 Fermentation start-up of the bioreactors

Every experiment starts with a precise characterization of the reactors. Their exact volume is determined by water filling. Their mass (empty) with and without top is registered. The different solutions and substrates are then added: 11 ml of mineral medium, 20 ml of inoculum, the necessary volume of COF (determined on the basis of the targeted final COD and the inoculum COD (see section 5.3)) and the demineralized water necessary to reach 220

ml. Every single addition is weighed and registered. Thereafter, the reactor is tightly closed, and the headspace is flushed with a mixture of H_2 and CO_2 in the proportions 77%-23% (v/v) at a flow rate of approximately 0.5 l/min, for 1 minute. The gas is supplied through the liquid sampling tube and leaves the headspace by opening the gas sampling two-way valve. After flushing, a gas sample is taken from the headspace with a 20 ml polypropylene syringe (BD Plastipack, USA) and its composition is analysed to ensure the absence of O_2. A UNIK 5000 manometer (GE, USA) with a three-way valve is connected to the gas sampling valve and the headspace pressure is increased to an absolute pressure of 1.3 bar with the H_2+CO_2 mixture. A second gas sample can be taken for analysis.

The reactors are then incubated in the dark at 35°C on an orbital shaker at 120 rpm (Kühner Shaker Lab-Shaker).

5.2.3 Fermentation management & monitoring

The fermentation status is monitored thrice a week following the steps described below for the Experiment 2.

1) The reactor is weighed.

2) At the time of the sampling, the initial pressure in the reactor is measured with a UNIK manometer.

3) A gas sample is collected (about 20 ml) with a 20 ml polypropylene syringe (BD Plastipack, USA) equipped with a three-way valve for gas analysis. The gas chromatogram is immediately checked, especially for the presence of N_2 and CH_4.

4) The pressure is measured again after the gas sampling.

5) A sample of mixed liquor (ML) is collected (about 7 ml if batch fermentation and 76 ml if semi-continuous fermentation) with a polypropylene syringe (60 or 100 ml) (BD Plastipack, USA) that is previously weighed to know its tare. One specific syringe is attributed to each individual reactor.

6) The syringe with the ML sample is weighed.

7) The ML sample is transferred into a plastic Falcon tube of 15 ml (Falcon™ 15 ml Conical Centrifuge Tubes). The syringe is then weighed.

8) The volume of ML in the Falcon is determined by eye with the graduations and recorded. The mass is recorded as well.

9) 2 ml of the ML of the Falcon 15 ml are transferred into a test tube for the pH analysis (cfr 15)).

10) 2 additional ml of the Falcon 15 ml are transferred into a 5 ml Eppendorf tube for a later microbial analysis. It is stored in a -80°C freezer.

11) The syringe with the remaining ML is weighed again.

12) The Falcon 15 ml (from steps 7, 9 and 10) is centrifuged at 4700 rpm for 10 min in a centrifuge (ThermoScientific Heraeus Megafuge 40 centrifuge).

13) The supernatant is transferred into a 5 ml Eppendorf tube for further COD (see section 5.4.3) and metabolite analysis (see section 5.4.4) while the solid fraction remains in the centrifugated Falcon.

14) Both fractions are weighed.

15) The pH of the ML is measured with a pH-meter (WTW pH-Electrode SenTix® 41, WTW Multiline P4 universal meter). If the ML has a pH difference of more than 0.2 compared to the targeted pH, the pH is readjusted. The pH targeted is 5.5 if methane is detected in the gas composition and 6.8 if there is no CH_4. The ML is titrated with KOH 2M if the pH is too acidic, or H_3PO_4 1M if the pH is too alkaline.

16) The amount of KOH 2M or H_3PO_4 1M needed in each reactor to adjust the pH is calculated from the respective volumes and added in the corresponding syringe. The syringe is then weighed.

17.a) If the experiment is a batch fermentation at constant volume, a specific volume of distilled water is added to the syringe to compensate the volume sampled for the analysis. If it is a real batch fermentation, no H_2O is added to the reactor. The weight of the syringe is recorded in both cases.

17.b) If the experiment is a semi-continuous fermentation, the adequate quantity of fresh substrate (BSG, AML, or H_2O) is added to the syringe. The weight of the syringe is recorded.

18) The mixture of substrate and/or KOH/H_3PO_4 + water is reinjected in its corresponding reactor.

19) The headspace pressure is measured.

20) The reactor is flushed with the headspace gas mixture H_2/CO_2 77/23 v/v for 30 seconds.

21) The pressure is adjusted to 1.3 bar.

22) A gas sample is collected (±20 ml) for gas analysis.

23) The pressure is measured again after the gas sampling.

24) The reactor is finally weighed.

For Experiment 1, the protocol is the same, except that no fraction of ML is collected for microbial analysis (point 10).

For Experiment 3, the targeted pH is 6.0 instead of 6.8, and it still adjusted to 6.0 in case of methanogenesis (point 15).

5.2.4 End of fermentation

At the end of the fermentation, all the operations of section 5.2.3 until 14) are conducted. The pH is only measured. Once it is done,

1) The reactor is weighed.

2) The reactor is opened under a hood.

3) The cap (with the PVC tubes) is taken out and weighed.

4) The reactor with its content is weighed.

5) The content of the reactor is transferred into a 0.75 L bucket previously tared with its top.

6) The 0.75 L bucket is weighed.

7) The empty bioreactor is weighed.

8) If ML remains on the sides of the bioreactor:

    8.1) A wash bottle filled with demineralized water is tared.

    8.2) The remaining ML is diluted with added water.

    8.3) The mixture is transferred into the previous 0.75 L bucket.

    8.4) The 0.75 L bucket is weighed again.

    8.5) The bioreactor is weighed again.

    8.6) The wash bottle is weighed.

9) The 0.75 L bucket is centrifugated at 4700 rpm for 10 min (ThermoScientific Heraeus Megafuge 40 centrifuge).

10) The supernatant is transferred into a 50 ml Falcon tube for liquid analysis.

11) The solid fraction is weighed and then transferred into another 50 ml Falcon tube.

12) The emptied bucket is weighed.

13) Both supernatant and solid fraction are characterized for dry, volatile matter and COD (see section 5.4.2)

5.3 Conditions tested in each experiment

5.3.1 Experiment 1 - Batch fermentation

This first experiment investigated the efficiency of different types of substrate; crude BSG (cBSG), pressed BSG (pBSG), AML of 4 days and no organic feedstock (NOF). The BSGs came from the brewer batch 2 (see Table 6 section 5.1.2). The 4-day AML used was the AML 1 (see Table 7 section 5.1.3). The substrate was mixed with the inoculum, mineral medium and demineralized water to reach a ML volume of 220 ml with a concentration of 100 g_{COD}/l, except for the NOF (H_2O) and AML reactors. All the fermentations were performed with the mixture H_2/CO_2 77/23 v/v supplied at 1.3 bar and a pH of 6.8. They were conducted as batches since only a few KOH or H_2SO_4 was added at each monitoring to adjust the pH (see section 5.2.3). Table 10 presents the initial composition of the reactors.

Table 10: Initial composition of the mixed liquor contained in the reactors of Experiment 1.

N° Reactor	Substrate	Mass of substrate (g/reactor)	Mass of substrate (g_{COD}/reactor)	Cc[6] of substrate (g_{COD}/l_{ML}[5])	Mass of inoculum (g/reactor)	Mass of inoculum (g_{COD}/reactor)	Cc of inoculum (g_{COD}/l_{ML})	Mass of mineral medium (g/reactor)	Mass of additional H_2O (g/reactor)	Total cc (g_{COD}/l_{ML})
1	cBSG[1]	75.4	22.09	88.91	19.7	0.2009	0.809	11.30	140.4	89.72
2	cBSG	75.5	22.12	89.15	20.9	0.2132	0.859	11.45	138.5	90.01
3	cBSG	75.6	22.15	89.38	20.5	0.2091	0.844	11.43	138.3	90.22
4	pBSG[2]	40.4	22.02	96.34	20.0	0.2040	0.893	11.53	154.5	97.23
5	pBSG	40.2	21.91	97.21	20.5	0.2091	0.928	11.46	151.1	98.14
6	pBSG	40.4	22.02	96.68	21.6	0.2203	0.967	11.45	152.3	97.64
19	AML[3] 1	173.9	3.31	14.76	20.5	0.2091	0.931	11.41	14.8	15.69
20	AML 1	173.9	3.31	14.68	21.5	0.2193	0.972	11.46	14.8	15.50
21	AML 1	173.2	3.30	14.73	20.6	0.2101	0.938	11.47	14.8	15.67
13	NOF[4]	/	0.00	0.00	20.3	0.2071	0.940	11.41	186.4	0.94
14	NOF	/	0.00	0.00	20.8	0.2122	0.960	11.45	186.8	0.96
15	NOF	/	0.00	0.00	20.2	0.2060	0.939	11.44	186.1	0.94

[1]cBSG = crude brewer's spent grain

[2]pBSG = pressed brewer's spent grain

[3]AML = acidogenic mixed liquor of 4 days

[4]NOF = no organic feedstock

[5]ML = mixed liquor

[6]cc = concentration

5.3.2 Experiment 2 - Fed-batch fermentation

The 2nd experiment was initially a classic fermentation of crude BSG from batch 1 (see Table 6 section 5.1.2). This corresponds to phase 1. The initial conditions are summarized in Table 11. The fermentations were performed under the same conditions as Experiment 1, except that they were performed at constant volume. This means a continuous dilution was exerted since water was added at each monitoring to replace the sampled volume.

At the end of the 2nd MCCA production phase (see section 2.6), on day 32, a change of regime was applied on four reactors. This corresponds to the start of phase 2. The mixed liquors of these four reactors were retrieved and distributed in two reactors each (50/50 v/v). Each half (around 110 g) was supplemented with 110 g of fresh feed. The fresh feeds were H_2O (i.e., NOF), EtOH, BSG suspension or AML. The BSG used was the crude BSG from batch 1. The AML used was the AML 2 (see Table 7 section 5.1.3). The concentrations of the EtOH and BSG suspensions were adjusted to the COD value of the AML 2 in order to add the same quantity of COD in all the reactors, except in the H_2O ones. The two split halves of each ML were fed with different fresh feeds, in order to avoid correlations between phase 1 and phase 2. The reactors entering this second feeding regime (phase 2) are presented in Table 12. In addition to these fed reactors, control reactors stayed under the first regime (i.e., the phase 1 regime). Once these new regimes launched, the experiment kept going in batch mode at constant volume, as in phase 1.

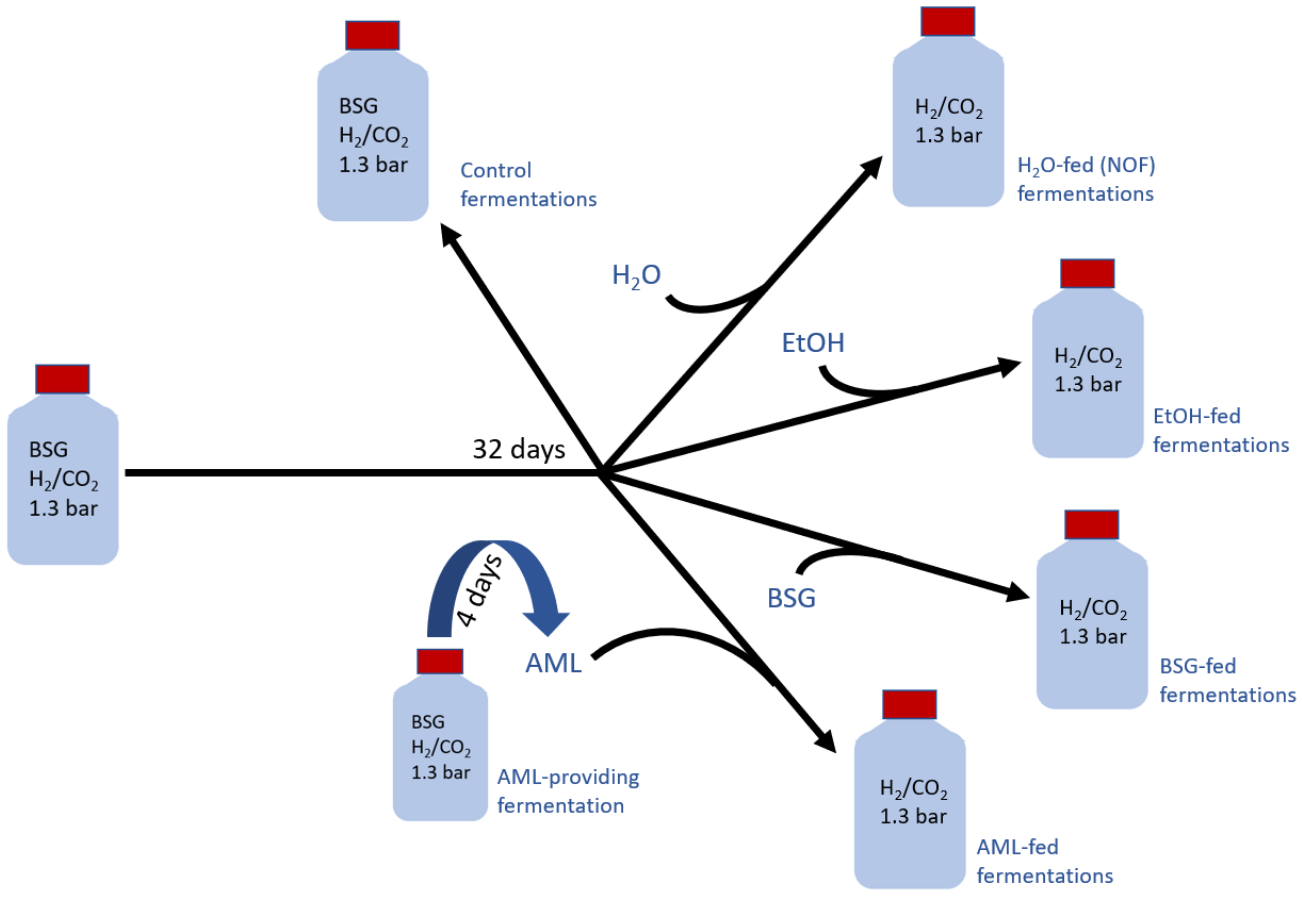

Figure 6: Scheme of the 2nd experiment.

Table 11: Initial composition of the mixed liquor contained in the reactors of Experiment 2 (phase 1).

