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Bio-electro-elongation of small and medium chain fatty acids

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I. ABBREVIATION LIST

C2	Acetic acid
C3	Propionic acid
iC4	Isobutyric acid
C4	Butyric acid
iC5	Isovaleric acid
C5	Valeric acid
C6	Caproic acid
C7	Heptanoic acid
C8	Caprylic acid
C9	Nonanoic aci
C10	Decanoic acid
CoA	Coenzyme A
EF	Electro fermenter
EtOH	Ethanol
H2A, H2B	Partial pressure reactor pressurised with hydrogen
N2A, N2B	Partial pressure reactor pressurised with nitrogen
S-MCFAs	Short and medium chain fatty acid

II. LIST OF FIGURES

1 INTRODUCTION

The end of the 20th century and the 21st century have raised the greatest challenge humanity has ever been confronted to : climate change. Since our fossil-carbon-based economy is responsible for major parts of the greenhouse gases emissions, dramatic changes have to be made in our societies.

Nevertheless, fossil-oil is still the main energy source for transportation (demand will keep growing at least until 2040 ("OPEC's 2015 World Oil Outlook » Peak Oil Barrel," n.d.) and the basis for almost the whole chemical industry providing synthetic polymers, medicines, food additives, etc. This intensive use of fossil-oil releases enormous amounts of greenhouses gases and other pollutants in the atmosphere. Replacing oil by a sustainable alternative is therefore a major concern.

Solutions are however massively investigated among the scientific community. Concepts like green chemistry and biorefineries try to replace fossil-oil-based fuels and chemicals by bio-based alternatives. In a biorefinery, biomass will first be broken down and processed by chemical and biological means into elementary building blocks : the platform chemicals. These narrow range of chemicals will then be post-processed into numerous useful chemicals. Several types of platforms are already implemented in the industry, like the sugar platform for the production of bioethanol.

The carboxylates platform has recently gained interest. This platform includes short and medium chain carboxylates (S-MCFAs), with carbon chains from 2 to 10 carbons. They can be derived from waste streams, natural microbial communities can replace the expensive catalysts, and they can lead to the production high-value chemicals.

An interesting process the synthesis of S-MCFAs is an uncomplete anaerobic digestion, stopped before the methanogenesis in order to accumulate acetic acid. This acetic acid is then further metabolised along with an electron donor (e. g. ethanol) into longer S-MCFAs. The metabolic pathway which is suspected to lead to the elongation of S-MCFAs is β- reverse oxidation cycle. S-MCFAs production through β-reverse oxidation cycle have been reported with concentrations up to 8,6 $g.L^{-1}$ of caproate when ethanol and acetate are used as a feed (Yin et al., 2017) or 8,27 $g.L^{-1}$ (Agler et al., 2011).

In this study, an alternative cheaper source of reducing equivalents was investigated : electricity. The purpose was to obtain MCFAs through elongation by the means of a bio-electrochemical process and identify the key parameters influencing this elongation.

The second aim of this work was to investigate the suspected role of in situ synthesised molecular hydrogen coming from the water hydrolysis was also investigated by the means of molecular hydrogen pressurised reactors. This was done by the means of reactors pressurised with dihydrogen and dinitrogen for the control.

2 STATE OF THE ART

2.1 INTRODUCTION

In the global context of climate change and fossil resources depletion, significant efforts have been made towards sustainability, especially in the industrialised countries. However, humanity still depends greatly on fossil resources and sustainable solutions struggle to replace them. The two major challenges that humanity has to overcome in order to eliminate its dependence to fossil resources are the production of energy on one side and the production of useful chemicals on the other.

Concerning sustainable energy sources for the production of electricity, numerous solutions have been found, the most well-known being solar panels, wind energy and geothermal energy. However, these three sustainable energy sources taken together only account for 2% of the worldwide electricity production, while coal, the most polluting electricity production technology, accounts for 40% of the worldwide electricity production (Evans et al., 2009).

The situation is even worse in the transportation sector. While fossil fuel accounts only for 7% of the worldwide electricity production (Evans et al., 2009), it is the major energy source for all types of transportations (Adbul-Hamid, O, et al.)[1]. Transportation is the most demanding sector in terms of fossil fuel and has also the highest demand growth. It is estimated that fossil fuels demand for transportation will keep increasing at least until 2040, mostly in the developing countries. (Adbul-Hamid, O, et al.)

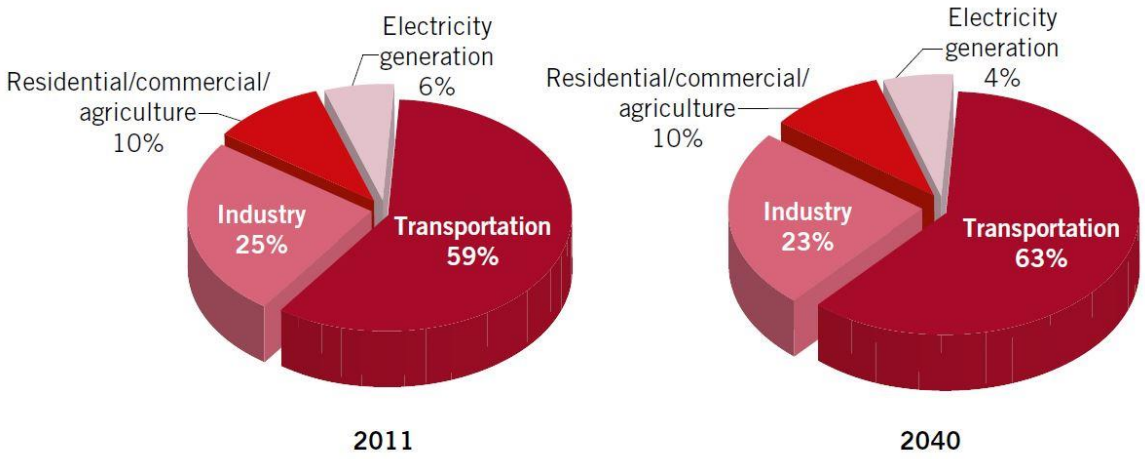

Figure 2-1 : Fossil oil demand for each sector in 2011 and 2040 (forecast).

[1] Adbul-Hamid O., Tapia Solis O., Ghanimi Fard H., Odulaja A., Hamel M., 2014. World Oil Outlook 2014.

Nevertheless, while alternatives for the sustainable production of energy or transportation do already exist and are being implemented all around the world, until recently, no large scale solution seems to be found for the replacement of oil based chemicals.

At the end of the 90's a new concept emerged in chemistry in order to combine scientific work towards a goal of sustainability : Green Chemistry. Green chemistry can be defined as : "The utilisation of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture and application of chemical products." (Anastas and Warner, 2000). Green chemistry could answer both challenges : the production of a renewable fuel for transportation and the sustainable production of chemicals, replacing the petrochemical derivates.

2.2 BIOMASS

Recently, a new renewable source of energy gained attention : Biomass. While the IUPAC defines biomass as "material produced by the growth of microorganisms, plants, fungi or animals" (Nagel et al., 1992), when used in the industry, the term biomass refers to biological material emerging from renewable sources, thus excluding fossil fuels for example. In its environmental policy, EU states that the term biomass when used in this context, refers to organic materials such as trees, plants, agricultural and urban waste ("Biomass - Energy - European Commission," n.d.).

In this context, biomass has been the first energy source ever used by humans through the combustion of wood. While it still is the main source of energy for domestic use worldwide, especially in developing countries, developed countries are reconsidering the combustion of wood and wood industry waste for the production of electricity. In 2010, 49% of the share of energy from renewable sources in the EU gross final energy consumption were produced by wood and wood waste combustion. It is therefore presented as being a key factor to reach the 2020 target of 20% of renewable energy. ("Biomass - Energy - European Commission," n.d.)

However, wood combustion is not the only biomass energy source exploited and other types of bio-based energy sources have also been used. Among them, a widely spread technology is biogas production, or biomethanisation. Although it was already discovered for several decades, this technology has gained major interest when oil prices were skyrocketing in the 1970's making the research for alternative energy sources more profitable. (Bond and Templeton, 2011)

Biomethanisation is an industrial process that uses anaerobic digestion biomass to produce a fuel gas composed of methane and CO_2 which can be turned into energy by combustion.

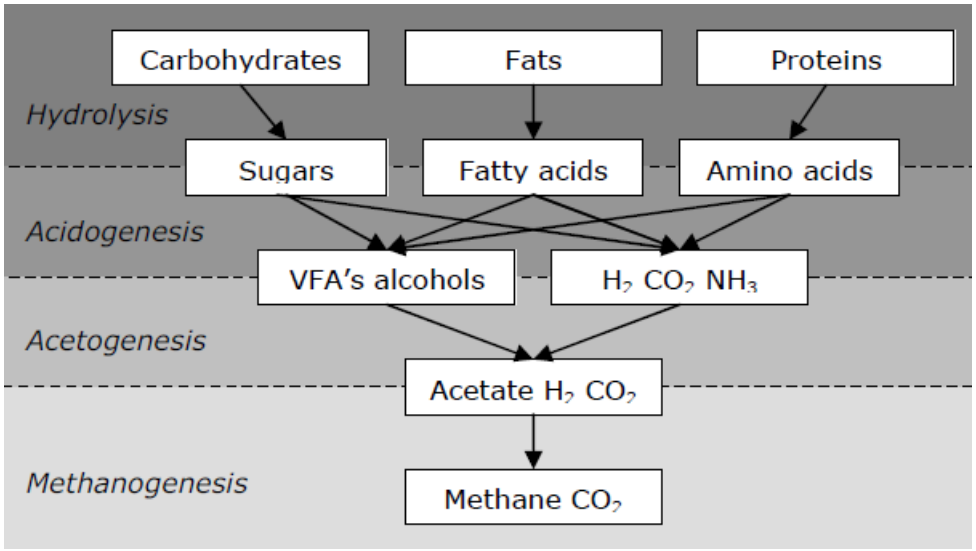

Figure 2-2 :Schematic view of the anaerobic digestion process. (Steinbusch, 2010)

Anaerobic digestion itself is a microbial multistage process when biomass gets degraded by microorganisms in anaerobic conditions summarised on figure 3-2.

During this anaerobic digestion, the complex polymers contained in the biomass are first broken down by the means of enzymes produced by the microorganisms into smaller mono- and oligomers in the first phase called hydrolysis. The second phase is the first proper fermentation phase where the monomers are metabolised by the anaerobic bacteria into VFAs, alcohols, H2, CO2 and NH3. This second phase is called acidogenesis. The third phase, called acetogenesis, is the further braking down of the previously produced mixture of acids and alcohol into acetic acid, H2 and CO2. Finally, methanogens, which are exclusively archaebacteria, convert the biomass into its final state : methane and CO2. (Bond and Templeton, 2011; Steinbusch, 2010)

While the integration of anaerobic digestion into a biogas producing facility may result in the sustainable production of electricity, methanogenesis can also have detrimental effect. Spontaneous methanogenesis occurring in natural environments, in animal digestion processes or in landfills for example is responsible for the yearly release of 590 to 800 million tons of methane, a powerful GHG, into the atmosphere (Bond and Templeton, 2011).

Once the biogas is produced, it can either be directly burned to produce energy, it can be purified and upgraded into biomethane (>90% of methane), and fed into the gas supply network (Bond and Templeton, 2011) or even compressed and used as an alternative transportation fuel (Rotunno et al., 2017).

However, biomethane, due to its low energy density and economical value is still far from being the perfect alternative for fossil fuel-based transportation (Steinbusch, 2010) on one hand and does not answer at all the problem of alternative sources for chemicals on the other hand. (Reddy et al., 2017)

However anaerobic digestion seems to have more potential than the degradation of biomass towards its final products...

2.3 BIOREFINERIES AND PLATFORM CHEMICALS

As said before, the replacement of fossil oil presents two major challenges : the replacement as an energy source, especially for transportation, and the replacement as a source for petroleum-based chemicals. Recently another concept closely linked to biomass has been defined : biorefineries. Biorefineries are defined as “facilities that integrate biomass conversion processes and equipment to produce fuels, power and value-added chemicals from biomass” (Demirbas, 2010) The major advantage of biorefineries is that they apply the concepts of the petrochemical industry on sustainable biomass. The aim is, just like in the petroleum refineries, to separate a complex stream of biomass into several valuable fluxes. These different fluxes can than either be directly used as fuel for the production of energy, or can be transformed into a other chemicals, known as platform chemicals. Platform chemicals are another concept brought from the petrochemical industry. The aim is to produce out of the complex biomass a narrow group of similar, small molecules, which can then serve as the building blocks for the production of more complex chemicals as presented on figure (Cherubini, 2010).

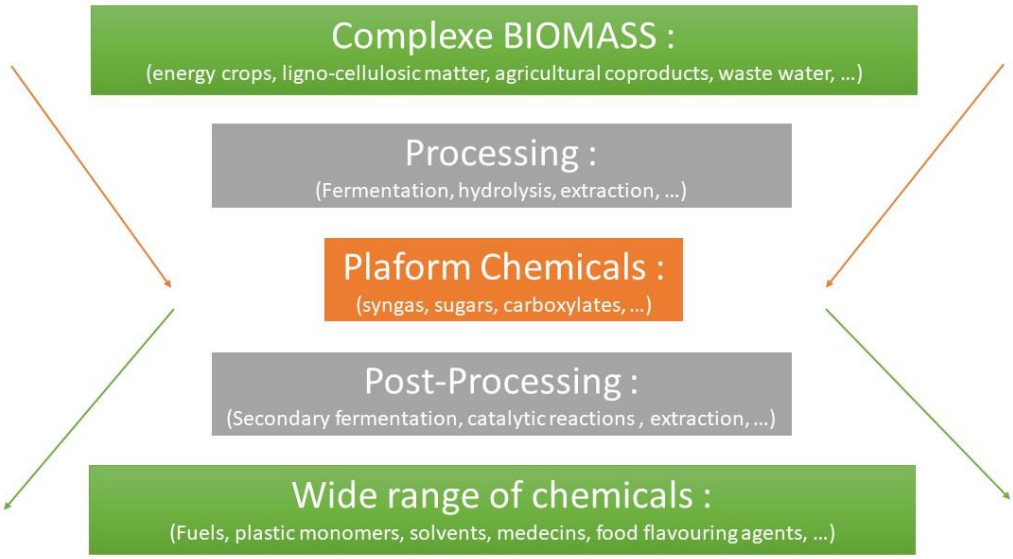

Figure 2-3 : Schematic view of the platform chemicals processes

One of the first platforms to be explored and implemented on a large scale is the sugar platform which is based on the fermentation of sugars mainly extracted from sugar and starch cultures into ethanol. After distillation of the ethanol is the mixed with conventional fuel for the transportation sector. Bioethanol and sort like fuels are called first generation biofuels. However, first generation biofuels rise ethical, political and environmental issues due to their competition with food producing crops. Second generation biofuels however solve this problem by only using raw materials that are not in competition with food production like waste residues or non-food crops biomass. (Cherubini, 2010)

The second platform that was intensely studied in the syngas platform. Syngas is a fuel gas mix of CO, H_2 and other gases. It is already well-known in the petrochemical industry where it can be produce through a process called “steam reforming”. It can either be burned for the direct production of energy or used to produce more valuable chemicals like fuels through the Fisher-Tropsch synthesis (Wilhelm et al., 2001). Syngas is also a product of the pyrolysis or gasification were biomass in heated up to high temperatures in the absence of oxygen. This process is mainly applied on ligno-cellulosic biomass in bio-refineries. (Kamm, 2007)

2.4 THE CARBOXYLATE PLATFORM

The carboxylate platform was the third platform to be studied and will be presented more in detail.

2.4.1 Carboxylates

Carboxylates, or carboxylic acids, are organic acids characterized by the presence of at least one carboxyl group. The carboxylates which are of specific interest as platform chemicals are the small and medium chain fatty acids (S-MCFAs) with an even number of carbons. Hence, acetic acid (C2), butyric acid (C4), caproic acid (C6), caprylic acid (C8) and decanoic acid will be presented in the following section.

2.4.1.1 Acetic acid

Acetic acid, or ethanoic acid, (C2) is a two carbons chain carboxylic acid, and is the shortest studied S-MCFA.

It’s market value ranges between 1200 and 1600 $/t with a global demand which will reach 18 million tons by 2020 (Pal and Nayak, 2017). Its uses vary from food preservation and flavouring (as vinegar), to intermediate ingredient for a variety of commercial grade chemicals or as an organic solvent for the production of terephthalic acid, the raw material for

polyethylene terephthalate (PET). But its main use is as raw material for the monomer vinyl acetate (Pal and Nayak, 2017; Yoneda et al., 2001).

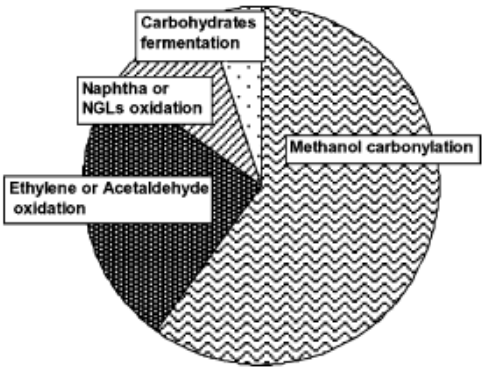

Figure 2-4 : Different industrial processes for the synthesis of acetic acid. Methanol carbonylation accounting 60%, Ethylene or Acetaldehyde oxidation 25%, Naphta or NGLs oxidation 10% and carbohydrates fermentation 5% for worldwide acetic acid production (source : Yoneda et al. 2001)

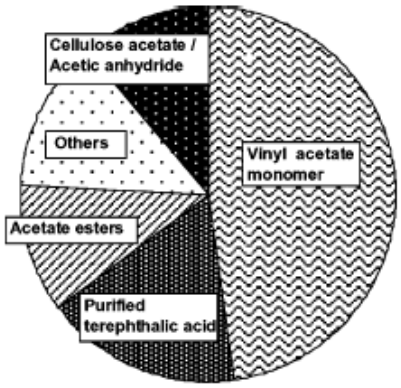

Figure 2-5 : Different industrial uses of acetic acid and their percentage regarding the worldwide acetic acid production. The use as a vinyl acetate monomer accounting for 47%, purified terephthalic acid for 17%, Acetate esthers for 12 %, other application for 14% and Cellulose acetate/acetic anhydride for 10%. (source : Yoneda et al. 2001)

While many different chemical routes are employed for the synthesis of acetic acid, the carbonylation of petroleum-derived methanol, is the most widely used process and accounts nowadays for 60% of the world's acetic acid producing capacity (see figure 1 below). This process was invented in the 1960's by Monsanto and improved by BP Chemicals in 1996, who renamed it the Cativa process, with the use of an iridium catalyst instead of the rhodium catalyst initially used by Monsanto. (Jones, 2000) Ethylene, acetaldehyde, naphtha and even natural gas liquids can also be oxidized into acetate but all these processes are also based on fossil oil and because of their lower productivity, are gradually abandoned for the Cativa process.

However, while they reach a very good yield, these oil-based processes still have major drawbacks like high energy consumption, the need for a huge chemical plant to perform the large amount of operations, the use of expensive catalysts, … These factors, along with the global goal of green chemistry, have mobilized a rising interest among scientist towards more sustainable ways of acetic acid production, mainly microbial fermentation. (Yoneda et al., 2001)

Several production pathways are being investigated or are already in use. For example, the food industry produces vinegar on a large scale through the process of submerged aerobic oxidative fermentation of ethanol by acetic acid bacteria, called the German method. While the yield of production of this process is high, the process still has a rather long time of reaction (approximately one week), making unsuitable for a massive production of acetic acid

for non-food applications. Nevertheless, majority of the other studied alternatives involve the use of carbohydrates or ethanol as feed and different types of microbial culture achieving different step of the fermentation process. While a lot of improvements and bacteria selection have already been made, these systems still encounter difficulties in terms of control of the mixed culture system, of product and substrate inhibition and of separation of the produced acetic acid. (Pal and Nayak, 2017).