N° Reactor	Mass of BSG (g/reactor)	Mass of BSG (g_{COD}/reactor)	Cc of BSG (g_{COD}/l_{ML})	Mass of inoculum (g/reactor)	Mass of inoculum (g_{COD}/reactor)	Cc of inoculum (g_{COD}/l_{ML})	Mass of mineral medium (g/reactor)	Mass of additional H_2O (g/reactor)	Total cc (g_{COD}/l_{ML})
1	65.7	23.06	93.13	20.2	0.1139	0.4601	11.0	149.8	93.59
2	65.5	22.99	93.19	20.0	0.1128	0.4572	11.0	149.3	93.64
3	65.9	23.13	93.57	20.0	0.1128	0.4563	11.0	149.4	94.02
4	65.8	23.10	93.24	20.0	0.1128	0.4554	11.0	150.0	93.69
5	65.5	22.99	91.96	20.0	0.1128	0.4512	11.0	152.6	92.41
6	65.6	23.03	93.78	20.0	0.1128	0.4594	11.0	148.0	94.24
7	65.7	23.06	93.36	20.0	0.1128	0.4567	11.0	149.4	93.81
8	65.7	23.06	93.58	20.1	0.1134	0.4601	10.9	148.8	94.04
9	66.0	23.17	93.82	20.1	0.1134	0.4591	11.0	148.9	94.28
10	65.5	22.99	93.11	20.0	0.1128	0.4568	10.9	149.6	93.57
11	65.6	23.03	93.44	20.0	0.1128	0.4578	11.0	148.9	93.90
12	65.6	23.03	93.44	20.1	0.1134	0.4601	11.0	148.8	93.90
13	65.7	23.06	93.81	20.0	0.1128	0.4589	11.0	148.2	94.27
14	65.5	22.99	93.19	20.0	0.1128	0.4572	10.9	149.4	93.64

Table 12: Phase 2 of Experiment 2: feeding regime of each bioreactor, applied once.

Feeding with	Initial bioreactor (identification n°)	Final bioreactor (identification n°)	Added mass of fresh feed (g/reactor)	Added mass of fresh feed (g_{COD}/reactor)	Additional H_2O added (g/reactor)
H_2O	5	5	110	0	0
	6	6			
EtOH (0.01627 g_{COD}/g_{FM})	4	4	110	2.21	0
	11	11			
AML 2 (0.02010 g_{COD}/g_{FM})	5	18	110	2.21	0
	11	20			
cBSG (0.351 g_{COD}/g_{FM})	4	17	6.29	2.21	105.14
	6	19			

5.3.3 Experiment 3 - Semi-continuous fermentation

Based on Experiment 2, the 3rd experiment investigated further the effect of renewing the mixed liquor during the 2nd MCCA production phase (export and dilution of accumulated metabolites, supply of fresh feeds). The initial parameters of the fermentations were identical, except that the substrate used was pBSG from batch 2 and that the pH targeted was 6.0. Table 13 shows the initial content of the bioreactors (phase 1).

The mixed liquors of the reactors were renewed (phase 2) at the middle of the 2nd MCCA production phase, on day 17, instead of the end (as in Exp 2 (see section 5.3.2)). The fresh feeds were H_2O (NOF), AML or BSG suspension. The BSG used was the pressed BSG from batch 2 (see Table 6 section 5.1.2). The AML used was AML 3 (see Table 7 section 5.1.3). The concentration of the BSG suspension was adjusted to the COD value of the AML 3 in order to add the same quantity of COD in all the reactors, except in the H_2O-fed ones. Table 14 presents the bioreactors entering this second feeding regime. The bioreactors were pooled in such a way that no particular fermentation profile of phase 1 corresponded to a specific fresh feed.

The feeding was operated in a semi-continuous way, replacing one third of the ML three times a week, so that the hydraulic retention time (HRT) was 7 days. In addition to these semi-continuously fed reactors, three control reactors stayed under their batch state, or i.e., their phase 1 state.

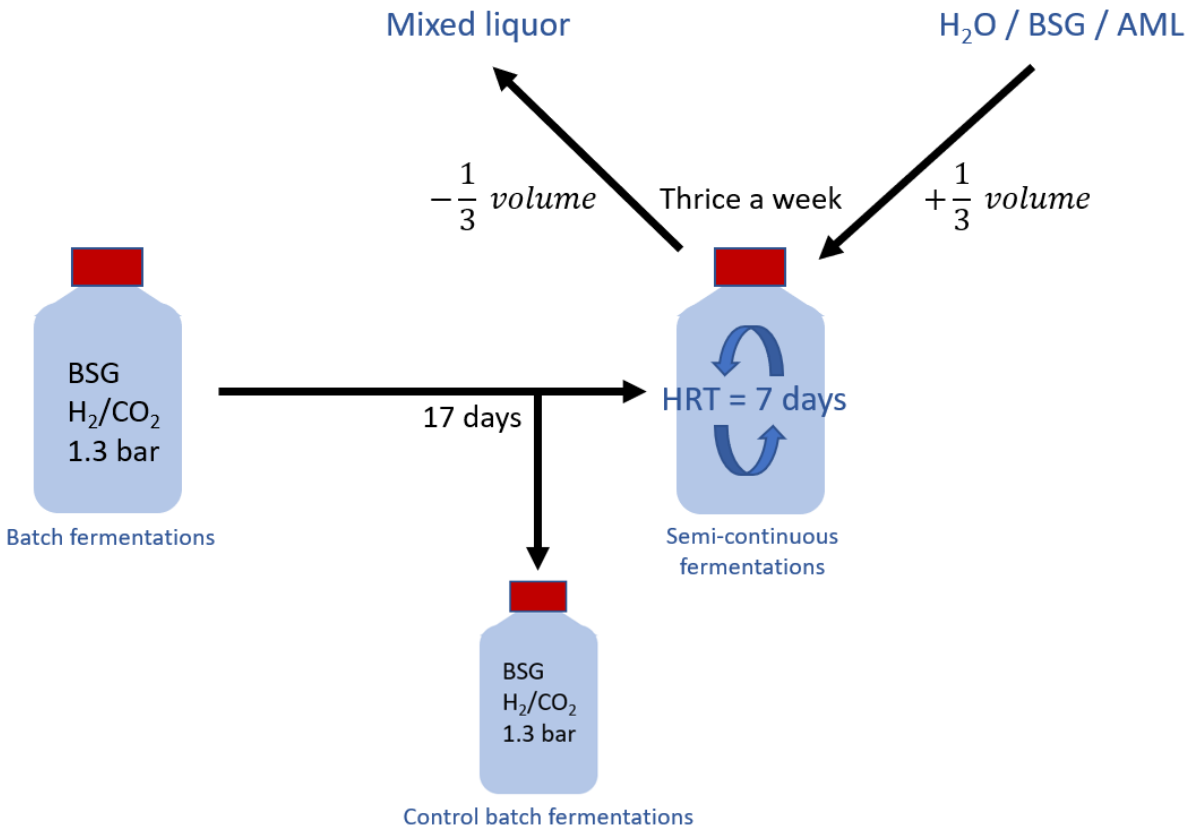

Figure 7: Scheme of the 3rd experiment.

Table 13: Initial composition of the mixed liquor contained in the reactors of Experiment 3 (phase 1).

N° Reactor	Mass of BSG (g/reactor)	Mass of BSG (g_{COD}/reactor)	Cc of BSG (g_{COD}/l_{ML})	Mass of inoculum (g/reactor)	Mass of inoculum (g_{COD}/reactor)	Cc of inoculum (g_{COD}/l_{ML})	Mass of mineral medium (g/reactor)	Mass of additional H_2O (g/reactor)	Total cc (g_{COD}/l_{ML})
1	40.5	22.07	97.09	19.8	0.2020	0.8883	11.6	153.7	97.97
2	40.6	22.13	97.33	19.9	0.2030	0.8928	11.4	153.6	98.22
3	40.4	22.02	97.38	19.7	0.2009	0.8887	11.3	152.8	98.27
4	40.4	22.02	97.12	19.8	0.2020	0.8909	11.3	153.3	98.01
5	40.7	22.18	97.89	19.8	0.2020	0.8913	11.3	153.0	98.78
6	40.3	21.96	97.27	19.7	0.2009	0.8899	11.3	152.7	98.16
7	40.5	22.07	96.85	19.8	0.2020	0.8862	11.3	154.5	97.74
8	40.3	21.96	96.76	19.7	0.2009	0.8852	11.3	153.9	97.64
9	40.5	22.07	97.49	19.7	0.2009	0.8875	11.3	153.1	98.38
10	40.8	22.24	98.04	19.7	0.2009	0.8860	11.3	153.2	98.93
11	40.3	21.96	97.31	19.6	0.1999	0.8858	11.3	152.7	98.20
12	40.3	21.96	97.05	19.8	0.2020	0.8924	11.3	153.1	97.95

Table 14: Phase 2 of Experiment 3: feeding regime of each bioreactor, applied thrice a week.

Semi-continuous feed with	Bioreactors (identification n°)	Retrieved mass of ML (g/reactor)	Added mass of fresh feed (g/reactor)	Added mass of fresh feed (g_{COD}/reactor)	Additional H_2O + pH corrector (g/reactor)
H_2O	R8, R11, R12	70	60	0	10
AML 3 (0.02035 g_{COD}/g_{FM})	R1, R4, R6	70	60	1.22	10
pBSG (0.545 g_{COD}/g_{FM})	R2, R5, R10	70	2.24	1.22	67.76

5.3.4 Overview of experimental conditions

Table 15 summarizes the conditions tested in the 3 experiments.

Table 15: Overview of the experimental conditions tested in each experiment.

Exp	Reactor	Initial operating mode	Substrate	pH	Change of regime ?	Feeding frequency	timing	feeding factor	feed
1	R1->3	Batch with loss of ML at each sampling	cBSG from batch 2	6.8	no	/			
	R4->6		pBSG from batch 2						
	R19->21		AML 1						
	R13->15		NOF						
2	R5, 6	Constant volume batch with dilution at each sampling	cBSG from batch 1	6.8	yes, to fed-batch	only once	on day 32	1/2	H2O
	R4, 11								EtOH
	R18, 20								AML 2
	R17, 19								cBSG
	R1, 2, 3, 7, 8, 9, 10, 12, 13, 14				no	/			
3	R8, 11, 12	Constant volume batch with dilution at each sampling	pBSG from batch 2	6.0	yes, to semi-continuous	thrice a week	from day 17	1/3	H2O
	R1, 4, 6								AML 3
	R2, 5, 10								pBSG
	R3, 7, 9				no	/			

5.4 Analytical methods

5.4.1 Gas composition

Gas samples were analysed by gas chromatography (GC) with a thermal conductivity detector (TCD) (Compact GC, Global Analyzer Solutions, Interscience, The Netherlands). This gas analyser can detect H_2, N_2, O_2, CO_2, and CH_4.

Compact GC characteristics:

The GC includes two channels (the front and back channels) both composed of an injection loop of 25 µl and a TCD. The front channel is equipped with an RI-QBond column (10 m length, 0.32 mm ID, Restek, France) in a 60°C oven and uses Helium as carrier gas at a flow rate of 1 ml/min. The front channel can only separate and quantify CO_2. The back channel is equipped with two columns placed in series in an oven at 70°C with Argon as a carrier gas at a flow rate of 1 ml/min. The first column is a RI-QBond column (2 m length, 0.32 mm ID, Restek, France) and the second is a Molsieve5A column (7 m length, 0.32 mm ID, Restek, France). A 3-way valve is placed between the two columns. It is switched just after H_2, O_2, N_2 and CH_4 enter in the second column and while CO_2 remains in the first column. CO_2 is consequently backflushed out of the GC. As the other gases continue to progress in the second column, they are separated and quantified by the TCD of the back channel. Retention times of H_2, O_2, N_2 and CH_4 were 21.7, 27.7, 35.0 and 51.0 seconds, respectively.

Gas sample analysis:

The gas sample contained in the syringe is gently injected through an injection valve into the GC and passes then successively through each injection loop before leaving the GC through an exit valve. Chromatograms are then automatically integrated; the peak areas are converted to gas concentrations using experimentally calibrated conversion factors. The gas composition is given as percentage of the different gases (H_2, O_2, N_2, CH_4 and CO_2) analysed by the Compact GC.

Calibration of the Compact GC:

The gas analyzer is calibrated with five gas mixtures: four artificial gas mixtures with known composition and air. Each calibration gas mixture was collected using a 50 ml syringe equipped with a three-way valve. During the sampling procedure, the syringe was first flushed twice with the gas mixture to be analysed to eliminate any trace of remaining air. Injections of each sample were repeated three times and the EZ-Start program automatically determined the calibration factor (ratio between gas concentration and peak area on the chromatograms) for each gas component. The detection limit of the GC is 0.1% V/V.

5.4.2 Contents of dry matter and volatile matter

The contents of dry matter at 105°C and volatile matter at 550°C were determined gravimetrically.

1) The crucibles are washed and heated in the oven at 550°C for 2 hours.

2) The crucibles are transferred from the oven to the desiccator to remain dry and to cool down.

3) Once the crucibles are at room temperature, they are tared. A fresh sample is then introduced in each crucible.

4) Each crucible is weighed with its content and then placed in the oven at 105°C to constant mass.

5) The crucibles are transferred from the oven to the desiccator until they reach room temperature.

6) The cooled crucibles with their content are weighed and then placed in the oven at 550°C to constant mass.

7) The crucibles are transferred from the oven to the desiccator until they reach room temperature.

8) Each cooled crucible is weighed with its content.

Each sample is analysed in triplicate.

The fresh matter (FM) is the mass of sample transferred in the crucible before it is introduced in the oven at 105°C. The dry matter (DM), expressed in g_{DM}/g_{FM}, is the mass of sample that remains in the crucible after the crucible stayed in the oven at 105°C until constant mass and has cooled down to room temperature. The ashes are the mass of sample, expressed in g_{ashes}/g_{FM} or g_{ashes}/g_{DM}, that remains in the crucible after the crucible stayed at 550°C until constant mass and has cooled down to room temperature. The volatile solid or volatile matter (VM), expressed in g_{VM}/g_{FM} or g_{VM}/g_{DM}, is the difference between the FM and the ashes or the DM and the ashes. It corresponds to the mass lost when the $DM_{105°C}$ was put at 550°C until constant mass.

5.4.3 Chemical Oxygen Demand (COD)

The oxidizable matter is oxidized by an excess of potassium dichromate in an acidic solution in the presence of mercury sulfate, which forms a complex with chlorides, and of silver sulphate, which serves as a catalyst. The excess unreacted dichromate can be measured either

by colorimetry or by titration with iron(II)-ammonium sulfate solution using iron orthophenanthroline as indicator.

5.4.3.1 Liquid homogeneous samples

COD measurement kits (Spectroquant, Merck KGaA, Germany) for samples between 500 and 10 000 mg_{COD}/l were used. Depending on the expected sample COD, a dilution is applied to ensure the final COD is well in the range of the kit. The volume of appropriately diluted sample added in the test tube is 1.000 ml. The tubes are then closed and strongly shaken before being heated up for 2 hours at 148°C in a preheated thermoreactor (Spectroquant TR620, Merck KGaA, Germany). The tubes are then cooled at room temperature for 10 minutes, strongly shaken and then cooled again until they reach room temperature. Once the tubes are cooled down, they are wipped and absorbance is measured at 605 nm with a spectrophotometer (Spectroquant Pharo300, Merck KGaA, Germany). Results are directly calculated through an internal calibration factor in the spectrophotometer and given in mg of chemical oxygen demand per litre of solution introduced in the tube with a confidence interval of ± 5 mg_{COD}/l. The obtained value is then multiplied by the dilution factor to get the COD concentration present in the initial sample.

5.4.3.2 Solid samples

Preparation of the needed reagents:

1) Distilled water without any organic matter

2) Solid mercury sulphate ($HgSO_4$)

3) Concentrated sulfuric acid (d = 1.83-1.84)

4) Sulfuric solution of silver sulphate: Commercial solution of 6.6 g/l of silver sulphate in concentrated sulfuric acid (AVS TITRINORM® for COD measurement).

5) Potassium dichromate solution 0.25 N: 12.259 g of dry $K_2Cr_2O_7$ are dissolved in distilled water to a final volume of 1 l.

6) Indicator solution: 1.48 g of 1-10 orthophenanthroline monohydrated and 0.7 g of $FeSO_4.7H_2O$ are dissolved in 100 ml of distilled water.

7) Iron(II) and ammonium sulfate solution 0.25 N (Mohr salt): 196 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ are dissolved in 1.6 l of water. 40 ml of concentrated H_2SO_4 are transferred in the solution before distilled water is added until the volume of solution reaches 2l. This solution is standardized before every use with the potassium dichromate solution. To do so, distilled water is added to 20 ml of the dichromate solution, until the total volume of solution reaches

250 ml and 20 ml of concentrated H_2SO_4 is then added to the solution. Once the solution has cooled down, 12 droplets of the indicator are added to the solution. The solution is then titrated with the Mohr salt solution. The normality ratio between the Mohr salt solution and the potassium dichromate is then given as f = m/n with m the volume (ml) of potassium dichromate 0.25 N solution introduced and n the volume (ml) of Mohr salt used for titration.

Sample oxidation:

An amount equivalent to 30 mg of volatile matter of the sample is introduced into a 500 ml spherical flask. 50 ml of 0.25 N $K_2Cr_2O_7$ solution and 0.4 g of $HgSO_4$ are then poured in the flask. 66 ml of sulfuric solution of silver sulfate are transferred in the 500 ml flask while gently shaking to make sure the water and concentrated sulfuric acid are well mixed and that only one liquid phase remains in the flask. The flask is connected to a 30 cm condenser and boiled at reflux for 30 min once boiling has begun. Once the 30 min are over, the flask is cooled down, the condenser is washed with about 184 ml of distilled water added to the spherical flask and the latter is left to cool down.

Titration:

12 drops of the indicator are added to the oxidized solution in the spherical flask. The excess of potassium dichromate is titrated with iron-ammonium sulphate solution until the solution turns from blue-green to red-brown.

For each set of determination, a negative control is analysed following the procedure described above.

The COD (g_{COD}/g) of the sample is then calculated thanks to the equation below:

$$COD = 2 * (A - B) * \frac{f}{m}$$

with m the mass of sample introduced (mg), A and B the volume of iron-ammonium solution used to titrate the negative control and the solution containing the sample, respectively. f is the normality ratio between the Mohr salt solution and potassium dichromate solution.

5.4.4 Analysis of carboxylates and alcohol metabolites

Reagents:

An internal standard solution is prepared by diluting 250.0 µg of 4-methylvalerate (4-MV) (Sigma Aldrich, USA), a carboxylic acid which is not present naturally in the fermentation broth, in 250.0 ml of NaOH 8g/l, in order to obtain a 4-MV solution of 1 g/l.

A 1 g/l of H_2SO_4 solution is prepared by diluting 55 µl of H_2SO_4 97% (ChemLab, Belgium) in a 100 ml flask with distilled water. The flask must be filled with 40 ml of water before adding the acid to prevent any excess heat emission.

Analyte extraction:

The analysis of the carboxylates and alcohols is performed on the supernatant of the centrifuged mixed liquor.

Metabolites are extracted from the ML supernatant in diethylether. Both samples and calibrating solutions undergo the same extraction process. For the extraction, glass screw top Pyrex test tubes with plastic caps and silicone seal are used. First, approximately 1 g of NaCl is introduced in the test tube (for salting out) along with 1.500 ml of distilled water and 1.000 ml of the sample or calibrating solution. Afterwards, 0.200 ml of the prepared 4-MV internal standard is added in each tube to correct for any variability of the injection-detection steps. Finally, 1.000 ml of the H_2SO_4 solution, immediately followed by 2.000 ml of pure diethylether (Sigma-Aldrich) are added into each test tube before closing them rapidly. The tubes are then intensely shaken for about 10 seconds to complete the extraction process. The tubes are centrifuged for 1 minute at 3000 rpm in the centrifuge to separate the water phase from the ether phase.

Gas chromatography:

Once centrifuged, the ether extract supernatant of each test tube is collected with a micropipette and transferred into a glass vial which is then sealed with a PTFE/silicone screw cap (VWR, Germany). The ether extracts are then analysed by gas chromatography (Trace 2000 Interscience, USA). The samples are injected with an autosampler in a split-splitless injector, in split mode 1/10, maintained at 230°C. The separation is performed in a nonpolar column (Agilent DB-WAX UI (30m x 0.25mm x 0.25µm), USA) by N_2 at 1.2 ml/min. The oven temperature is set at 40°C and increased by 10°C/min for 20 minutes until it reaches 240°C. It remains at this temperature for 22 minutes. The total run time is 43 minutes. The detection is performed by a flame ionization detector (FID) at 240°C. Chromatograms are then automatically integrated by the 'EZchrom' program; integration is manually checked; each peak area is normalized by the peak area of the internal standard. Normalized peak areas are converted to concentrations using the corresponding calibration factor.

Calibration:

Two calibrating solutions were prepared by distributing the targeted concentration of pure compounds in each solution. Every peak area obtained by the integration of the chromatograph is normalized by the peak area of the internal standard. This ratio can then be plotted as a function of the calculated concentrations of each fatty acid. A calibration line is then plotted for each analyte and the slope of a calibration line crossing the origin is

determined by linear regression. The calculated slope is used to determine the concentration of each analyte in the analysed sample.

5.5 Calculation

5.5.1 Cumulated production of metabolites

The cumulated production represents the net total amount of metabolites produced over time from the start of the experiment. This enables to get rid of manual additions or removals of metabolites during the experiment and thus to only consider the metabolites produced by the microorganisms. The cumulated production curves shown in the results section below are calculated as follow.

On day "t", the ML of the reactor has a total volume: $V_{t\_ML\_start}$ (l). A ML sample is then collected to analyse the composition of the ML liquid phase (see section 5.2.3). This sample has a volume of V_{t_sample} (l). The sample is analysed, and each metabolite concentration is determined: $C_{t\_metabolite\_start}$ (g_{COD}/l_{ML}). KOH or H_2SO_4 1M and water are added to the reactor. $V_{t\_returned}$ (l) is the volume returned to the reactor. The final volume of ML in the reactor $V_{t\_ML\_end}$ (g_{COD}/l_{ML}) is calculated as follow:

$$V_{t\_ML\_end} = V_{t\_ML\_start} - V_{t\_sample} + V_{t\_returned}$$

The concentration of the metabolites in the reactor after monitoring $C_{t\_metabolite\_end}$ (g_{COD}/l_{ML}) can then be calculated as:

$$C_{t\_metabolite\_end} = C_{t\_metabolite\_start} * \frac{V_{t\_ML\_start} - V_{t\_sample}}{V_{t\_ML\_end}}$$

On day "t+1" the ML of the reactor has a total volume: $V_{t+1\_ML\_start}$ (l). A ML sample is taken to analyse the liquid phase of ML composition. This sample has a volume of V_{t+1_sample} (l). The sample is analysed, and each metabolite concentration is determined: $C_{t+1\_metabolite\_start}$ (g_{COD}/l_{ML}).

The net cumulated production at day t+1 ($Prod_{t+1}$, in g_{COD}/l_{ML}) is calculated as follow:

$$Prod_{t+1} = Prod_t + \frac{(C_{t+1\_metabolite\_start} * V_{t+1\_ML\_start}) - (C_{t\_metabolite\_end} * V_{t\_ML\_end})}{V_{t+1\_ML\_start}}$$

Since the net cumulated production is an amount of COD normalized to the volume of the ML in the reactor, a positive slope from day t to day t+1 means that the metabolite is produced, while a negative slope indicates a consumption of the metabolite.

5.5.2 Theoretical COD

The theoretical COD of the monitored metabolites is obtained by the calculation developed in section A of the appendix.

6 Results

The fermentation profiles of all the individual fermentations are presented in section B of the appendix, respecting the order of the experiments.

6.1 Experiment 1 - Batch fermentation - Influence of substrate on metabolite profile

This experiment was designed to investigate the influence of the substrate composition on the metabolite profile, with the mixture H_2/CO_2 77/23 v/v supplied at 1.3 bar in the headspace. The fermentation procedure is described in section 5.3.1.

6.1.1 Crude BSG

Figure 8 shows the mean of the fermentation profiles of the three cBSG reactors.

The production of MCCAs started after day 2, and their concentration reached a maximum of 5.2 g_{COD}/l on day 16 (Fig 8.e,f). Concerning the gases, H_2 and CO_2 were significantly consumed from day 4, whereas methanogenesis started on day 11 (Fig 8.b,c).

6.1.2 Pressed BSG

Figure 9 shows the mean of the fermentation profiles of two out of the three pBSG reactors; R4 and R5. R6 presented a different behavior, it was then removed from the mean and is presented in the appendix (Fig VI).

The production of MCCAs started around day 3 (Fig 9.e,f). The MCCA concentration reached its maximum of 4.9 g_{COD}/l on day 11. Methanogenesis did not occur (Fig 9.b,c).

6.1.3 Acidogenic mixed liquor

Figure 10 shows the mean of the fermentation profiles of the three AML reactors.

The AML fermentations presented a very slow but continuous MCCA production (Fig 10.e,f). The latter operated simultaneously with methanogenesis, which started around day 2 (Fig 10.b,c).

6.1.4 No organic feedstock

Figure 11 shows the mean of the fermentation profiles of the three NOF reactors.

A rapid acetate production started after day 2 and stopped on day 7 (Fig 11.d,f). Then, C2 was consumed, a clear C4 production took place until day 18 and C4 reached a concentration of 2.0 g_{COD}/l. After day 18, C6 and C8 were produced, and the experiment stopped on day 21. In parallel, methanogenesis started around day 9 (Fig 11.b,c).

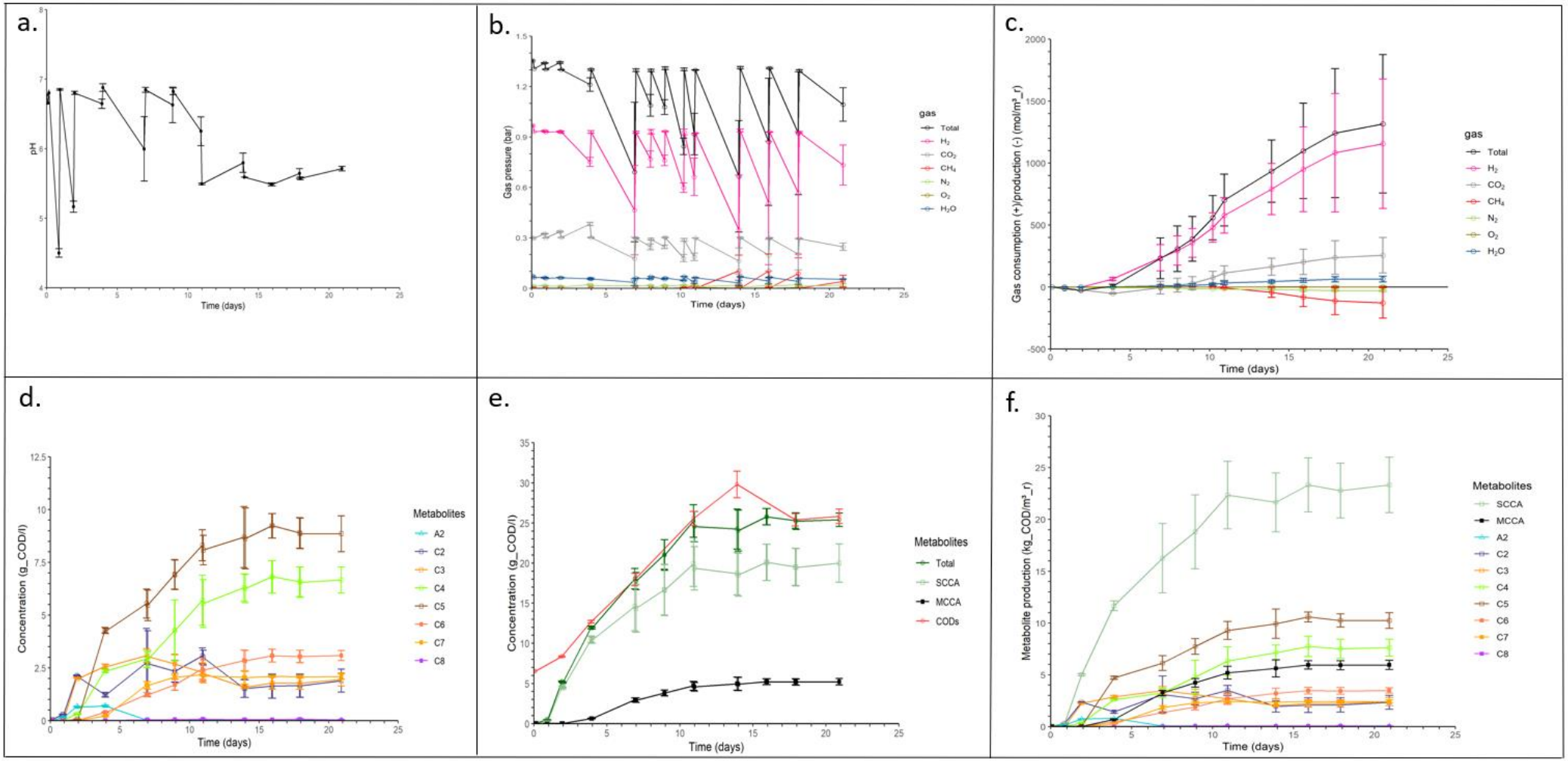

Figure 8: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations (A2 = ethanol) ; e) the mean grouped metabolite concentrations and total COD_s (soluble COD) ; f) the mean metabolite productions and consumptions, for the cBSG reactors R1, R2 and R3.