2.4.2 Butyric acid

Butyric acid, or butanoic acid, is a four carbon chained carboxylic acid. Up to now, butyric acid is mainly produced through a process of oxidation of butyraldehyde, which is obtained from propylene, which is itself derived from crude oil. It is widely used in the chemical, food and pharmaceutical industries. It is also used in the manufacturing of emulsifiers, disinfectants and esters. It is used in the form of pure acid in food flavours, as precursor of butyric ether, an additive used to increase fruit fragrance, and as aromatic compounds in perfumes (Brar et al., 2016). But the main application of butyric acid is the manufacture of cellulose acetate butyrate plastics which is used for textile fibre production.

However, besides the many current uses in the chemical industry, the energy sector could become even more important as butyric acid is one of the most studied precursor for biofuel, either in the form of *n*-butyl either in the form of butanol or even butane (Dwidar et al., 2012). An alternative source to fuel based chemistry has however to be found for the sustainable production of butyrate in the first place. Nevertheless, the increasing demand for bio-based butyrate didn't came first from the green-energy industry but from the pharmaceutical and food industry which wanted non-oil-derived butyrate and derivates for their products. Hence, fermentation pathways were studied and more than 10 different organisms were found being capable of butyrate synthesis with a good yield, mostly with glucose as a feed as presented in the following reaction (Zhang et al., 2009).

$$\text{Glucose} \longrightarrow 0{,}85\ \text{butyrate} + 0{,}4\ \text{acetate} + 2\ CO_2$$

2.4.3 Caproic acid

Caproic acid, or hexanoic acid, is a 6 carbon saturated carboxylic acid and is therefore the shortest of the medium chain fatty acids.

It can be synthesised through several petrochemical ways like oxidation of hexanal, direct oxidation of hydrocarbons or carbonylation of ethylene with carbon monoxide and water (Wasewar and Shende, 2011). But even if these processes have high conversion yields, they

are generally not preferred because of their high operational costs. Hence, caproic acid is used on a small scale as a precursor for high value chemicals or as a food additive. To answer this small demand for caproic acid, extractions from plant and animal oils are sufficient. However, caproic acid, along with the other medium chain FA rise interest since they would be the perfect precursors for future biofuel production (Angenent et al., 2016).

The current market value of caproic acid ranges between 4100 and 4900$/ton

2.4.4 Caprylic acid

Caprylic acid, or octanoic acid, is an 8 carbon saturated carboxylic acid. It is very similar to caproic acid in its production and uses. Currently, the main source of caprylic acid are palm and coconut oil out of which the caprylic acid is extracted to be used mainly in the organic cosmetic and pharmaceutical industry. Recently, interest for caprylic acid has been growing because of the increasing demand for natural personal care products. This interest will probably keep on growing since caprylic acid is one of the most promising bio-fuel precursors, along with C6 and C4 and can be produced by fermentation. Due to their higher hydrophobicity, the longer MCFAs can be easily separated from water (in opposition to bio-ethanol which needs an energy intensive distillation). Another major advantage of these medium chain FA is their higher energy density compared to shorter FA, due to their lower oxygen/carbon ratio.(Van Eerten-Jansen et al., 2013).

2.5 S-MCFAs POST TREATMENTS

Once produced, the S-MCFAs can undergo several types of post-treatments to give them better fuel and chemical properties.

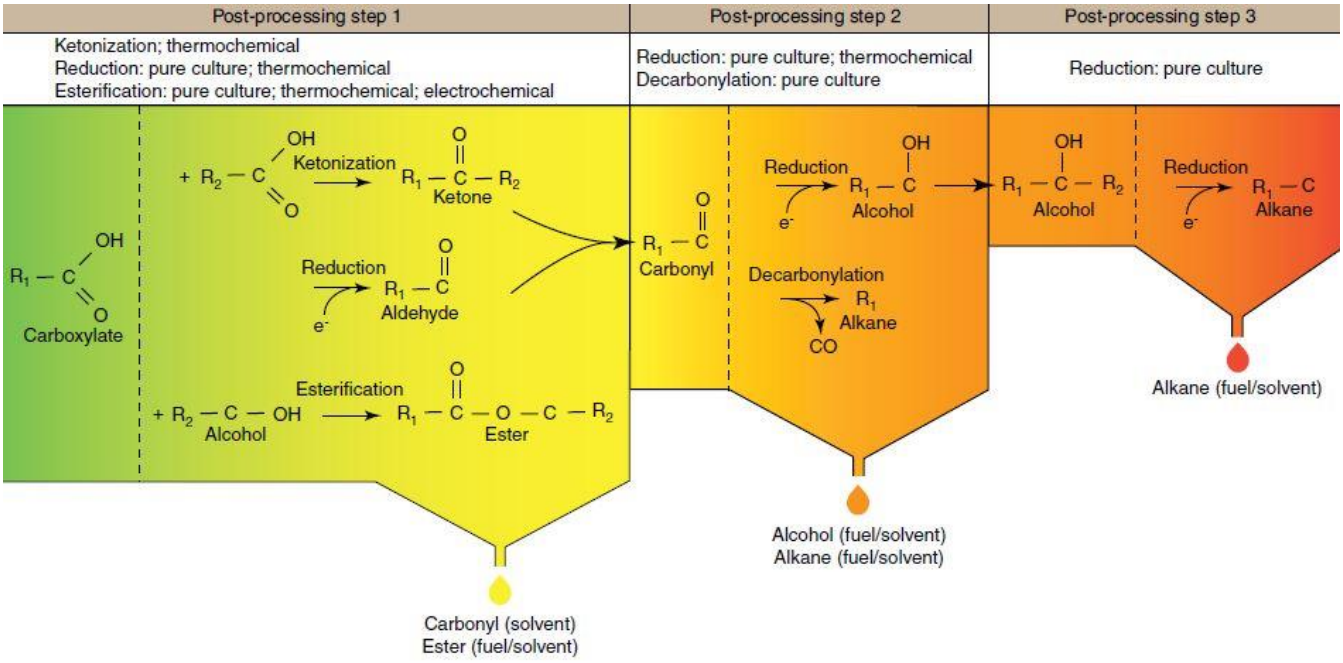

Figure 2-6 :Summary of the chemical, thermochemical, electrical and biological post treatment processes for carboxylates. (Agler et al., 2011)

Figure 6 shows different steps for the production of a valuable fuel or a solvent starting from a S-MCFA. These operations can be performed by chemical, thermochemical, electrochemical and biological ways. Post processing can also be either achieved within the same fermentation broth as the S-MCFAs synthesis or once the S-MCFAs are separated from the broth. The first option could be interesting especially for the conversion of S-MCFAs into their respective alcohol and afterwards into their respective alkane through biological reduction when, for instance, undefined mixed cultures are used for the valorisation of complex waste streams. However incompatibility between optimal conditions for both processes make this type of design unsuitable for industrial scale-up. Hence, post treatments of synthesised carboxylates will surely come after an extraction phase, making the hydrophobicity of the carboxylates a key parameter since extraction will be easier with longer S-MCFAs. (Agler et al., 2011)

When the purpose is to use S-MCFAs as fuel precursors, the longer the chain is, the more energy the fuel will carry (as shown on figure 7), thus increasing the value of the fuel. However, even with longer MCFAs, a post treatment is still needed before the chemical can be used as fuel in order to increase even more its energetic density. When a S-MCFA undergoes the post-treatment operations presented on figure 6, its energy content increases at each stage as presented in table 1 where butyric acid was taken as an example.

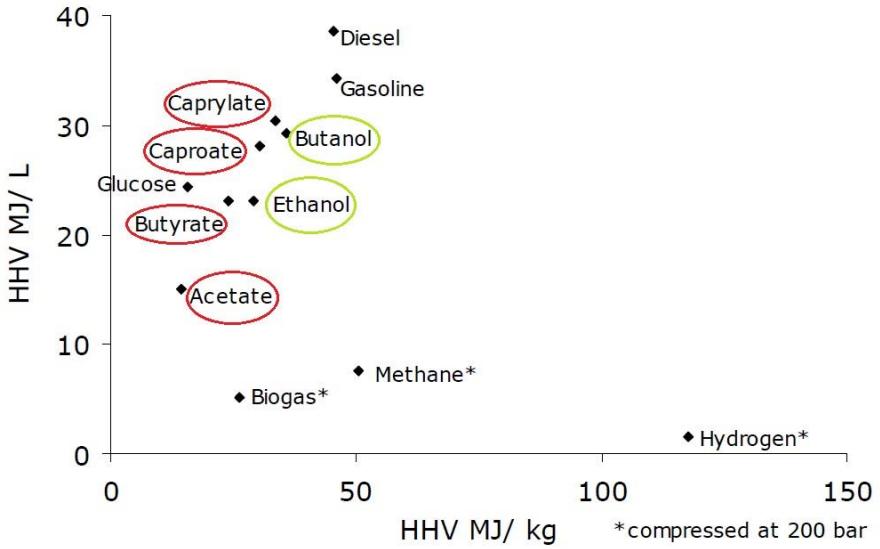

Figure 2-7 : Comparison of energy density by mass and by volume for S-MCFAs, ethanol, butanol, biogas and conventional fuel. (Steinbusch, 2010)

Table 2-1 : High Heating Value at each step of the post processing of butyric acid towards butane.

	Butyric acid	Butanone/Butanal	*n*-Butanol	Butane
High heating value (MJ/kg)	24,78	33,89/34,25	36,1	49,4

The fact that the energy increases with the carbon chain length for a S-MCFA and increases with the post-processing operations presented in figure 6 can be explained by a higher C/O ratio, resulting in a higher energy release during the combustion. (Steinbusch, 2010)

In addition, an important feature of S-MCFAs whether they are used as precursors for fuels or other chemicals, is that their hydrophobicity increases with the chain length of the carboxylate. Hence separation processes from water will be easier and cheaper. (Agler et al., 2012)

2.6 SHORT AND MEDIUM-CHAIN FATTY ACIDS ELONGATION

As S-MCFAs are an interesting alternative of precursors for both bio-based fuels and chemicals and that longer carbon chains are easier to extract and have higher energy content, a process has quickly gained interest : the elongation of S-MCFAs. To produce a S-MCFA through S-MCFAs elongation, a carbon and an reducing equivalent source is needed. Methanogenesis also needs to be inhibited to prevent the anaerobic digestion to reach its final state : the production of methane, thus competing with the targeted process.

2.6.1 The carbon source

The carbon source is a critical factor when a sustainable process is targeted. Many studies focusing on the production of S-MCFAs, and especially the elongation of S-MCFAs, were working on the use of waste (mostly wastewater) as a carbon source (Agler et al., 2011; Steinbusch, 2010; Van Eerten-Jansen et al., 2013). Since waste contains carbon under many different forms, it first needs to be transformed in order to produce one particular type of chemical that can then be further used as a carbon source. Just like in the biomethanization process used to convert waste biomass into energy, the anaerobic digestion (figure 2) occurring during biomethanisation can be used to produce the building block for S-MCFAs elongation : acetic acid. Acetic acid is indeed produced during the penultimate step of the anaerobic digestion process : acetogenesis. If the ultimate stage of the anaerobic digestion (methanogenesis) could be avoided, large amounts of acetic acid could be produced and accumulated in the waste stream (Steinbusch, 2010).

2.6.2 Methanogenesis inhibition

Methane needs to be avoided at two stages of the process : during the anaerobic digestion of the biomass to ensure the acetic acid accumulation and during the elongation process to prevent the competition between methanogenesis and the targeted process.

Several methods to avoid methanogenesis during a S-MCFAs production experiment are already in use, ranging from the simple addition of chemical inhibition agents, to the pH lowering and thermal choc.

2-bromoethanesulfonic acid (BESA) is a well-known specific methanogenesis inhibitor due to its targeted competitive inhibition of a key enzyme of the terminal phase of the methanogenesis cycle. It is a very effective methanogenesis inhibitor and has been used for several S-MCFs elongation experiments (Liu et al., 2011). When compared to non-inhibited fermenters, the fermenters containing BESA were found to have produce significantly more

S-MCFAs compared to non-inhibited reactors (Liu et al., 2011). However, while the use of these methanogenesis inhibitors may work on lab scale, they do not eliminate the methanogens and the levels of chemical inhibitors drop with time. Hence, other ways have to be investigated to prevent the production of methane (J. Steinbusch et al., 2011).

Methanogenesis can also be prevented by controlling the pH and keeping it low enough (pH< 5) to inhibit methanogens (Kim et al., 2004). This can be a cheap way to effectively control methanogens without reducing the S-MCFA production rates. An n-caproate rate of production of 3,38 g/l per day could be obtained while methane production was significantly limited in an experimental system where the pH was controlled at a value of 5,5 as sole methanogenesis inhibitor. However, the methanogens are still present in the medium and the risk still exists that methanogenesis will start as pH may vary during the fermentation (Ge et al., 2015).

Another way to avoid methanogenesis would be a pre-treatment of the mixed culture at higher temperatures. Since all the methanogens are archaea, none of them are capable of sporulating. By heating up the media for a certain period of time, the mesophilic methanogens are eliminated while the other, sporulating organisms like *Clostridium kluyveri* were kept. However, heating the fermentation broth may be problematic for the scaling up of the technique in a continuous process (J. Steinbusch et al., 2011).

Finally, a very interesting way to block methanogenesis is by the means of MCFAs. While the role of SCFA in methane production inhibition is still controversial, caprylate can inhibit methanogenesis to some extent (Koster and Cramer, 1987). This could be very interesting for S-MCFAs elongation since caproate and caprylate could be the targeted product and facilitate the production by inhibition of the methanogenesis at the same time meaning that the product produced by β-reverse oxidation could be used directly in the acetic acid production process. (Angenent et al., 2016)

2.6.3 The S-MCFA elongation pathway

The formation of butyrate and caproate through mixed culture anaerobic fermentation of ethanol combined or not with acetate was already described in the XIXth century. Further specific research with *Clostridium kluyveri* enlightened the biological pathway through which S-MCFA are elongated : the β-reverse oxidation cycle. (Angenent et al., 2016)

Beta reverse oxidation is a cyclic metabolic pathway where an acetyl-coA, derived from the oxidation of an ethanol molecule, is added to a carboxylate thus increasing its carbon number by 2 (figure 3-8). As an overall balance, for the β-reverse oxidation cycle, with 4 acetate and 6 ethanol molecules, 5 molecules of n-butyrate and 5 molecules of water are produced. Moreover, 2,5 Adenosine triphosphate (ATP) units are converted from 2,5 Adenosine diphosphate (ADP) molecules by proton pumping ATP synthase complexes, counting as an energy production. Finally, the redox coenzyme NAD^+/NADH is overall balanced since the same amount of NAD^+ and NADH + H^+ are consumed and produced respectively (Angenent et al., 2016).

Numerous types of bacteria can achieve β-reverse oxidation to generate ATP under anaerobic conditions : *Clostridium, Eubacterium, Megashphaera, Rhodospirillum*,… However, *Clostridium kluyveri* is the most studied micro-organism for this biological pathway both in pure and mixed culture. (Agler et al., 2011)

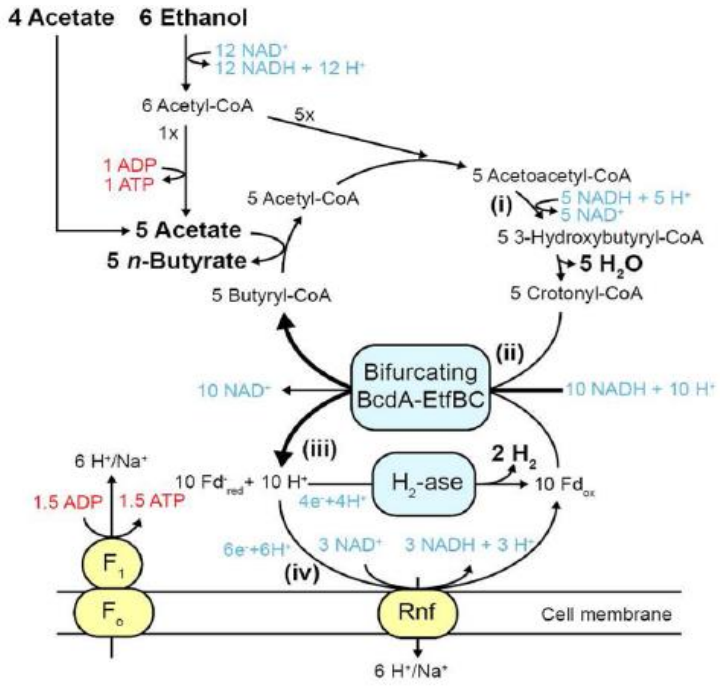

Figure 2-8 : β-reverse oxidation cycle with acetate and ethanol as substrate and n-Butyrate as a product. Redox-factors are highlighted in blue; Classical energy conservation in red; and more recent described mechanisms of energy conservation in yellow; roman numerals explained in main text. Abbreviations: BcdA-EtfBC = butyryl-CoA dehydrogenase-electron transferring flavoprotein complex; CoA = Coenzyme A; F_0F_1 = H_+/Na_+-pumping ATP synthase complex; Fd_{ox} = oxidized ferredoxin; Fd_{-red} = reduced ferredoxin; H_2-ase = hydrogenase; Nfn= NADH-dependent reduced ferredoxin:NADP oxidoreductase; Rnf = ferredoxin-NAD reductase complex.

It is suggested that elongation of longer MCFAs, occurs by the combination of acetyl-coA with butyryl-coA and caproyl-coA for the production of respectively caproic acid and caprylic acid (Reddy et al., 2017).

2.6.4 The reducing equivalent source

Along with a carbon source, the elongation process also needs a source of reducing equivalents. In most published scientific work, these reducing equivalents come from ethanol which is either added into the fermentation broth (Ge et al., 2015; J. Steinbusch et al., 2011), either initially present in the substrate stream (Angenent et al., 2016). Other reduced chemicals have also been reported as effective electron donors for the β-reverse oxidation cycle like lactate, sugars like glucose or fructose, methanol, propanol, amino acids in peptides, or polyols (Agler et al., 2011; Reddy et al., 2017). Along with providing reducing equivalents to the fermentation broth, the addition of these chemicals also furnishes an additional carbon source. However, when acetic acid is used as a carbon source, reducing equivalents need to be added.

2.6.4.1 Molecular hydrogen

Molecular hydrogen has been studied as an electron donor. However, during the assimilation of molecular hydrogen, microbial pathways are initiated which use ethanol as an intermediate chemical, not changing the next part of the process compared to situations where ethanol was added (Agler et al., 2011).

2.6.4.2 Electricity as an electron donor

Another alternative comes from the waste water treatment (WWT) research. Since the end of the XXth century, WWT research has been focusing on the production of energy through the treatment process. Similarly to the anaerobic digestion of biomass, waste water can be degraded in the absence of air into methane which can then be burned to produce energy. However, while these types of systems are already widely in use and effective, a new, potentially more effective energy production technology is being developed : Microbial Fuel Cells (MFCs). A MFC is a “bioreactor that converts chemical energy in the chemical bonds in organic compounds to electrical energy through catalytic reactions of microorganisms under anaerobic conditions” (Du et al., 2007). The electricity is produced by separating the microorganisms from the O2 and other electrons accepting molecules to force the electrons produced by the biomass degradation to be exchanged with the anode. In the cathodic compartment, the arriving electrons can then reduce O2 to complete the cycle. Both electrodes are connected via an electrical circuit where the electricity can be used.

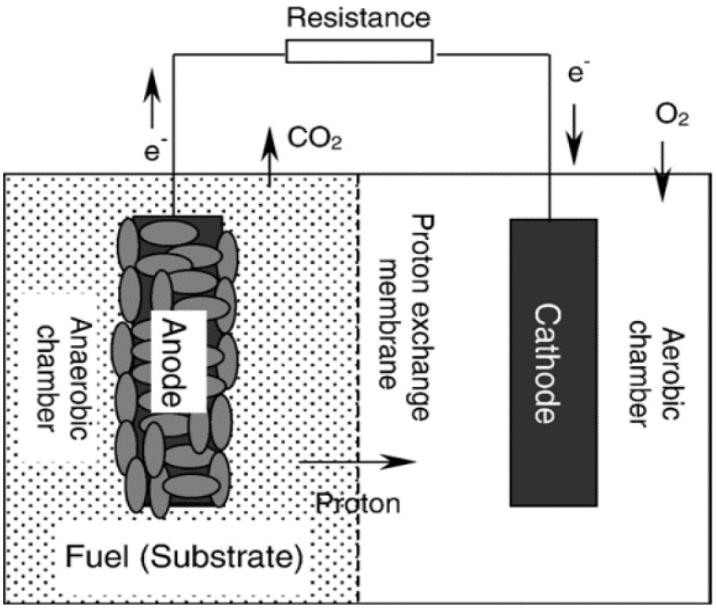

Figure 2-9 : Schematic view of the Electro-fermentation process where electricity is used to produce valuable chemicals out of biomass and its possible inputs and outputs. (Schievano et al., 2016)

However, power densities produced by these systems are still quite low making and MFC are hardly used in full scale installations yet. Recently, their application field was increased by using them in the opposite direction : current was applied to the system to promote bioreactions at the electrodes (Harnisch and Schröder, 2010). By having an anaerobic fermentation broth at the cathode instead of the anode, microorganisms are able to get electrons directly from the current, thus making MFC the perfect replicant for conventional electron donors (Schievano et al., 2016).