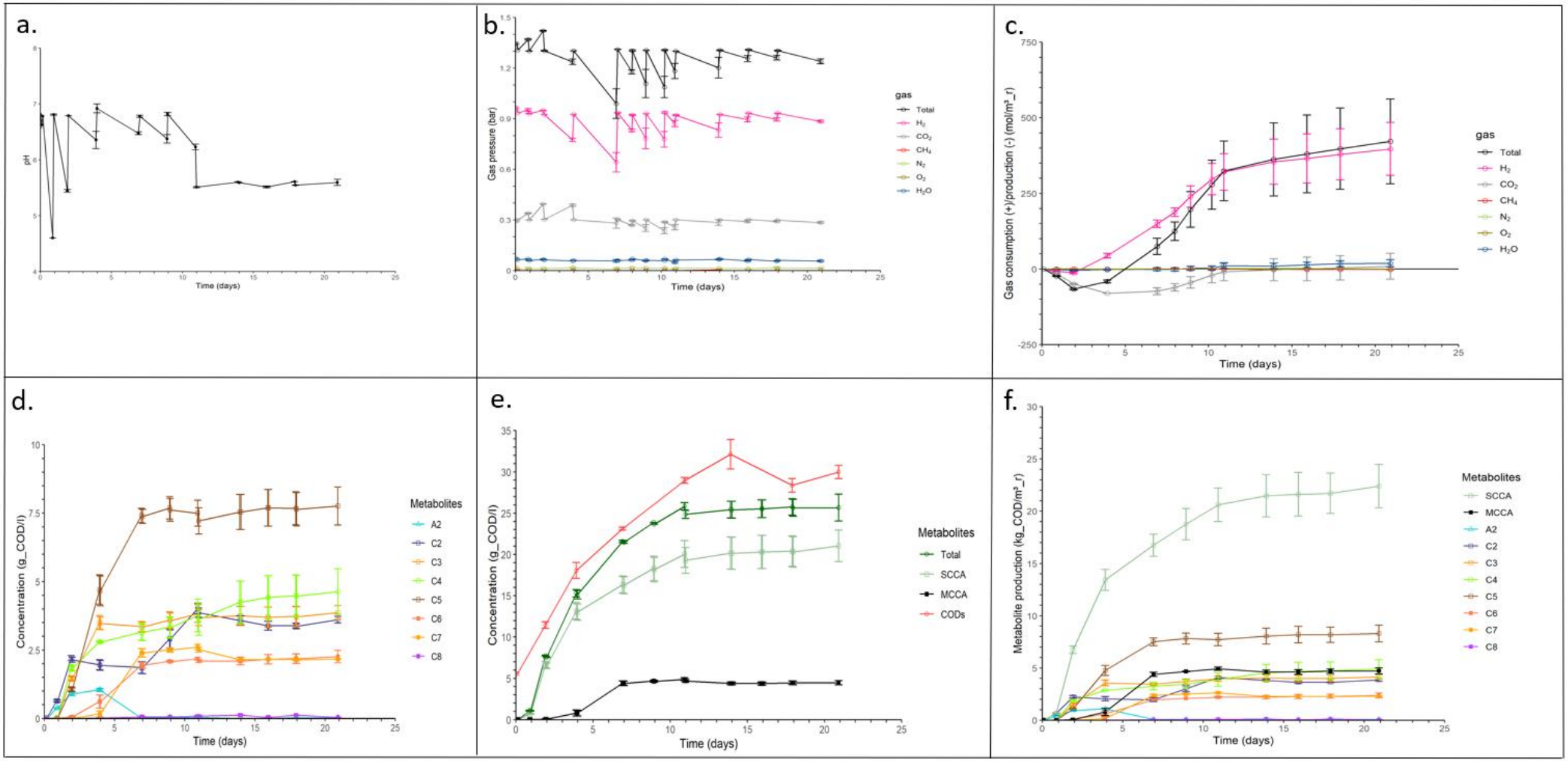

Figure 9: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the pBSG reactors R4 and R5.

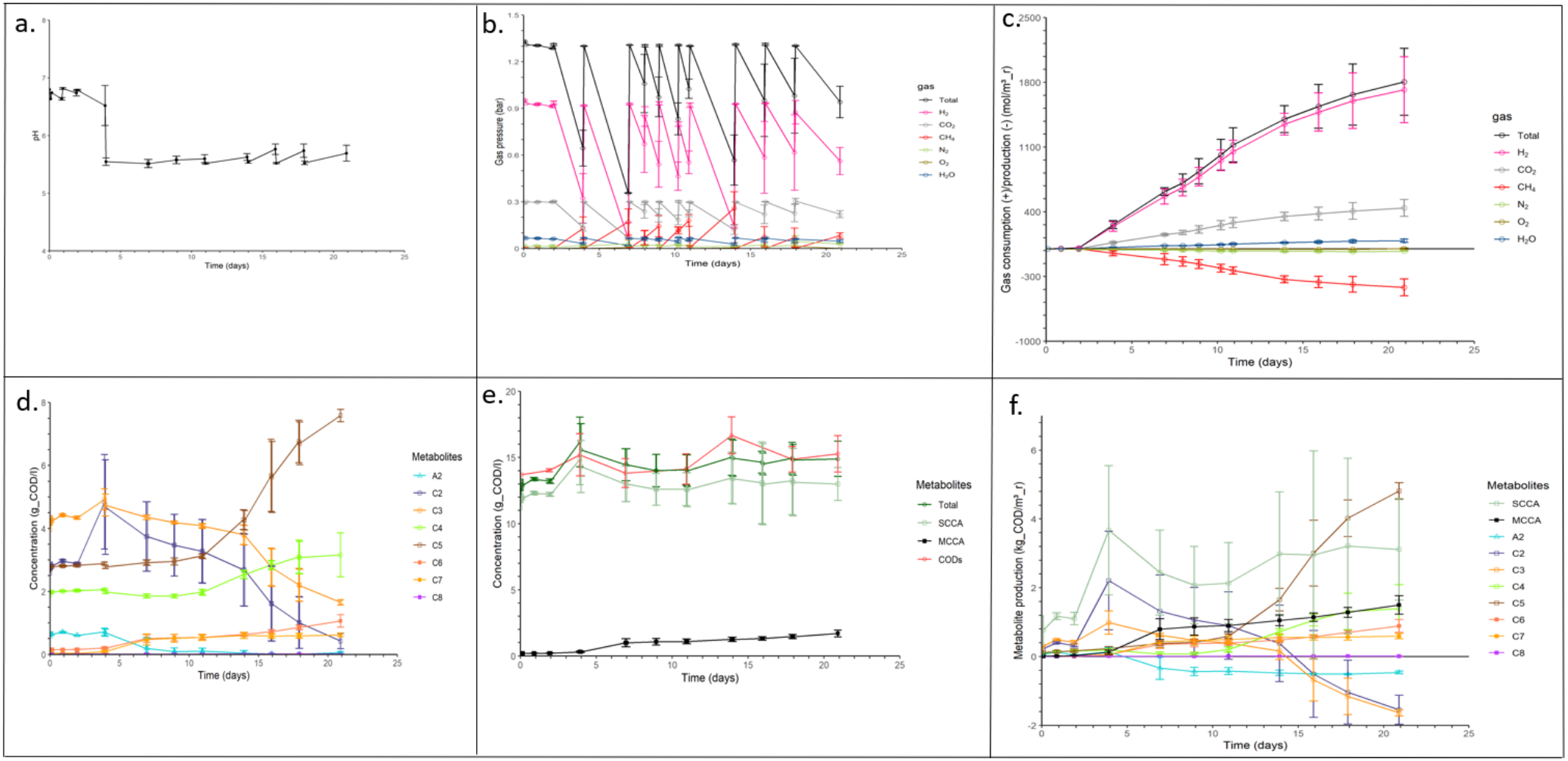

Figure 10: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the AML reactors R19, R20 and R21.

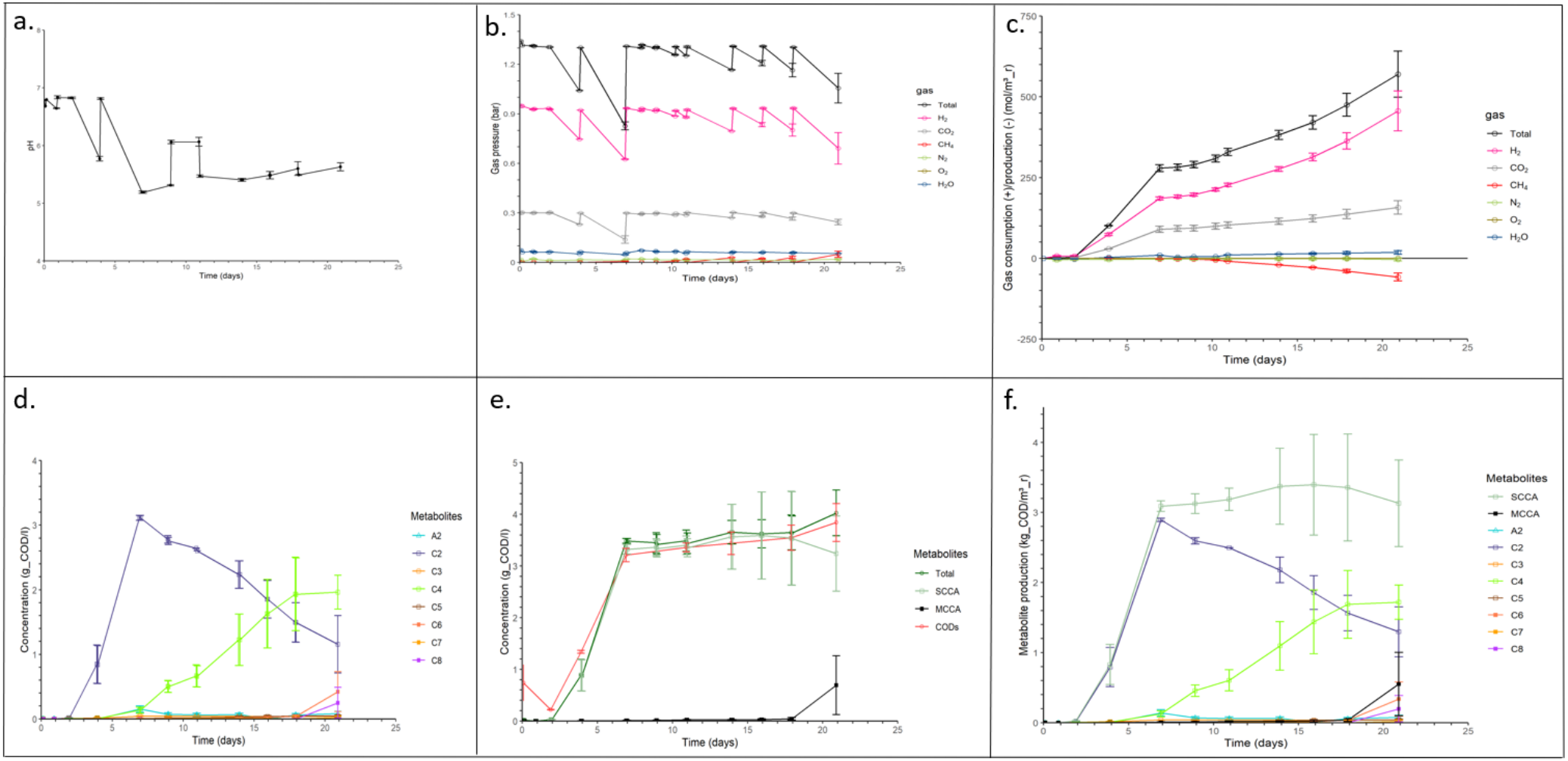

Figure 11: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the NOF reactors R13, R14 and R15.

6.2 Experiment 2 - Fed-batch fermentation - Influence of fed substrate on MCCA production

This experiment was designed to investigate the 2nd phase of MCCA production previously obtained with the mixture H_2+CO_2 supplied at 1.3 bar (in an experiment preceding Exp 1 (see section 2.6)), but which was not obtained with Exp 1 (see section 6.1). The goal was to recover this 2nd MCCA production phase, and then potentially reach a 2nd plateau, to conclude at which MCCA concentration the MCCA production stops again. The second part of this experiment was focused on identifying whether a substrate depletion or a MCCA accumulation is a limiting factor of this 2nd MCCA production phase.

Fermentations were started in batch mode with crude BSG as substrate (phase 1 (see section 5.3.2)). On day 32, 6 of the 14 initial reactors did and ended or were ending a 2nd phase of MCCA production. Two of them were kept as control reactors, while the 4 others were shifted to the fed-batch mode (phase 2) and fed once with different types of fresh feeds: water (NOF), ethanol, 4-day AML and BSG. The specific conditions are described in section 5.3.2. The 8 remaining reactors remained under the initial conditions of phase 1.

6.2.1 Control batch fermentation

Figure 12 shows the mean of the fermentation profiles of the two control reactors.

H_2 and CO_2 were both consumed between day 4 and day 11, and then after day 15 (Fig 12.c). The reactors proceeded a first MCCA production until day 11 (Fig 12.f), followed by a lag phase. On day 15, C2 started to be produced. Then, a second MCCA production phase occurred, from day 18 to day 42, with maximal C6 and MCCA concentrations of 10.7 g_{COD}/l (Fig 12.d) and 16.1 g_{COD}/l (Fig 12.e), respectively. One of the two control fermentations, R7, reached a MCCA concentration as high as 19.3 g_{COD}/l (Fig XIX.e Appendix).

6.2.2 Fed-batch fermentations

6.2.2.1 Control fed-batch fermentation with water

Figure 13 shows the mean of the fermentation profiles of the two reactors fed with water (NOF).

In phase 1, a great C6 production followed C4 and C2 productions, leading to maximal C6 and MCCA concentrations of 10.6 g_{COD_C6}/l and 17.0 g_{COD_MCCAs}/l on day 32, when the production was still going on (Fig 13.d,e,f). Following the dilution on day 32, a great C2 production operated (Fig 13.d), simultaneously to H_2 and CO_2 consumptions (Fig 13.c). No clear production of C4 or C6 can be observed after the dilution.

6.2.2.2 Fed-batch with ethanol

Figure 14 shows the fermentation profiles of one of the two EtOH-fed reactors (R11).

EtOH was fed on day 32, as identifiable on Fig 14.d. EtOH was consumed in the next days while acetate was produced. Then, from day 38, C4 was produced, and from day 48, C6 was produced as well (Fig 14.f).

The second EtOH-fed reactor (R4) rapidly started methanogenesis after the ethanol feeding. It then showed a very different profile and did not produce again MCCAs. It is presented in the appendix (Fig XVI).

6.2.2.3 Fed-batch with AML

Figure 15 shows the mean of the fermentation profiles of the two reactors fed with AML.

After feeding with AML on day 32, the MCCA production kept going. At day 42, the MCCA concentration topped at a level of 10.5 g_{COD}/l, which is lower than the concentration of 16.0 g_{COD}/l reached before the dilution associated with feeding on day 32 (Fig 15.e). After day 42, a few C2 was continuously produced until day 56. No C4 or MCCA production followed (Fig 15.f). Finally, a slow methanogenesis started around day 46 (Fig 15.b,c).

6.2.2.4 Fed-batch with BSG

Figure 16 shows the fermentation profiles of one of the two reactors fed with BSG (R17).

Two distinct phases of MCCA production, before the feeding, can be distinguished (Fig 16.e,f). The first took place between day 2 and day 11 while the second did between day 19 and 28. On day 28, the MCCA concentration reached a maximum of 15.5 g_{COD}/l (Fig 16.e). 12 days after the feeding, the MCCA production started again (Fig 16.f). Over the whole monitoring, the C6 productions closely followed the C4 productions, which themselves followed the C2 productions.