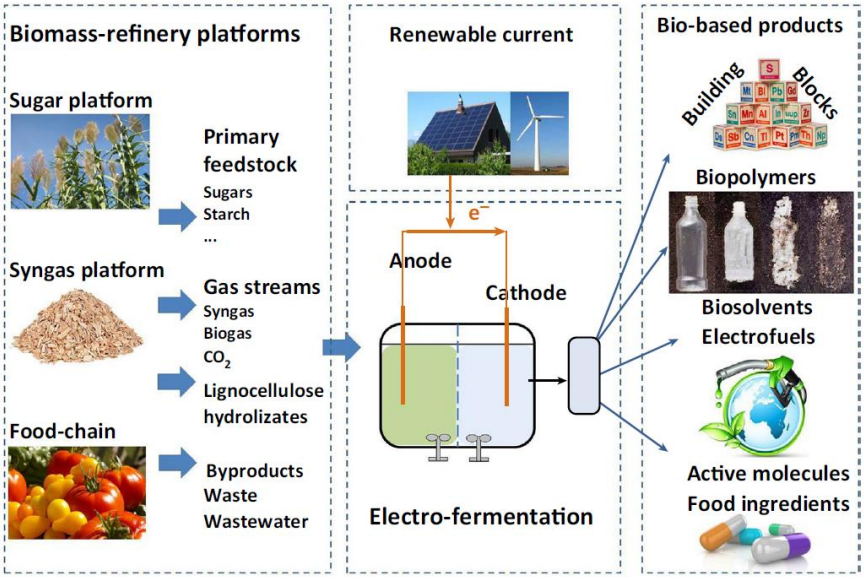

Figure 2-10 schematic view of the electro-fermentation process with its inputs and outputs. (Schievano et al., 2016)

2.6.4.3 Electron transfer mechanisms

Although the understanding of the mechanisms for extracellular electron transport in pure cultures has known some major advances at a single-cell level, the interactions between the mixed microorganisms or even the pure biofilms remain still largely unexplained. Key parameters like electrode surface, conduction among the different biofilm layers, influence of the distance to the electrode, … must still be studied (Harnisch and Rabaey, 2012). However, five possible mechanisms may explain the electron transfer from the electrode to microorganisms :

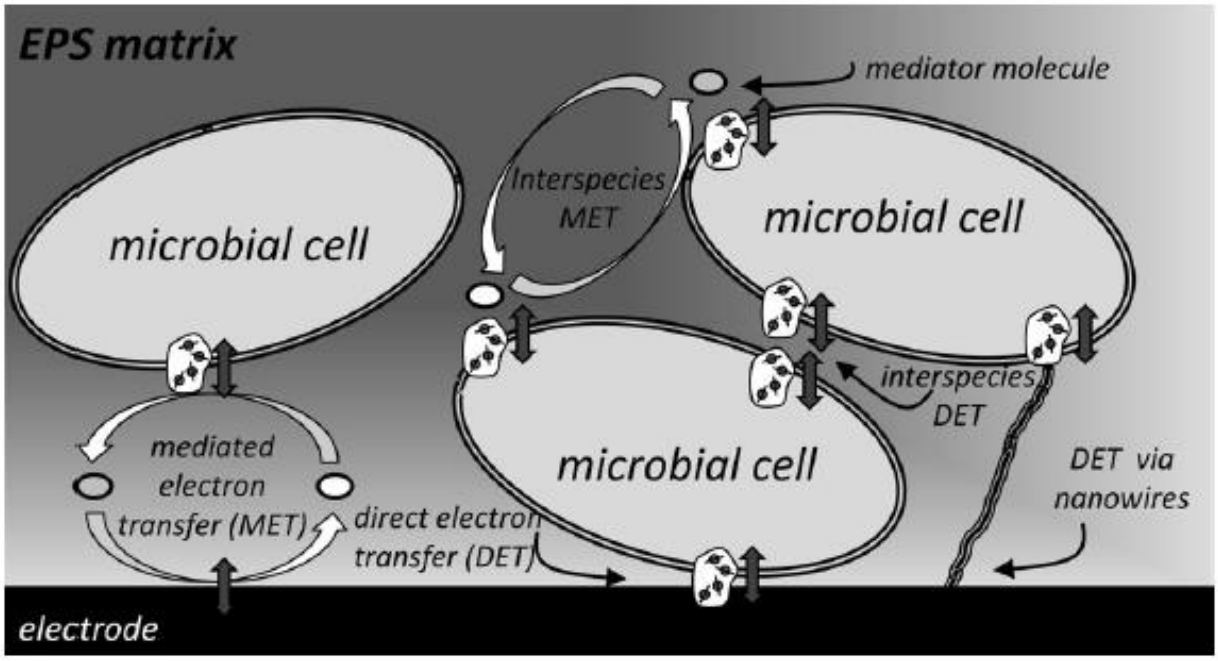

Figure 2-12 : Schematic view of the different potential electron transfer mechanisms. (Harnisch and Rabaey, 2012)

1) The transfer might be direct through the contact surface between the electrode and the microorganisms. This mechanism alone, however could not explain the electron transfer to the outer layers of the biofilm.
2) The electron could be transferred through an intermediate chemical which could be reduced the cathode and oxidized by the microorganism.
3) A specific case of this mechanism could be linked to the reduction of water into hydrogen at the cathode. This hydrogen could then be taken up by the bacteria as a source of electrons. This is the most likely mechanism of electron transfer. (Rabaey and Rozendal, 2010)
4) A pilus could also be the intermediate between the electrode and the microorganism.
5) Finally, in a community of microorganisms, several interactions could occur where only a particular type of microorganism in contact with the electrode takes up the electrons and transfers them to the rest of the biofilm microorganisms through a way given above.

2.7 PREVIOUS RESULTS OF BIO-ELECTRO ELONGATION OF S-MCFAS

A first experiment was led by Van Eerten-Jansen et al. in 2013 where plane electrodes, one in platinum coated titanium for the anode, one in graphite for the cathode where used. The inoculum was taken from a microbial culture already elongating S-MCFA up to C4-C8 and where Clostridium kluyveri was supposed to be predominant. Electric current was adapted to obtain a potential of -0,9 V vs normal hydrogen electrode (NHE) between the cathode and the reference electrode.

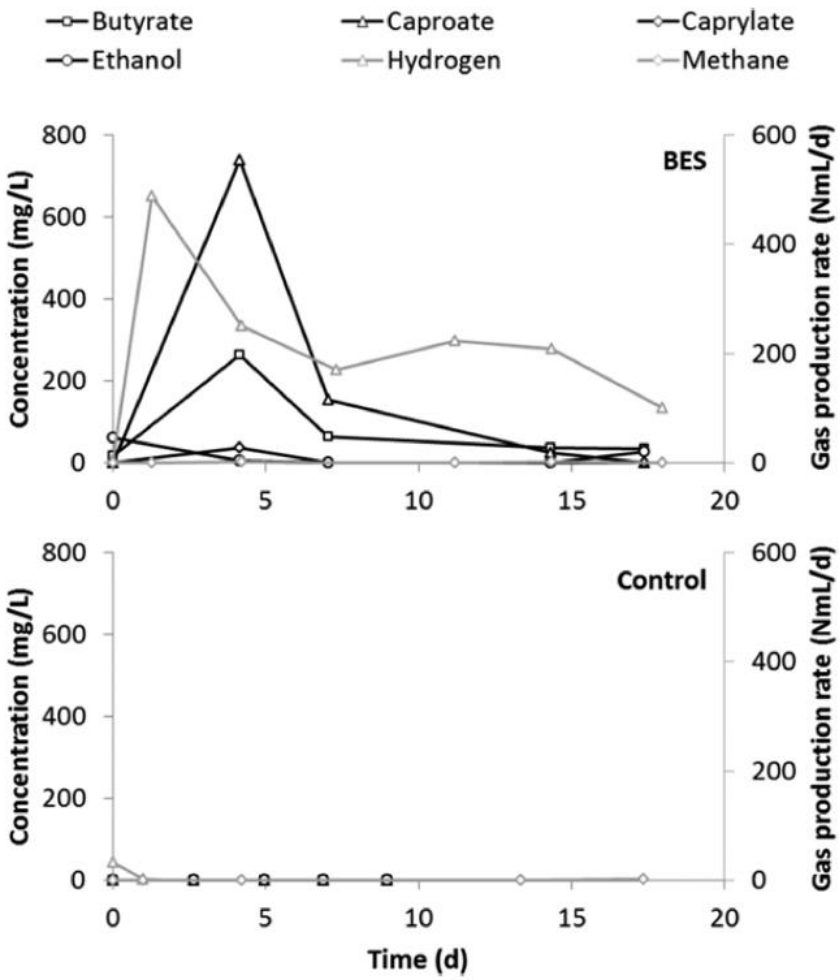

Figure 2-13 : results of S-MCFAs elongation : time variation of the concentrations in the fermentation broth. (Van Eerten-Janses et al., 2013)

Results showed a maximum production rate of carboxylate at day 4 of the experiment and the highest S-MCFAs concentrations reached 739 mg/l, 263 mg/l and 36 mg/l for caproate, butyrate and caprylate respectively. (Van Eerten-Jansen et al., 2013)

3 MATERIALS & METHODS

3.1 EXPERIMENTAL PART

To test the influence of the source of reducing equivalents, we designed two types of reactors: electrical and non-electrical reactors. Bio-electro systems, also named electro-fermenters (EF) were design to investigate the bio-electro-elongation of VFAs. On the other hand, non-electrical reactors pressured reactors (PR), were designed for two different purposes. Two of these PR were dedicated to testing the hypothesis of molecular hydrogen being able to provide the needed electrons for VFA elongation. The two other PR were used as control reactors for the two first ones and pressurised with nitrogen.