The second reactor fed with BSG (R19) did not produce again MCCAs after the feeding. It is presented in the appendix (Fig XXVII).

6.2.3 Other batch fermentations

Among the 8 reactors which did not start a 2nd MCCA production phase before day 32, different behaviors could be detected in such a way that no trend can be identified (R1, 3, 8, 9, 10, 12, 13, 14). Their fermentation profiles are presented in the appendix (Fig XIII, XIV, XX, XXI, XXII, XXIII, XXIV, XXV).

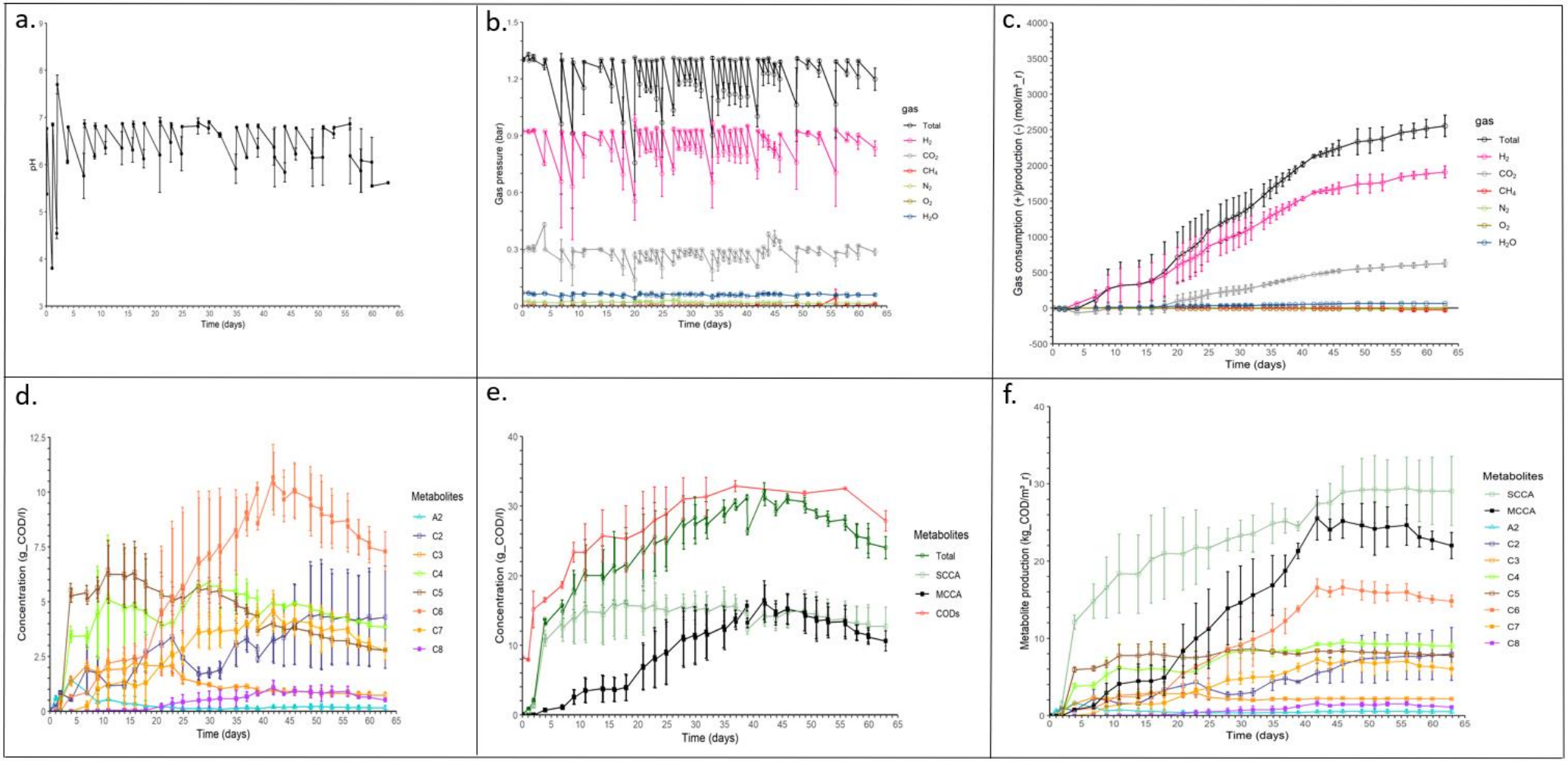

Figure 12: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the control reactors R2 and R7 (batch without feeding).

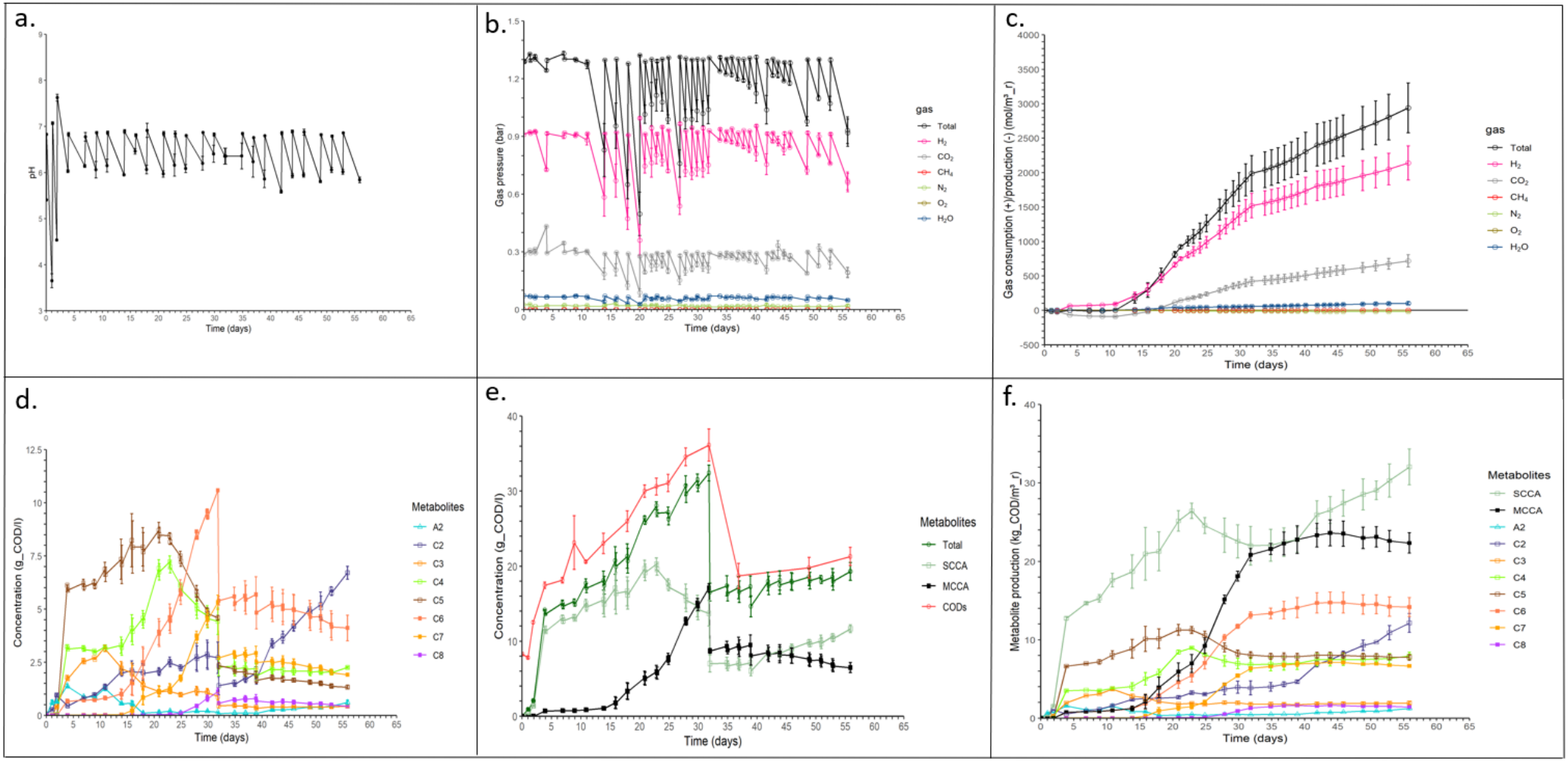

Figure 13: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the water-fed reactors R5 and R6.

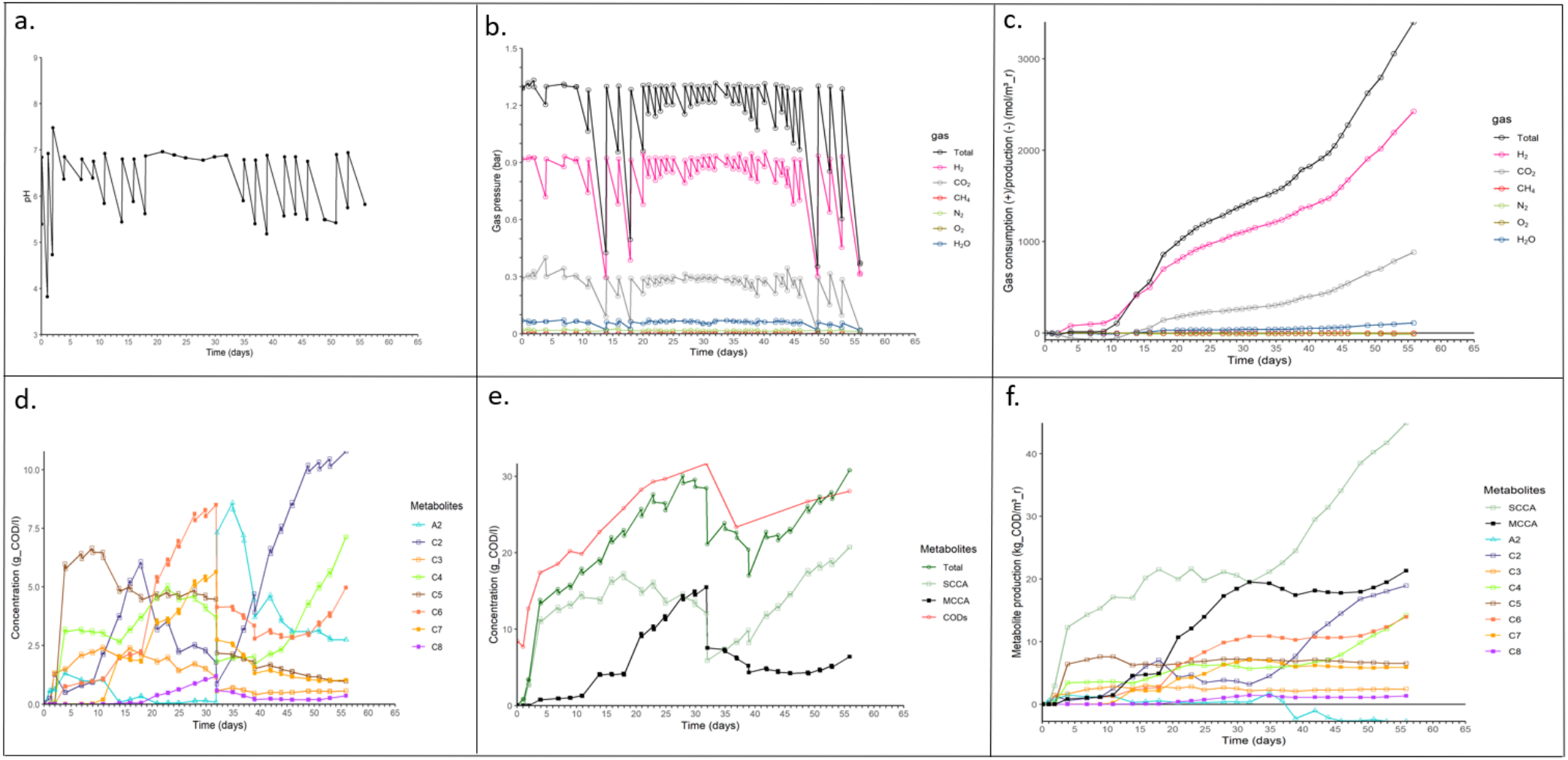

Figure 14: Evolution over time of a) the pH ; b) the gas partial pressures ; c) the gas productions and consumptions ; d) the individual metabolite concentrations ; e) the grouped metabolite concentrations and total CODs ; f) the metabolite productions and consumptions, for the EtOH-fed reactor R11.

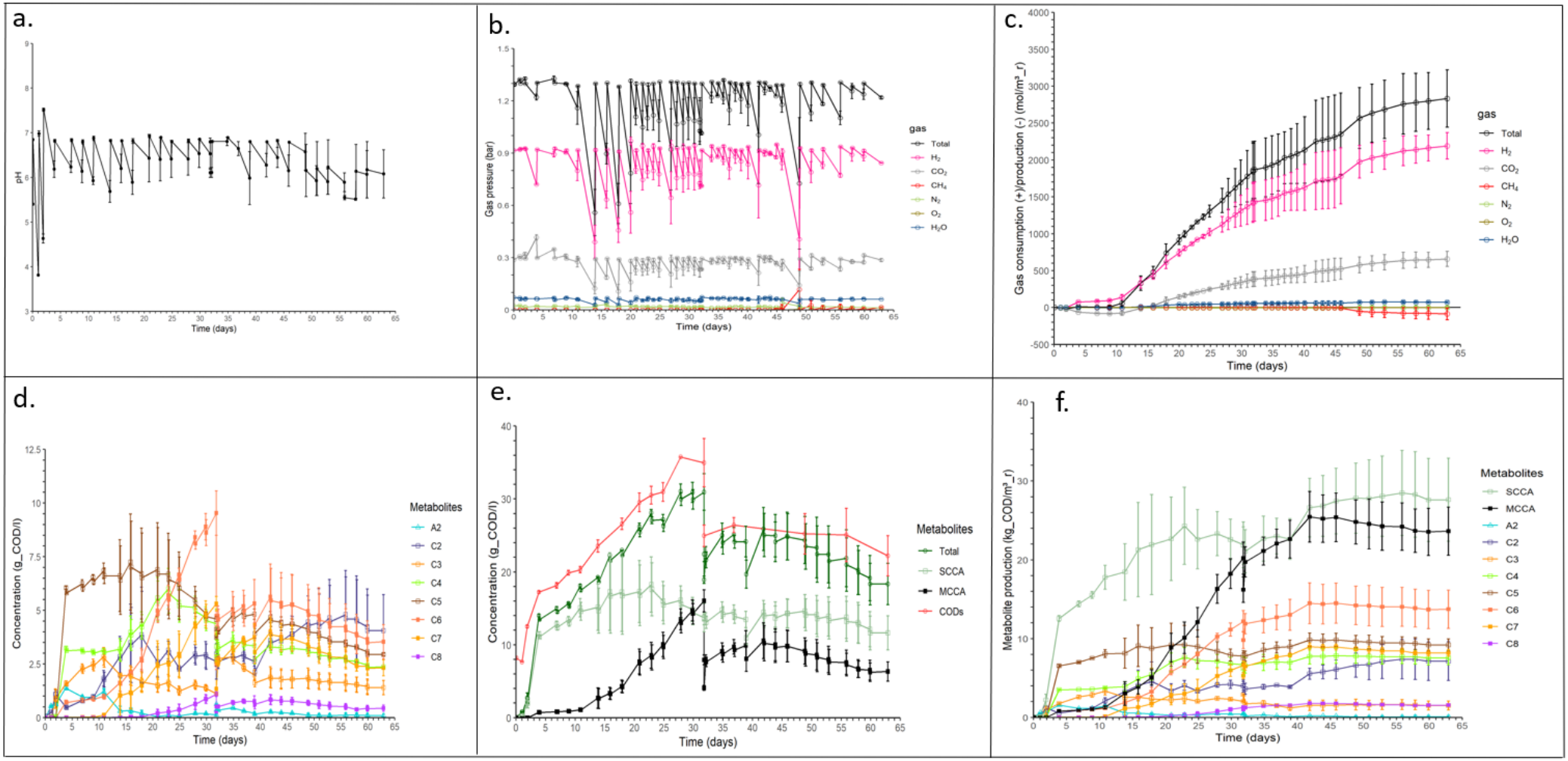

Figure 15: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the AML-fed reactors R18 and R20.

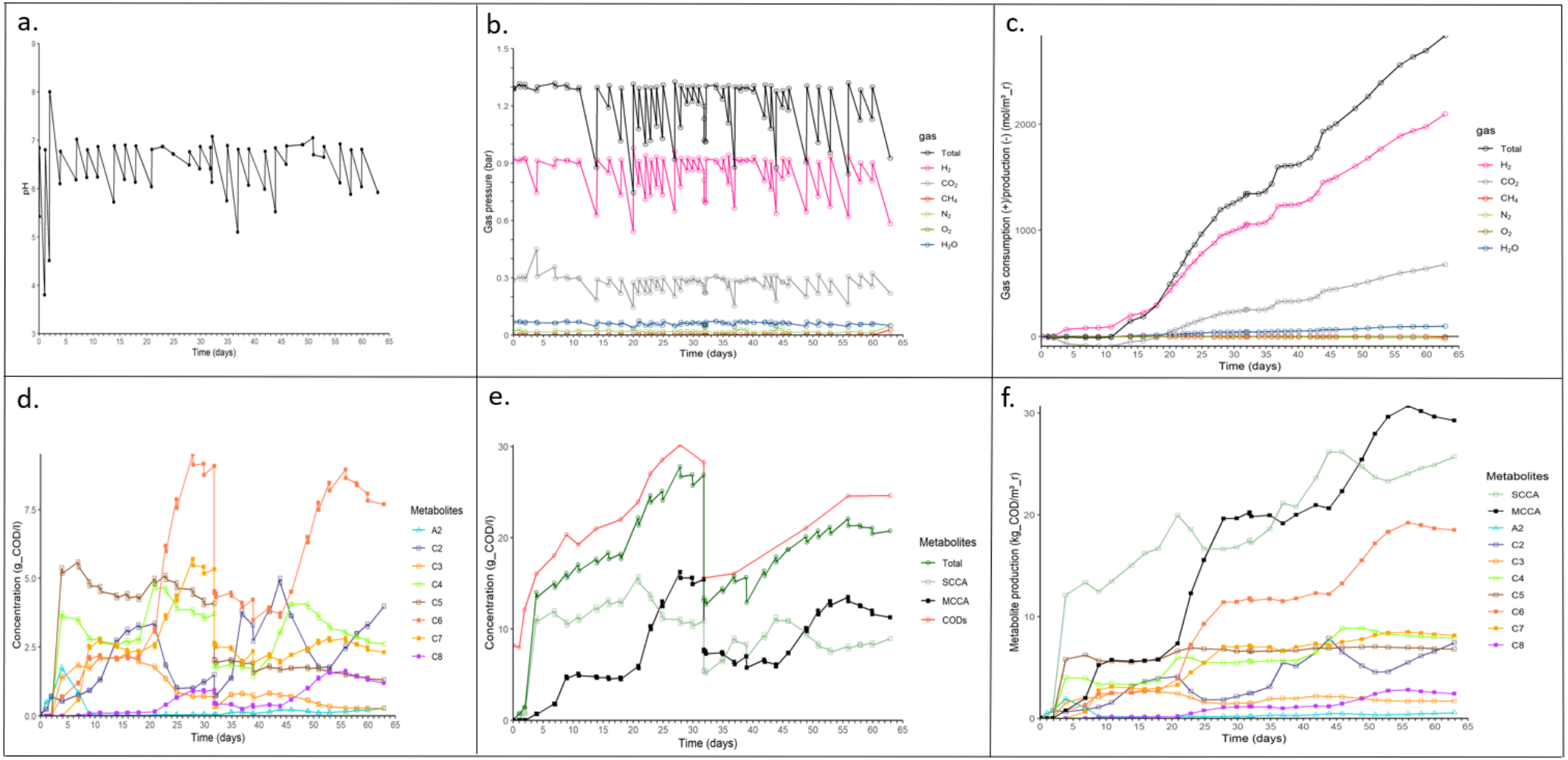

Figure 16: Evolution over time of a) the pH ; b) the gas partial pressures ; c) the gas productions and consumptions ; d) the individual metabolite concentrations ; e) the grouped metabolite concentrations and total CODs ; f) the metabolite productions and consumptions, for the BSG-fed reactor R17.

6.3 Experiment 3 - Semi-continuous fermentation

This experiment was designed to further investigate the reasons of the interruption of the 2nd MCCA production phase, obtained with the mixture H_2/CO_2 77/23 v/v supplied at 1.3 bar. By moving to a semi-continuous system (phase 2 (see section 5.3.3)) during this 2nd MCCA production phase, the idea was to reduce the MCCA concentration, and then avoid the potential inhibition of microorganisms by MCCAs. Moreover, the feedings of BSG and AML would provide information about the possibility of a lack of organic substrate (non-gaseous) causing the interruption of the MCCA production.

6.3.1 Control batch fermentation

Figure 17 shows the mean of the fermentation profiles of the three batch control reactors.

These reactors produced MCCAs until day 30 (Fig 17.f), reaching an overall MCCA concentration of 15.1 g_{COD}/l and a C6 concentration of 7.1 g_{COD}/l. The maximal concentrations obtained among the control reactors were 16.3 g_{COD_MCCAs}/l and 9.9 g_{COD_C6}/l.

6.3.2 Semi-continuous fermentation

6.3.2.1 Control semi-continuous fermentation with water

Figure 18 shows the mean of the fermentation profiles of the three semi-continuous reactors fed with water.

Two distinct phases of MCCA production cannot be identified (Fig 18.f) but Figures 18.d, e and f show that the feeding regime change of day 17 was well applied during a phase of clear MCCA production. This production kept going until day 28. On this day, the total MCCA production was around 15 g_{COD}/l (Fig 18.f), or 3.3 g_{COD_MCCAs} (ML volume = 220 ml). After that, SCCAs were significantly produced (Fig 18.f), while their concentration remained inferior to 5.0 g_{COD}/l (Fig 18.e).

6.3.2.2 Feeding with AML

Figure 19 shows the mean of the fermentation profiles of the three AML-fed semi-continuous reactors.

After the feeding regime change on day 17, the MCCA production kept going on until the end of the experiment (Fig 19.e,f). The production rate was relatively constant between day 17 and day 28 (Fig 19.f), with about 0.93 g_{COD_MCCAs}/l_{ML}*d. A MCCA concentration of around 6.5 g_{COD}/l (Fig 19.e) was maintained. The production rate then progressively diminished from day 28, when methanogenesis started (Fig 19.b,c). Furthermore, after day 28, the SCCA concentration remained relatively constant, with concentrations ranging between 10 and

12 g_{COD_SCCAs}/l (Fig 19.e). On day 35, the total MCCA production was around 21 g_{COD_MCCAs}/l (Fig 19.f), which corresponds to 4.6 g_{COD_MCCAs}.

6.3.2.3 Feeding with BSG

Figure 20 shows the mean of the fermentation profiles of the three BSG-fed semi-continuous reactors.

After the feeding regime change on day 17, the MCCA production remarkably slowed down but stabilized around day 25. It then kept going until the end of the experiment at a rate of 0.45 g_{COD_MCCAs}/l_{ML}*d (Fig 20.f). It resulted in a stable MCCA concentration of around 3.15 g_{COD}/l (Fig 20.e). Parallelly, a great and constant SCCA production set up after day 17 (Fig 20.f). It resulted in a SCCA concentration always superior to 10.5 g_{COD}/l (Fig 20.e). On day 35, the total MCCA production was around 15 g_{COD}/l (Fig 20.f), or 3.3 g_{COD_MCCAs}.

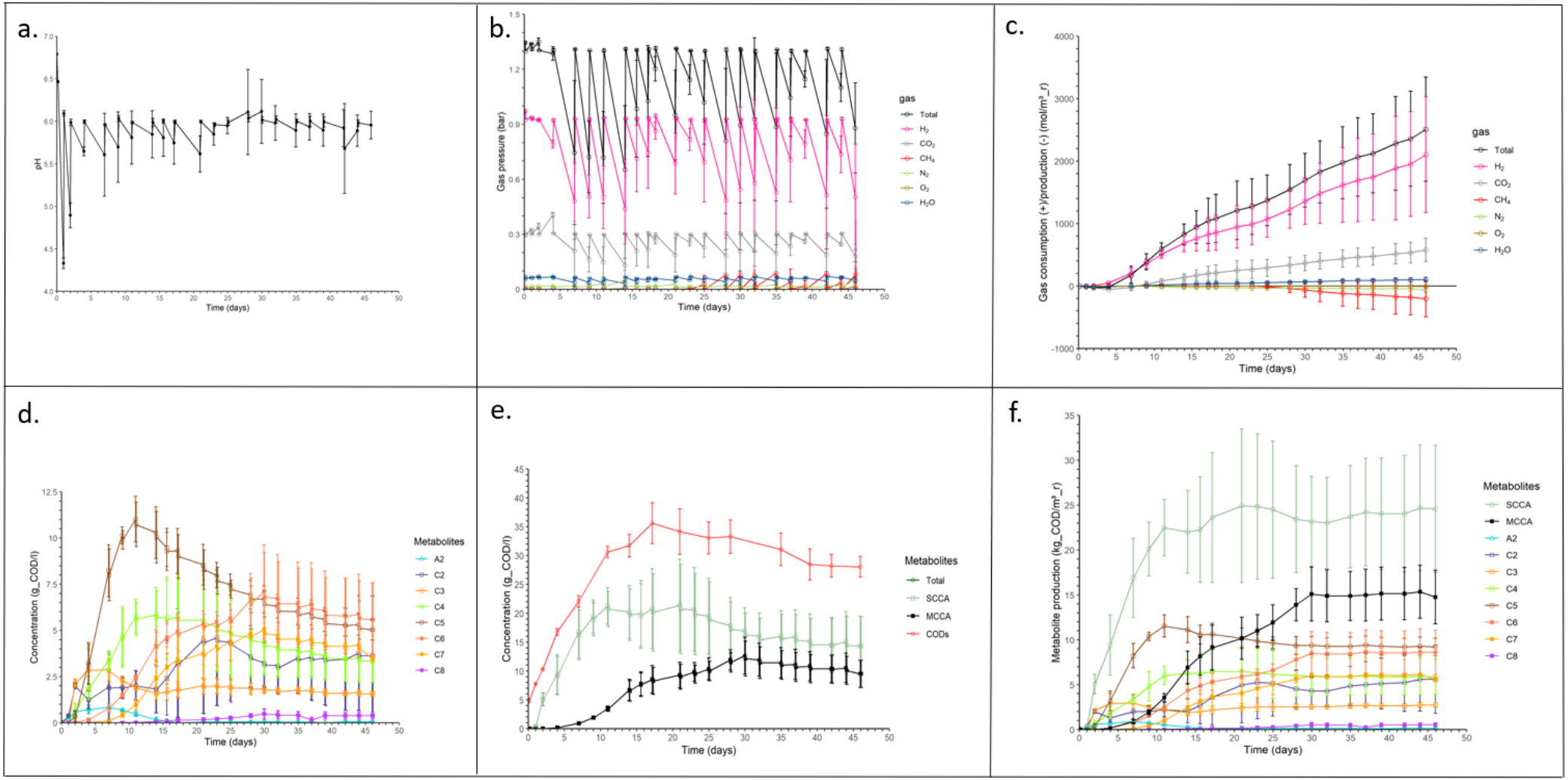

Figure 17: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the control batch reactors R3, R7 and R9.

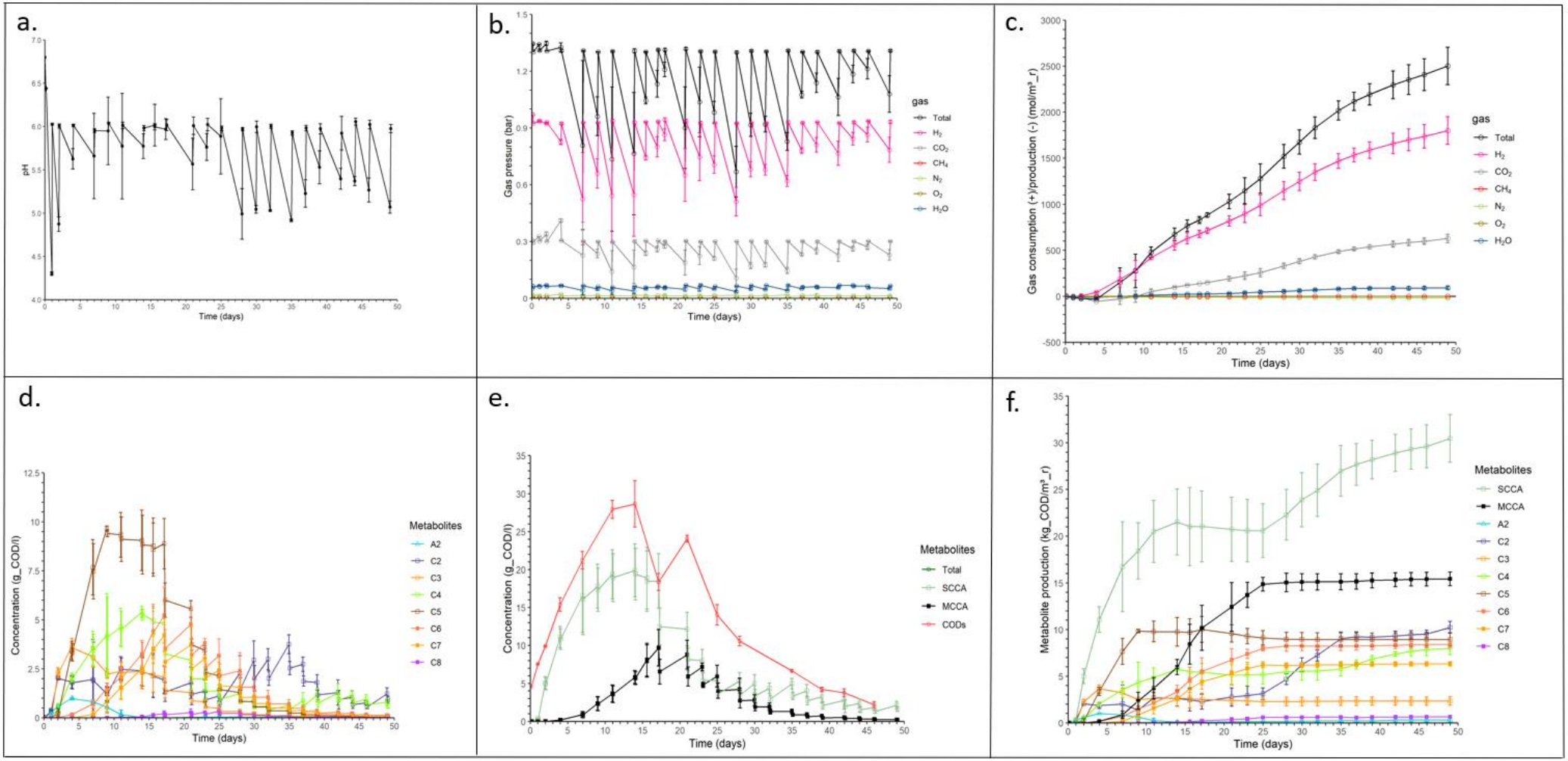

Figure 18: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the semi-continuous reactors fed with water R8, R11 and R12.