3.1.1 Set up

3.1.1.1 Pressure Reactors (PR)

3.1.1.1.1 Bottle design

The four PR all have the same design. Each reactor consists in a 1l Scott bottle (GL45). The cap of each bottle is made of a stainless steel headpiece with 2 hole nozzles. The headpiece is pressed on nylon seal by a pierced screw cap. On the outside of the headpiece, two flexible PVC tubes are sealed with, at the other end, a two way Luer lock valve for sampling. On the inner side of the cap, a flexible PVC tube is sealed on one of the two hose nozzles. It is used as a liquid sampling tube. The sealing of the PVC tubes over the nozzles is done by the use of a short piece of silicone tube with a slightly smaller outer diameter compared to the PVC tube's interior diameter. The silicone tube is then fitted inside the PVC tube and its compression ensures the tube's gas tightness.

A magnetic bar is put at the bottom of the bottles and driven by a six positions stirrer at 200 rpm.

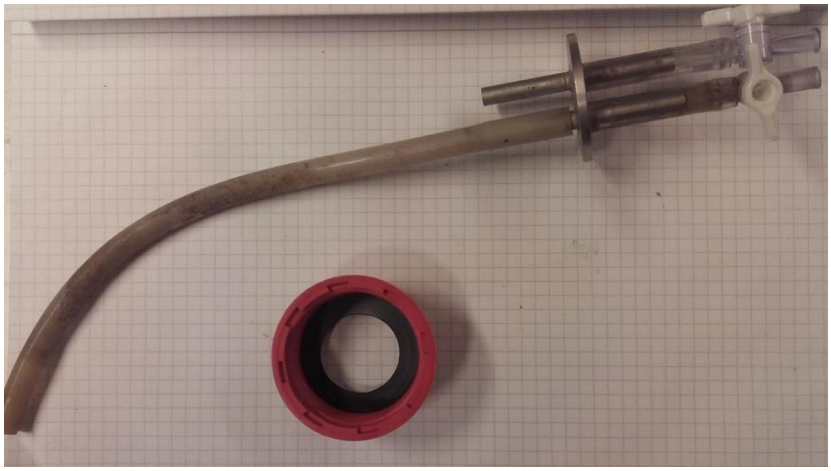

Figure 3-1 : Upper part : headpiece with its PVC tubes and two way valves. Lower part : hollow cap with the silicone seal inside. Used on top of the PR

3.1.1.1.2 Incubating conditions

A magnetic stirrer is put at the bottom of the bottles. The bottles are then disposed in a 30°C incubator and continuously stirred at 200 rpm by the means of a magnetic plate.

Figure 3-2 : The four PR on the stirring plate disposed inside the incubator

3.1.1.2 *Bio-electro fermenters (EF)*

3.1.1.2.1 *Vessel and the cap*

The bio-electro system (BES) is a 1l glass vessel (see figure 3-3 below) with a warming water jacket. An handmade acrylic cap is pressed on a silicone seal at the top of the reactor by a metallic ring. The cap has a 5,5 cm hole at its centre. In this central hole, a 5 cm inner diameter, 10 cm in long acrylic tube is inserted and glued with hard PVC glue (Pattex, Germany). Around the centre hole, 6 holes where made. One for the pH probe (diameter : 1,2 cm), one for the reference electrode, two for sampling and flushing (1cm) and two for the cathode connexion to the power supply. Finally, the EF is wrapped with aluminium foil to keep it from (sun)light and preventing unwanted photo-biochemical processes.

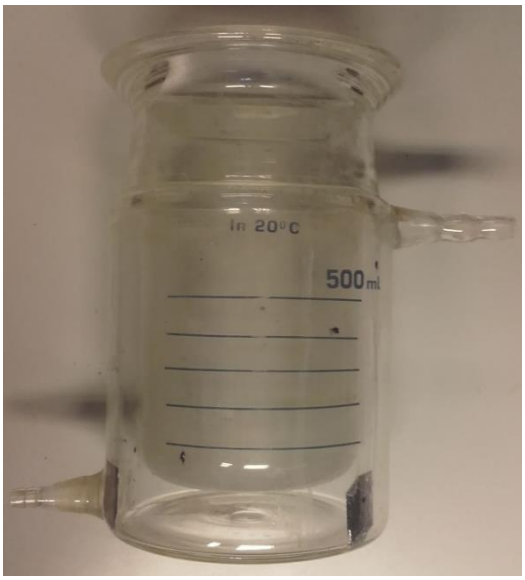

Figure 3-3 : Empty vessel first generation BES

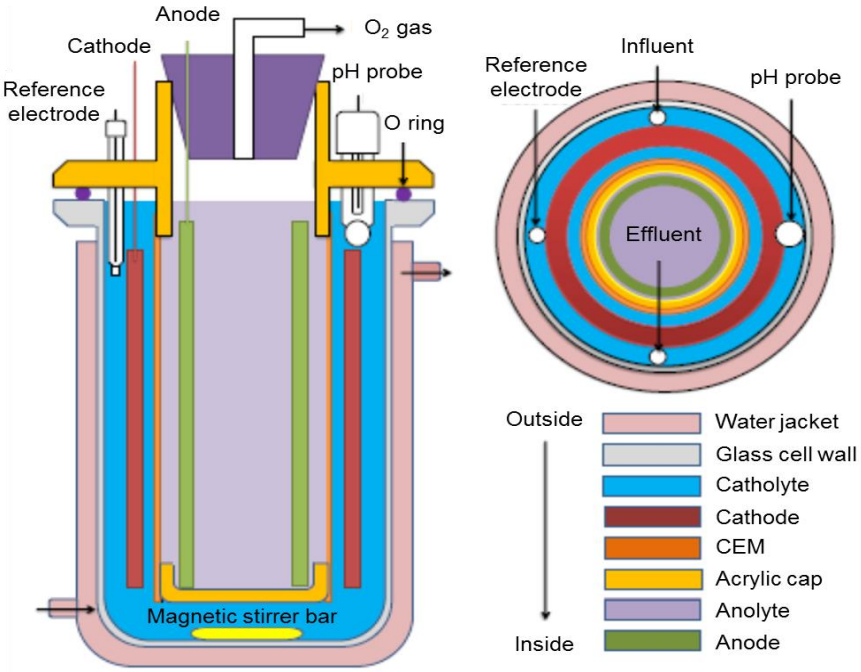

Figure 3-4 : First generation BES (Dr. Kun Guo LabMET (Ughent))

3.1.1.2.2 *Membrane*

The vessel is separated into to compartments by a tubular cation exchange membrane CMI-7000T (International Membrane, USA). This tubular membrane is fixed around the inner part of the acrylic tube passed through the cap. The lower part of the membrane is closed with an acrylic cap of the same diameter as the tube. Both extremities of the membrane are sealed with a stainless steel wire clamp.

3.1.1.2.3 Anode compartment

The inner compartment separated by the membrane is the anode compartment. Inside it, a platinum-iridium oxide coated anode (Zmanode, China) is submersed in 450 mL of anolyte solution. The anolyte solution is a 0,1M K_2SO_4 (VWR, Germany) aqueous solution. The upper side of the acrylic tube is sealed with a silicone plug. To prevent harmful accumulation of oxygen due to water hydrolysis in the anolyte, two tubes cross the this plug. The first one is an 0,5 inner diameter stainless steel tube, which discharges a pure nitrogen flux inside the anolyte headspace. The second one, a PVC tube with an inner diameter of 10mm, drives the gases (nitrogen + produced oxygen) out of the anode compartment.

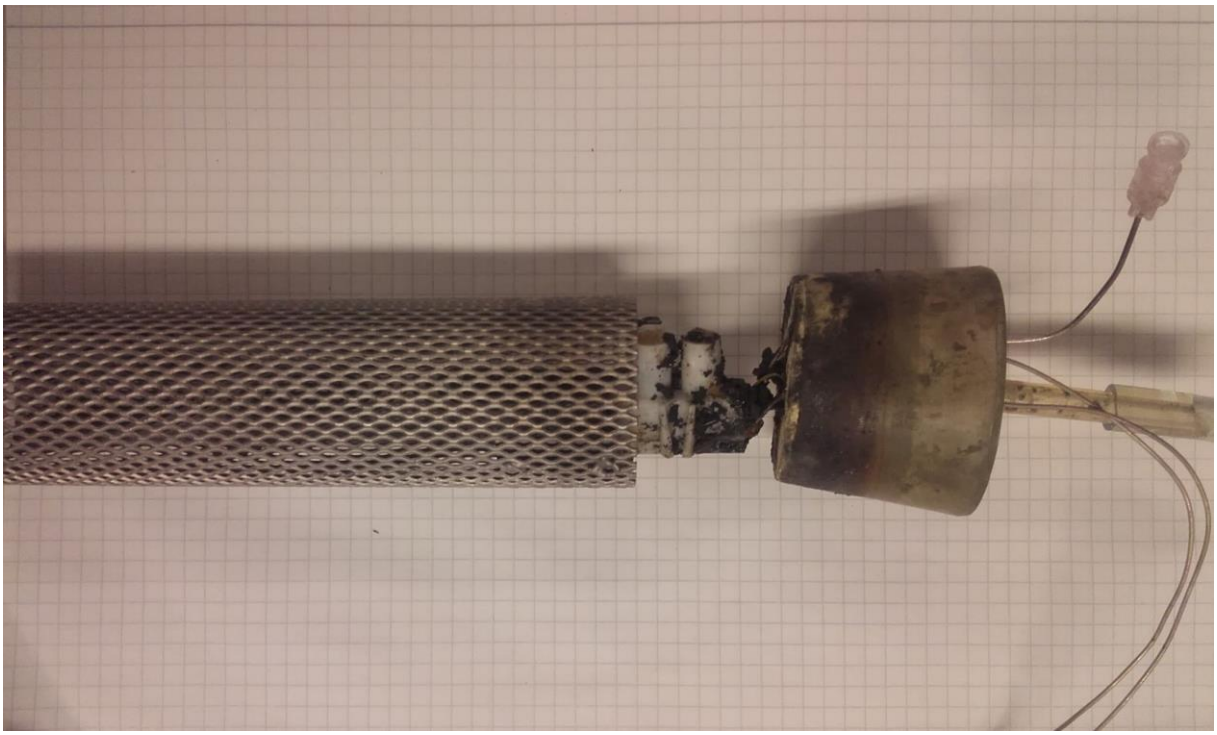

Figure 3-5 : anode connected to the silicone plug with the upper gas tube coming out at the upper side, the outflow tube in the middle and the connexion cable for the anode

3.1.1.2.4 Cathode compartment

The outer compartment is the cathode compartment. Inside of the cathode compartment, a non-woven stainless steel plate of 45cm x 10cm is folded around the membrane into an accordion shaped circle (to maximize specific surface) and is connected via the electrical connecters in the cap. The catholyte consists of 600ml of fermentation broth which is further explained is section 3.1.2.1.