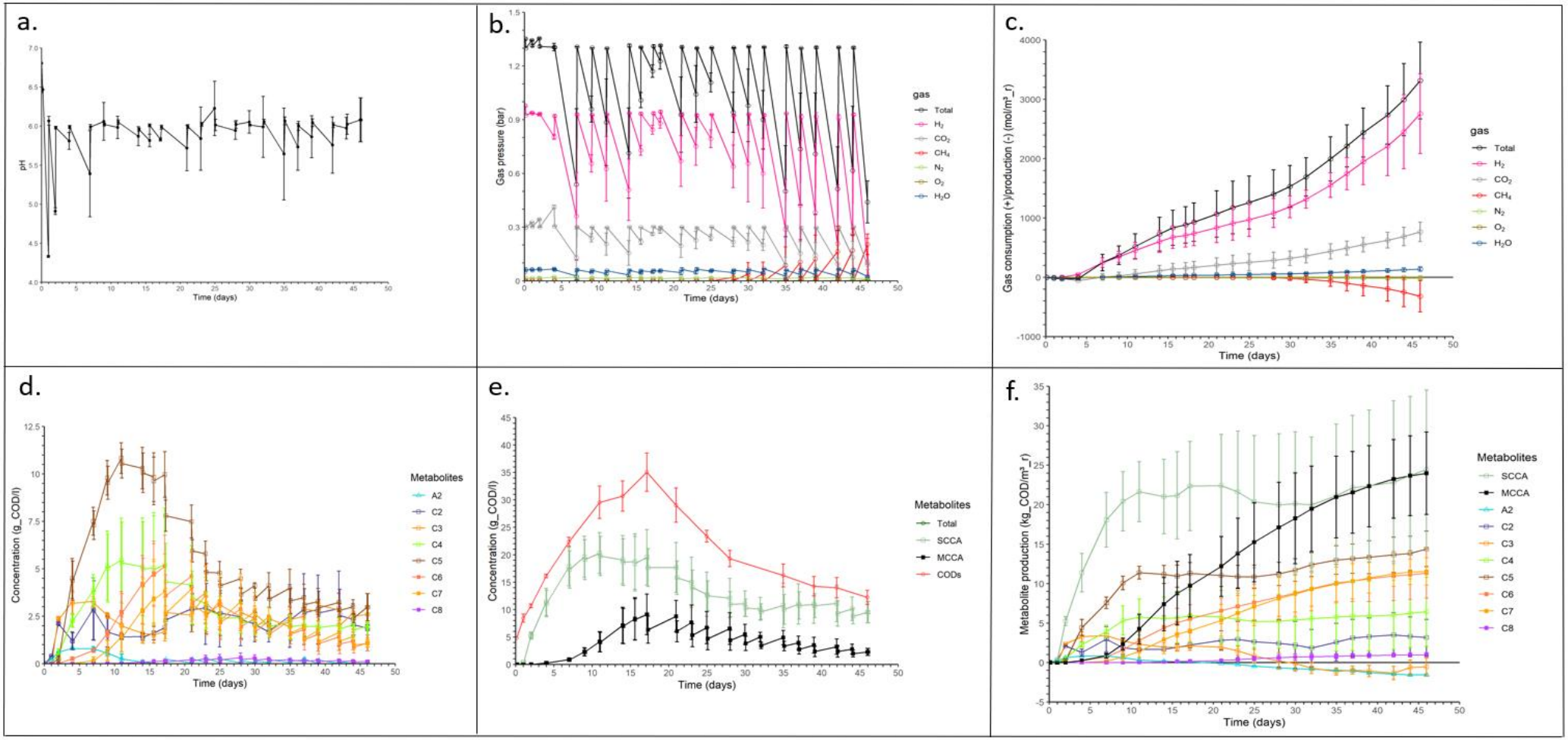

Figure 19: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the AML-fed semi-continuous reactors R1, R4 and R6.

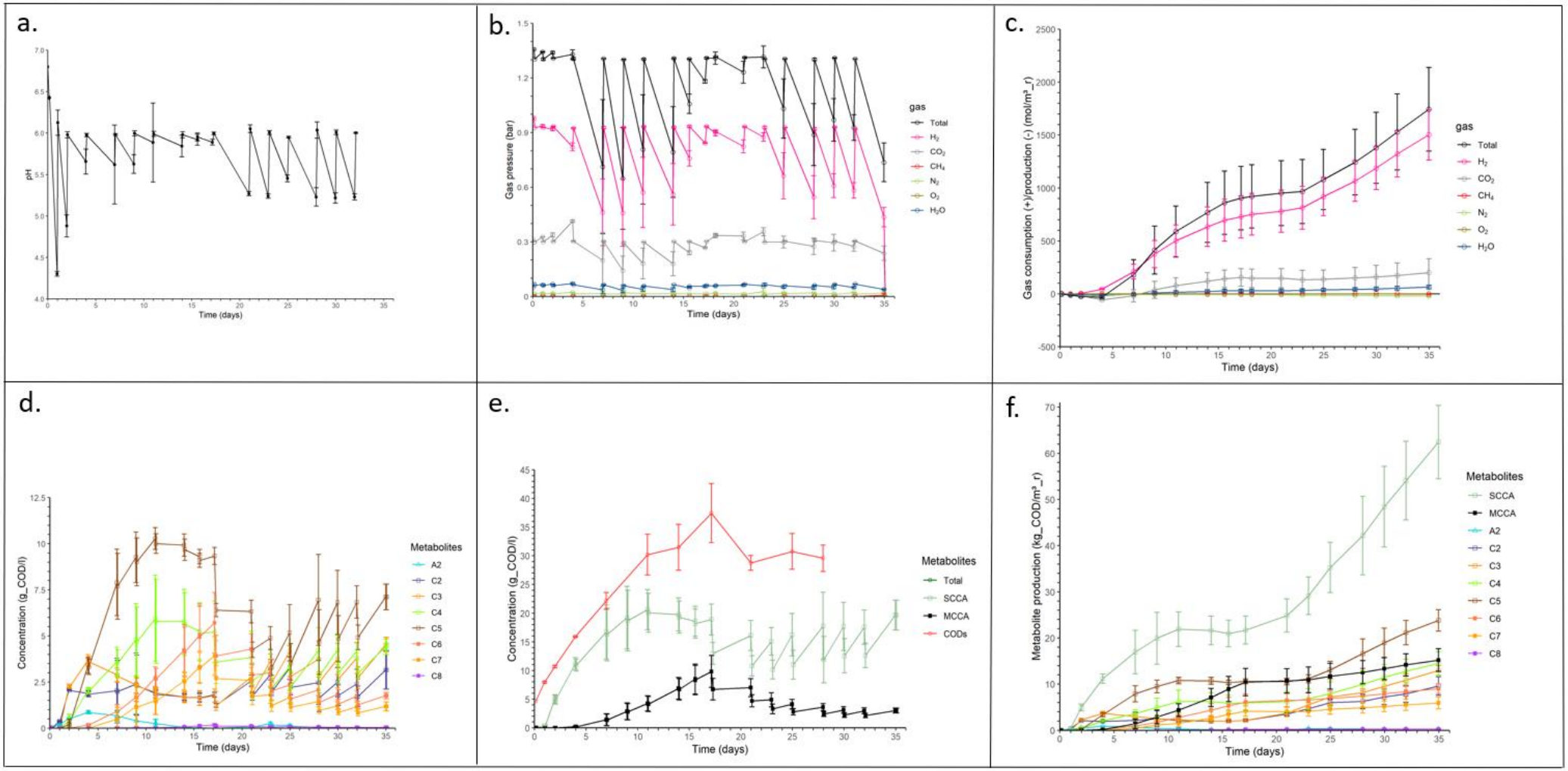

Figure 20: Evolution over time of a) the mean pH ; b) the mean gas partial pressures ; c) the mean gas productions and consumptions ; d) the mean individual metabolite concentrations ; e) the mean grouped metabolite concentrations and total CODs ; f) the mean metabolite productions and consumptions, for the BSG-fed semi-continuous reactors R2, R5 and R10.

7 Discussion

7.1 Second phase of MCCA production

The phenomenon of 2-phase MCCA production previously observed by the team only under H_2+CO_2 (see section 2.6) was reproduced under our conditions. The phenomenon is then reproducible.

Among replicates, some showed a 2nd phase of MCCA production while some others did not. Among replicates presenting a 2nd phase, different final MCCA concentrations were reached, at different times. The BSG from the brewer batch 1 (Exp 2) presented proportionally more two-phase MCCA production than the brewer batch 2 BSG (Exp 1 & 3). In brief, the 2-phase MCCA production entails much variability.

Despite this variability, the fermentations presenting a 2nd phase of MCCA production overall led to higher MCCA productions than fermentations with a unique phase. This suggests that this two-step phenomenon promotes the elongation process.

7.2 Substrate influence on the MCCA production

7.2.1 In batch mode

Among the organic substrates (non-gaseous) initially provided in batch (Exp 1), the best MCCA productions were obtained from the fermentations of BSG (Fig 8-9.e), followed by AML (Fig 10.e) and then by H_2O (NOF) (Fig 11.e).

In the absence of organic feed (NOF), MCCAs were produced after 19 days of batch fermentation. A maximal MCCA concentration of 1.3 g_{COD}/l was reached on the last day of experiment (day 21) while the production was still going on (R13 of Exp 1). This value can be compared to the results obtained by Zhang et al. (2013). They reported, under H_2/CO_2 60/40 v/v at 1.4 atm, a maximal MCCA concentration of 3.20 g_{COD}/l, after 80 days of incubation, while the production started on day 36 (see section 2.4.5.3). The MCCA production obtained way earlier in our experiment pushes to believe that the MCCAs would have risen up to concentrations as high as 3.20 g_{COD}/l. A longer study should be driven to ensure that the MCCAs would keep rising and to determine the maximal MCCA concentration obtainable in these NOF conditions.

7.2.2 In semi-continuous mode

In the semi-continuous mode with a HRT of 7 days, the continuous MCCA production was more efficient with AML than BSG. The NOF did not lead to a continuous MCCA production.

7.2.3 Efficiency of the different types of substrates

Overall, the results indicate that an organic (liquid or solid) substrate (like BSG or AML) is not required for the elongation process when H_2+CO_2 is supplied. However, the MCCA production process is promoted in presence of an organic substrate.

The efficiency ranking of the organic substrates is not the same in batch, where BSG is the best, as it is in semi-continuous mode, where AML is the best. This may be explained by the raw undegraded character of BSG organic matter, whose hydrolysis and acidogenesis take some time. 7 days seem to be insufficient for completing the acidogenesis and the conversion of the intermediate metabolites into MCCAs (see section 2.2.1). This would make the AML productivity higher than the BSG productivity. This suggests that fermentable organic substrates are the most suitable (liquid or solid) substrates for the elongation process, as long as they spend sufficient time in the fermenter to be sufficiently converted.

7.3 Interruption of the MCCA production

7.3.1 Observation in batch mode

All the batch fermentations (presenting a unique MCCA production phase as well as two phases) showed a stop of MCCA production, as expected. The highest MCCA concentration obtained was 19.3 g_{COD}/l and was accompanied by the highest C6 concentration of 12.2 g_{COD}/l (R7 Exp 2). The other effective[3] fermentations stopped at MCCA and C6 concentrations of 15.1-19.3 g_{COD}/l and 7.1-12.2 g_{COD}/l, respectively, around day 30.

7.3.2 Observation in semi-continuous mode

The stop of MCCA production in the H_2O-fed (NOF) semi-continuous reactors coincided with SCCA concentrations fallen below 5 g_{COD}/l (Fig 18.e,f). The AML and BSG-fed semi-continuous broths never fell below that value and they never stopped producing MCCAs. They reached continuous productions of 0.93 g_{COD_MCCAs}/l_{ML}*d and 0.45 g_{COD_MCCAs}/l_{ML}*d, respectively. However, when methanogenesis started in the AML-fed fermenters, the MCCA productivity decreased and was approaching a stop of MCCA production at the end of Exp 3.

7.3.3 Causes of the interruption of MCCA production

Figure 21 is a schematic representation of the MCCA production process from a COF and H_2+CO_2 supply, with the main parameters influencing each bioconversion step represented by a blue dot.