Three 5ml polystyrene serological pipettes (Greiner Bio-One, Austria) are put through the holes in the cap. Two of them are cut just behind the narrowing part of the pipette to have a constant diameter along the 20 cm tube that is left over. These two tubes go down into the fermentation broth, one is the nitrogen flushing tube and the other one is the liquid sampling tube. The last serological pipette is cut to only reach the headspace (±6 cm) and is used for gas sampling. The upper ends of those three serological pipettes, which are located outside of the reactor are connected by a flexible PVC tube to three way valves with Luer Lock tips to facilitate sampling, similarly to the sampling tubes of the PR. Both pipettes are sealed with a plastic cable gland, which is itself sealed to the cap of the reactor.

A pH probe (70701/2 TUNZE, Germany) and an Ag/AgCl reference electrode (+197mV vs SHE, SI Analytics GmbH, Germany) are also submerged in the fermentation broth.

Figure 3-6 : EF membrane sealed to the cap of the reactor. The black wire are the cathode connexions, the white screw is the pH probe hole, the two grey screw sampling tubes holes.

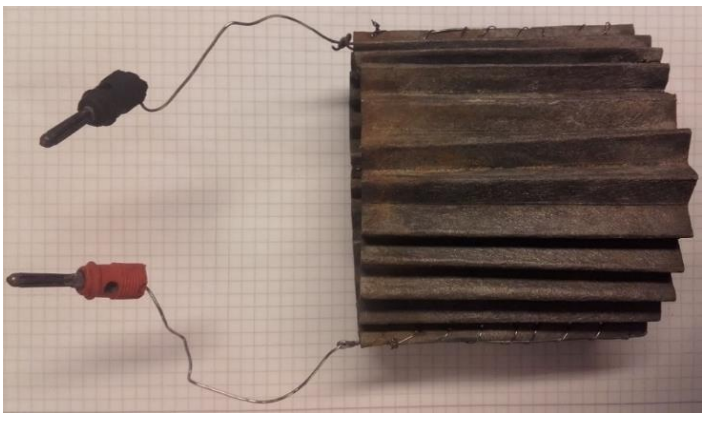

Figure 3-7 : Folded stainless steel cathode

3.1.1.2.5 Gas collecting and sampling device

To prevent the produced gas from accumulating in the cathodic compartment and thus increasing pressure, the gases produced by fermentation and water hydrolysis must be evacuated from the reactor. However, such an opening directly in the reactor would allow direct diffusion of air into the headspace, making anaerobic conditions no longer guarantied. To solve these problems, a gas collecting device has been set up. A flexible PVC tube is placed at the Luer Lock of the gas sampling tube coming from the cathodic compartment. On the other end of the PVC tube, a small needle (BD, USA) is fixed. The end of the tube with the small needle is put into a 50 mL syringe (without its piston) which is held upside down in a basin filled with water. The syringe is closed by a three way valve with Luer lock. The water level in the basin is just above the syringe bottom, so that the air inside the syringe is trapped. To collect gas samples or to evacuate the gases contained in the syringe, another syringe equipped with a three way valve is connected to the vertical syringe and the gases contained in the vertical syringe are vacuum pumped into the other syringe, thus elevating the water level into the vertical syringe as shown on figure 3-8.

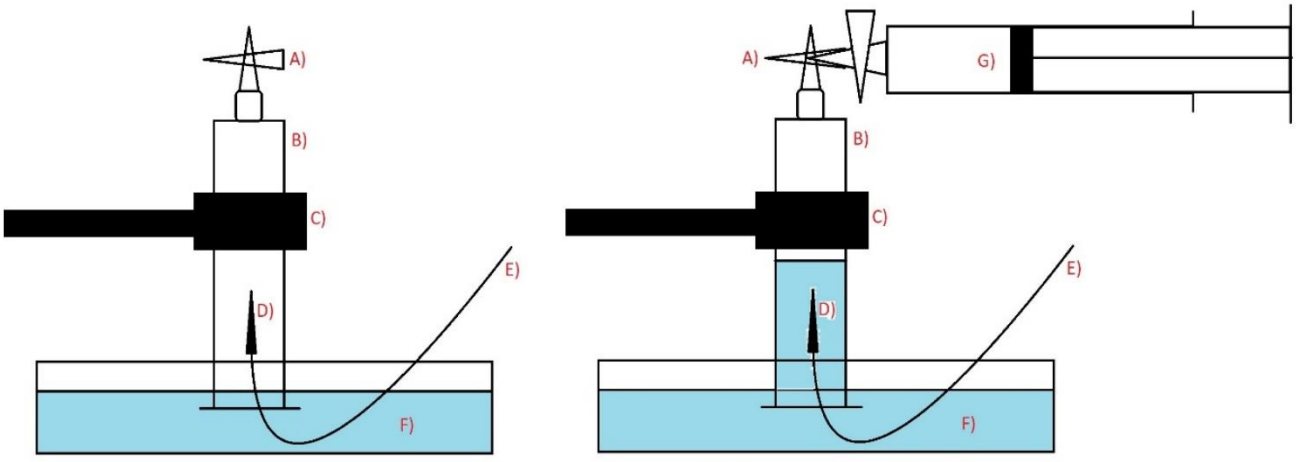

Figure 3-8 : gas collecting/sampling device. A) Three way valve, B) Vertical collecting syringe, C) Holding clamp, D) Small syringe, E) Gas tube from cathodic compartment, F) Water filled basin, G) Second syringe

3.1.1.2.6 Power supply

A constant current[2] of 0,04 A is applied to the system by the means of a classical lab power supply (RND Lab). One Power supply is needed for each reactor. The cathode is connected via the connecters on the cap of the vessel while the anode is connected via a wire passed through the silicone cork.

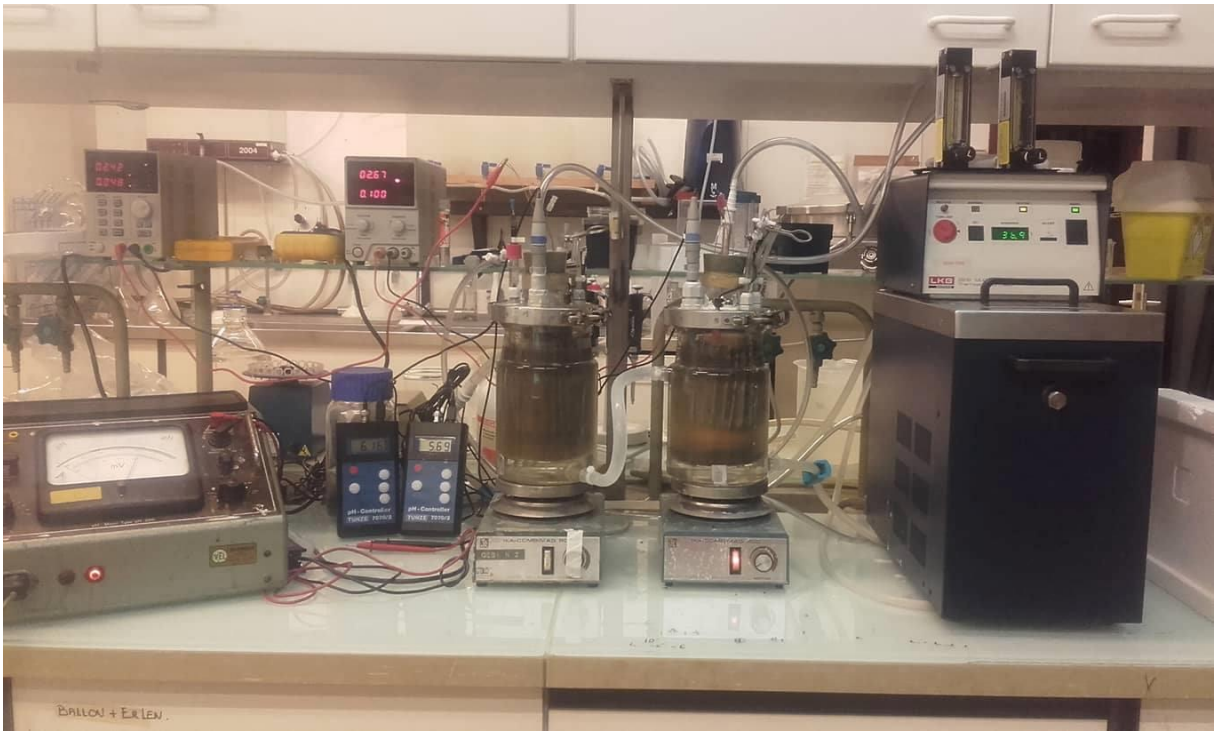

Figure 3-9 : BES first experimental setup

[2] We choose a constant current to facilitate energy and electron transfer calculations but applying a constant voltage can also be interesting.

3.1.2 Launching of the fermentations

3.1.2.1 Fermentation broth preparation

The fermentation broth used in the PR and EF is the same. Activated sludge from the waste water treatment plant in Mont-saint-Guibert is used as a base for the fermentation broth. It is first diluted to obtain a chemical oxygen demand (COD) between 5 and 10 g/l. The diluted sludge is then put in 1l GL45 Schott bottles and heated for 1 hour at 70°C in a water bath (one bottle the EF and one bottle for all the PR). By heating the medium up to 70°C we try to preselect the targeted organism which can sporulate and to eliminate non sporulating organisms like methanogenic archaebacteria which might compete the wanted elongation processes. Once the treated sludge is cooled at room temperature and an exact volume of 1l is left in the bottle, an acetic acid/acetate buffer is added. To buffer the fermentation broth at a pH of 5,5 , 2ml of acetic acid (Sigma-Aldrich, USA) and 16,58g of potassium acetate (VWR, Germany). The total concentration in acetic acid/acetate is 12g/l.