[3] without significant methanogenic activity

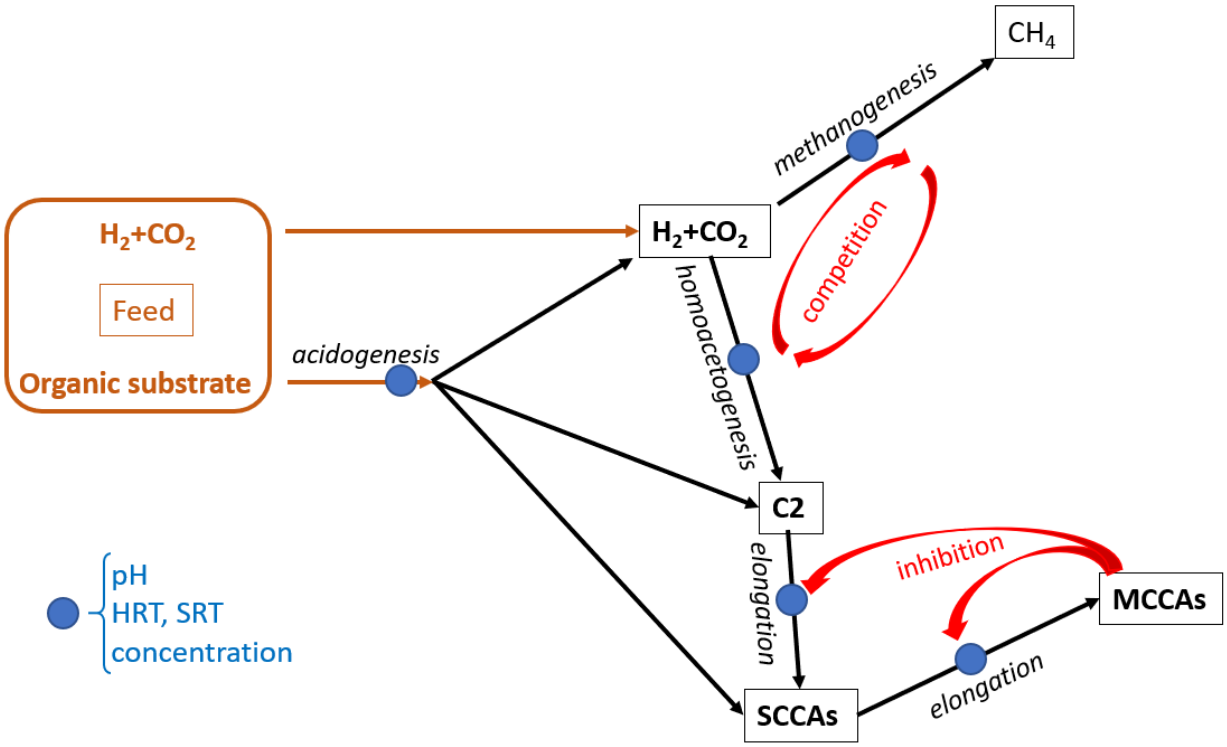

Fig 21: Scheme of the MCCA production process from a COF and H_2+CO_2, with the main parameters of influence.

7.3.3.1 Hypothesis 1: Depletion of SCCA substrates

The results of the semi-continuous fermentations (Fig 18-19-20.e,f) suggest that, when SCCA concentrations fall below 5 g_{COD}/l, the MCCA production stops. None of all the fermentations conducted contradicts this hypothesis. So, the MCCA production interruption could be attributed to a depletion of SCCAs. This hypothesis would be in line with the findings of several studies which concluded that the elongation process stop is the result of a lack of essential metabolites (De Groof et al. 2019; San-Valero et al. 2019) (see section 2.3.2). Those missing metabolites would correspond, in our case, to SCCAs, the electron and C acceptors.

7.3.3.2 Hypothesis 2: MCCA toxicity

In Exp 2 and Exp 3, the switch to a fed-batch or a semi-continuous feeding regime (see sections 5.3.2 & 5.3.3) involved a dilution of MCCAs. Since MCCAs are toxic (see section 2.3.2), their lower concentration resulting from the dilution could explain the restart or continuation of MCCA production in Exp 2 and 3. By contrast, the control fermentations - whose MCCAs were not diluted by feeding - did not restart to produce MCCAs after the MCCA plateau, supporting the toxicity hypothesis. Furthermore, the no-restart of MCCA production following the feeding in some fermenters of Exp 2 could be explained by the possibility that the elongating

microorganisms were already too inhibited by MCCAs when the new feeding regime was applied (plateau already reached).

The bioreactors that moved to the H_2O-fed semi-continuous mode go in the direction of this toxicity hypothesis. Indeed, MCCAs continued to be produced after a period longer than the HRT. This suggests that the MCCA production stop was not attributed to a depletion of supplied organic substrate, but rather to the MCCA toxicity.

Exp 2 and Exp 3 could thus indicate that the MCCA accumulation up to 15.1-19.3 g_{COD}/l leads to a stop of MCCA production, because of the MCCA toxicity.

7.3.3.3 Hypothesis 3: Competition by methanogenesis

In the semi-continuous feeding regime (Exp 3), in the AML-fed fermentations, the appearance of methanogenesis resulted in a progressive slowdown of MCCA production, leading to a complete stop. In some reactors in batch mode, methanogenesis operated along with MCCA production (e.g., R19, 20, 21 Exp 1 (Fig 10)). Those reactors presented quite lower MCCA productions than reactors with no methanogenesis.

Overall, these observations confirm that, in any system, when methanogenesis operates, it competes with the elongation process (see section 2.2.2). Moreover, this suggests that methanogenesis ends up totally preventing the elongation process in semi-continuous mode, whereas both processes could continuously and simultaneously operate in batch.

7.3.3.4 Synthetic interpretation of the causes of the interruption of MCCA production

In summary, the results point out three likely causes explaining the MCCA production stops encountered in these experiments. The MCCA toxicity seems to be the main cause of the interruption of MCCA production in batch, while the SCCA depletion seems to be the main cause in semi-continuous mode. Finally, methanogenesis seems, in any system, to push towards a MCCA production slowdown or stop. As represented in Fig 21, these causes could function lonely as well as in combination.

7.4 Maintaining a continuous MCCA production

7.4.1 Preventing the interruption of MCCA production

The results indicate that a continuous MCCA production can be obtained in a semi-continuous system. To reach that, SCCA concentrations should remain over 5 g_{COD}/l. The feedings with BSG and AML are capable to provide this minimal concentration with a HRT of 7 days. For the NOF (Fig 18), a longer HRT could allow to maintain this minimal SCCA concentration. More investigation could confirm this assumption and determine the minimal HRT required.

However, it must be emphasized that NOF fermentations anyway give lower yields than fermentations of an organic feedstock.

In parallel to the minimal SCCA concentration, the MCCAs should remain below 15.1 g_{COD}/l. To not exceed this maximal concentration, an appropriate combination of substrate concentration, substrate digestibility and retention time (RT) should be implemented.

Additionally, to maintain a continuous MCCA production, methanogenesis should be avoided. The H_2O (NOF) and BSG-fed semi-continuous systems indicate that a HRT of 7 days is short enough for that, in accordance with literature. In effect, researchers reported that methanogenic archea need a strictly minimal RT of 10 days in the pH range 6.0-7.0 to be able to survive (Taconi et al. 2008). By contrast, the AML-fed semi-continuous conditions seem to indicate that a HRT of 7 days is not sufficient to prevent the methanogenic archea development. This unexpected observation can be a consequence of an undesired long solid retention time (SRT) under the tested conditions. Large BSG solid particles (residues from the BSG used to start the fermentations) were segregated by the sampling tubes and remained in the reactor for longer than the liquid phase (SRT > HRT). Those particles may have served as carrier material hosting methanogenic archea. The latter could then stay and develop in the reactor, despite the short HRT, and produce CH_4, at the expense of SCCAs and MCCAs. Thus, ensuring that not only the HRT, but also the SRT, are shorter than 10 days could completely prevent methanogenesis, by effectively washing the methanogenic archea out of the reactor. To ensure the required SRT, the BSG should be thinly grinded before being introduced into the bioreactors.

7.4.2 Promoting the continuity of MCCA production

7.4.2.1 Supply of H_2+CO_2 & homoacetogenesis

The consumptions of H_2 and of CO_2 seem to be mutually linked (molar ratios H_2/CO_2 between 2.0 and 5.7, depending on conditions and time). The production of acetate seems also to be correlated to the consumptions of H_2 and CO_2 in some cases (e.g., Fig 12, 13.c,f). In the H_2O-fed reactors of Exp 2 (Fig 13), between day 39 and day 56, the consumed H_2/CO_2 molar ratio was close to 2.0 mols/mol, and the molar ratio (consumed_CO_2/produced_C2) was close to 1.6 mols/mol. Considering that some C2 can simultaneously be consumed (methanogenesis, elongation) these ratios are close to the ratios expected for homoacetogenesis:

$4\ H_2 + 2\ CO_2 \longleftrightarrow CH_3COOH + 2\ H_2O$ (see section 2.3.1.1)

Additionally, in most of the fermentation profiles, C4 and C6 productions happened simultaneously to C2 consumptions (e.g., Fig 14.d). This suggests that C2 promotes the elongation into MCCAs, in accordance with literature (San-Valero et al. 2019). This totally makes sense since acetate is integrated within the reversed β-oxidation pathway (see section 2.3.1.3).

Nevertheless, hydrogenotrophic methanogenesis consumes H_2 and CO_2 (see section 2.3.1.1) and competes with homoacetogenesis. As an illustration of this competition, elevated C2 productions were never obtained simultaneously with methanogenesis in the 3 experiments. So, to promote the MCCA production, especially under H_2+CO_2, measures to avoid methanogenesis and favor homoacetogenesis should be taken (e.g., SRT < 10 days).

7.4.2.2 Managing the pH

In line with literature (section 2.4.1), higher maximal MCCA concentrations were obtained, for our experiments, at pH 6.8 than at pH 6.0. This confirms that the elongation process happens best at neutral pH. This also corroborates the hypothesis of the microorganism inhibition by MCCAs. Indeed, the more acidic the pH, the more MCCAs are protonated and therefore toxic for elongating microorganisms (see section 2.3.2).

So, in a semi-continuous feeding regime - provided that the HRT and SRT are correctly conducted - a pH of 6.8 seems recommendable, in order to maximize the MCCA productivity.

8 Conclusion

The aim of this master's thesis was to investigate the influence of the substrate on the MCCA production and the causes of the interruption of the elongation into MCCAs process.

The two-step MCCA productions were obtained under several conditions, proving the reproducibility of this process, which was observed in previous experiments.

The fermentations performed with NOF produced MCCAs, demonstrating that an organic substrate (liquid or solid) is not required when supplying H_2 and CO_2 to the MMC. A concentration of 1.3 g_{COD_MCCAs}/l was reached at the end of experiment while the production was still going on, suggesting that higher concentrations can be obtained from only H_2 and CO_2. The MCCA production was however promoted by the supply of an additional organic substrate. Indeed, in batch mode, the best MCCA concentrations (19.3 g_{COD_MCCAs}/l) were obtained from BSG, followed by AML and then by NOF. This proves that fermentable organic substrates are the most suitable substrates in batch. On the other hand, in semi-continuous mode with a HRT of 7 days, the best yields (total concentration and productivity) were obtained with AML, then with BSG. The reason of this different ranking between the two systems most likely is that 7 days make a short HRT for fermentable organic substrates. This suggests that, in semi-continuous feeding regime with a higher HRT, fermentable organic substrates are also the most productive substrates.

The MCCA production was likely restrained by three main processes. The first process is the MCCA toxicity. In batch mode, the MCCA productions stopped at MCCA concentrations of around 15.1-19.3 g_{COD}/l. The feeding regime changes suggested that these interruptions were the result of the elongating microorganism inhibition by MCCAs. The second process is the SCCA substrate depletion. The stops of MCCA production in semi-continuous mode were associated to SCCA concentrations fallen below 5 g_{COD}/l. Since none of all the fermentations conducted contradicts this statement, this suggests that the elongation into MCCAs requires SCCA concentrations > 5 g_{COD}/l. The 3rd process is the competition by methanogenesis. It seems that this competition leads to a complete stop of MCCA production in semi-continuous mode, whereas methanogenesis and elongation can continuously and simultaneously operate in batch. The yields are anyway lower when there is methanogenesis.

Our experiments demonstrated that a continuous MCCA production can be ensured by a fermentation of BSG starting in batch mode, then shifted - before the MCCA inhibition level - to a semi-continuous system fed with either BSG or AML. With a HRT of 7 days, productivities of 0.93 $g_{COD\_MCCAs}/l_{ML}*d$ for AML and 0.45 $g_{COD\_MCCAs}/l_{ML}*d$ for BSG were obtained. Increasing the HRT would most probably allow the BSG-fed semi-continuous fermentations to reach productivities as high as the AML-fed. A higher HRT would also allow the H_2O-fed (NOF) fermentations to maintain a SCCA concentration > 5 g_{COD}/l and then ensure a continuous MCCA production. Further investigations would provide more certainty about these

assumptions. Besides, it must be noted that sufficiently small solid particles should be used as substrate to ensure the right management of the SRT and avoid methanogenesis.

Finally, our results indicated that homoacetogenesis and a pH of 6.8 enhance the MCCA production.

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Fermentation of a complex organic feedstock to medium chain carboxylates by a natural microbial consortium:

Investigation on the substrate influence and the process limitations

Submitted by Antoine de Halloy de Waulsort

The depletion of natural non-renewable resources pushes towards better using renewable bio-resources. Medium chain carboxylic acids (MCCAs, C6 to C10) are useful molecules so far produced by petro-chemistry. An opportunity to produce MCCAs from biowastes has recently been identified: bacteria can ferment organic matter into short chain carboxylic acids (SCCAs), then elongate them into MCCAs. In previous experiments in our team, MCCAs were produced in batch by a mixed microbial culture (MMC) from brewer's spent grain (BSG), used as complex organic feedstock. With the supply of a H_2+CO_2 gas mixture, the MCCAs were produced in two phases, and no plateau concentration was reached.

The aim of the present work was to know whether the two-step MCCA production is reproducible, when and why the MCCA production would stop, and how to avoid the end of the production. It was also to identify the influence of the substrate composition on the fermentation profile.

Batch fermentations supplied with H_2/CO_2 77/23 v/v at 1.3 bar and with a MMC as inoculum were performed with 2 different substrates: BSG and 4-day acidogenic mixed liquor (AML), in addition to fermentations with no organic feedstock (NOF) or, i.e., H_2O. These experiments allowed to reproduce the 2nd phase of MCCA production and to reach a 2nd plateau at 15.1-19.3 g_{COD_MCCAs}/l after about 30 days. The highest MCCA concentration was 19.3 g_{COD}/l. All conditions tested produced MCCAs, in descending order of production: BSG, AML, NOF.

After performing the fermentations under batch mode, the switch to fed-batch and semi-continuous modes at different times of fermentation was tested. The semi-continuous fermentations (HRT of 7 days) of BSG and AML presented a continuous MCCA production, the production from AML being larger. The H_2O-fed (NOF) semi-continuous fermentation stopped producing MCCAs when the SCCA concentration fell below 5 g_{COD}/l.

The results demonstrated that the two-step MCCA production is reproducible and stops when the MCCAs reach concentrations up to 15.1-19.3 g_{COD}/l, which may correspond to the inhibitory concentration for microorganisms. Passing to a semi-continuous system could allow to keep the MCCAs ongoing under this concentration. Continuous MCCA productions were observed as long as SCCAs remained > 5 g_{COD}/l. Mean production rates of 0.93 and 0.45 $kg_{COD\_MCCAs}/(m^3_{ML}*d)$ were obtained with AML and BSG, respectively.

UNIVERSITÉ CATHOLIQUE DE LOUVAIN
Faculté des bioingénieurs
Croix du Sud, 2 bte L7.05.01, 1348 Louvain-la-Neuve, Belgique | **www.uclouvain.be/agro**