3.1.2.2 Partial Pressure Reactors

200 mL of the buffered sludge is put into the each PR. The hydrogen and nitrogen PR are then respectively flushed with pure dihydrogen or pure dinitrogen gas to eliminate the air present in the bottle. This is done by connecting the gas supply line to the Luer Lock of the liquid sampling tube and by opening the gas sampling two way valve. After 10 minutes of continuous gas flow, the PR can be over pressurized[3]. To do so, an UNIK 5000 manometer (GE, USA) with a three way valve on top is used. The manometer is connected to the two way valve of the gas sampling tube while the gas supply line is connected to the liquid sampling line. The PR is then filled with their corresponding gas until it reaches a constant pressure of 0,1 bar compared to the atmosphere. A first gas sample can be taken to check if the gas in the PR was well flushed.

3.1.2.3 BES

600 mL of the buffered sludge is put into the catholyte compartment of each bio-electro-reactor. 300 mL of the anolyte solution is put into the inner compartment. To detect any leak and to prevent any damage to the anode in the case of a leaking membrane, the level of anolyte in the anode compartment has to be at any time higher than the level of catholyte in the catholyte compartment. A constant flush of nitrogen is then blown trough the catholyte solution via the flushing tube and evacuates the head space air through the gas sampling tube.

[3] Even if this has not been done during our experiments, the H_2 PRs should first be flushed with nitrogen to eliminate the oxygen present in the reactor's headspace. This way any dangerous contact between hydrogen and oxygen can be prevented.

After one night of nitrogen flushing, a gas sample is taken to check if oxygen is still present. If it doesn't, the nitrogen flush is brought to the anolyte compartment and the current is switched on (0,04A constant current), as well as the magnetic stirrer 200 rpm.

3.1.3 Monitoring and Sampling

3.1.3.1 Partial Pressure Reactors

Twice a week, pressure in the reactor is measured with the manometer. Then, several steps are taken to sample liquid and gases and to bring back to an over pressure. Gas samples are taken with 40 mL polypropylene syringes (BD Plastipack, USA) equipped with a three way valve as well. Liquid samples are taken with a 20 mL plastic syringe (BD Plastipack, USA) and put into a 15 mL falcon tube (BD Plasitpack, USA) for further analysis.

1) First pressure measurement : gives the initial pressure in the reactor
2) First gas sample collection (30 mL) : gives the initial gas composition in the reactor
3) Liquid sample collection (10ml) : gives the liquid composition in the reactor
4) Reactor over pressurization to 0,15bar. This is purposely higher than the targeted value because of the last gas sampling
5) Second gas sample collection (30 mL) : gives of the gas concentrations in the reactor for the next monitoring period
6) Second pressure measurement : gives the pressure in the reactor for the next monitoring period

3.1.3.2 Bio-electro fermenters

3.1.3.2.1 Electrical measurements

Every two days, the voltage applied by the constant current power supply is measured. The difference in electrical potential between the reference electrode immersed into the fermentation broth and the cathode is measured with a potentiometer.

3.1.3.2.2 pH measurements

pH is continuously monitored by the pH probe (70701/2 TUNZE, Germany) immersed into the fermentation broth. During the process, pH may go up and reach unwanted high levels. In case of a strong pH increase, pure phosphoric acid is added through the liquid sampling tube with a 20 mL syringe. However, when pH increase was linked with a drop in C2 concentration, acetic acid was added instead of phosphoric acid to lower the pH. It was decided that the C2 concentrations should never exceed 12g/l of mixed liquor at any time during the experiment, this to prevent any inhibition effect of acetic acid on the microorganisms.

3.1.3.2.3 Gas collection and gas flow measurement system

The gas present in the vertical syringe presented in figure 3-8 is first pumped up with the second syringe and evacuated. This gas is indeed no longer representative of the gas present in the head space of the reactor since the plastic tube and syringe are permeable to hydrogen. Once the water level is put back on maximum in the syringe, the gas flow coming from the cathodic compartment can be calculated by the time needed by the water to drop from a certain level to a lower one.

$$gasdebit\left(\frac{ml}{s}\right) = \frac{Volume\ of\ water\ displaced\ by\ the\ gas\ over\ a\ given\ period\ of\ time\ (ml)}{Time\ needed\ for\ the\ water\ level\ to\ drop\ (sec)}$$

Once 30 mL of gas are present in the collecting syringe, the gas can be pumped by the second syringe and can be analysed.

3.1.3.2.4 Liquid sampling

Two different kinds of samples were taken from the BES. A 10 mL sample is taken during the first stages of the EF (before the Fed-batch). It is taken through a 50ml polypropylene syringe (BD Plastipack, USA) connected to the liquid sampling tube and stored into a 15 mL Falcon tube (BD Plastipack, USA). A 50ml sample is taken in a similar way during the fed-batch period. It is then store into a 50ml Falcon tube. The syringe is washed several times with tap water and demineralised water before and after every liquid sampling. When the samples are taken, liquid is first flushed in and out of the syringe, through the sampling tube, to homogenize the liquid present in the sampling tube with the rest of the fermentation broth.

3.2 ANALYSES PART

3.2.1 Gas analysis

3.2.1.1 Compact GC characteristics

The gaseous samples are analysed with a compact gas chromatograph (GC) TCD-TCD (Compact GC, Global Analyser Solutions, Interscience, The Netherlands). This gas analyser can detect H_2, N_2, O_2, CO_2, and CH_4. The GC includes two channels (the front and back channel) both composed of an injection loop of 25µL and a thermal conductivity detector (TCD). The front channel can only separate and quantify CO_2. It is equipped with an RI-QBond column (10m length, 0.32 mm ID, Restek, France) and the carrier gas is helium. The back channel is equipped with two columns placed in series with argon as a gas carrier. The first column is a RI-QBond column (2 m length, 0,323 mm ID, Restek, France) and the second is a Molsieve5A column (7 m length, 0,32 mm ID, Restek, France). A 3-ways valve 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 and is consequently back-flushed out of the GC. As the other gases continue to progress in the second column, they are separated and quantified by another TCD. Retention times of H_2, O_2, N_2 and CH_4 were 21,7, 27,7, 35,0 and 51,0 seconds respectively.

Chromatographs are then automatically integrated to give a percentage of the different gases (H_2, O_2, N_2, CH_4 and CO_2) analysed by the Compac GC.

3.2.1.2 Sample injection

Gas samples, whether they come from PR or BES, are collected in 50 mL polypropylene syringes equipped with a three way valve. The gase 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.

3.2.1.3 Calibration of the Compac GC

The calibration of the compact GC is realised with five gas samples : four gas mixtures with known composition and one of air. Each 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 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 (rates between gas concentration and peak area on the chromatograph) for each gas component. The detection limit of the GC is 0,1%$_{v/v}$.

3.2.2 Liquid sample analysis

The liquid samples, whether they are in a 15 or 50 mL Falcon tube, undergo the same analyses. First 1,5 mL of sample is taken from the Falcon after homogenizing the content, put into an Eppendorf tube (Greiner Bio-one, Austria) and stored into a freezer at -20°C until total COD measurement. The rest of the sample tube is then centrifuged (Beckman Coulter, USA) in the Falcon at 3000 G for 6 minutes. A new Eppendorf is used to collect 1,5 mL of the supernatant from the centrifuged fermentation broth. This new Eppendorf is stored at -20°C as well and will be used for the measurement of the soluble COD while the rest of the sample remaining in the Falcon kept for fatty acids analysis.

3.2.2.1 COD measurements

The Chemical oxygen demand tests consists in the total oxidation of all the organic matter and other reduced compounds present in the sample. This is done by the means of an excess of hot sulphuric solution of potassium dichromate ($K_2Cr_2O_7$) as oxidizer, silver sulphate as catalyst, and mercury sulphate in order to complex chlorides ions present in the sample. This prevents the chlorides ions from precipitating silver ions and thus inhibiting their catalytic function. A photometric measurement at 620 nm then determines the concentration of the remaining yellow chromate ions, unconsumed during the oxidation reaction. The results are then calculated in g of oxygen needed for the total oxidation of the sample per liter of sample.

COD measurement kits (Spectroquant, Merck KGaA, Germany) for COD measurement between 500 and 10000 mg/l were used used. First, 1,5 mL of distilled water is put into each tube followed by 500µl of sample. 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, absorbance is measured 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 oxygen demand per litre of solution introduced into the tube with a confidence interval of ±0,005 g_{COD}/l. The obtained value is then multiplied by 4 to get the COD present in the initial sample.

3.2.2.2 Fatty acids analysis

3.2.2.2.1 Preparation of needed reagents

At first a solution of 8g/l of NaOH is prepared to dilute the fatty acids of the calibration solutions. To do so, 8 g of pure NaOH (VWR, Germany) are weighted and dissolved into a 1l volumetric flask which is then filled up to the mark with distilled water. The flask is then agitated until all the caustic pellets are dissolved.

Secondly, an internal standard is prepared using 4-methylvalerate (4-MV), a fatty acid which is not present naturally in the fermentation broth. 100,0µl of 4-methylvalerate (Sigma Aldrich, USA) are put into 100,0 mL of distilled water.

Finally a solution of 1g/l of H_2SO_4 is made by diluting 55 µl of H_2SO_4 97% (ChemLab, Belgium) into a flask of 100ml with distilled water. The flask must be filled with 40 mL of water before putting the acid in it to prevent any excess heat emission that may be dangerous.

3.2.2.2.2 Preparation of calibrating solutions

To prepare the calibrating solution, first, approximately 100 mL of the prepared NaOH solution are put into 3 volumetric flasks of 250 mL. Then, the quantities mentioned in table 2.1 of each fatty acid are added in each flask. After every addition of a new fatty acid, the exact quantity added must be weighted before taring the balance again for the next fatty acid.

Table 3-1 : Concentrations in fatty acids for calibration solutions

	Calibration Solution 1	Calibration solution 2	Calibration solution 3
C_2	400µl	800 µl	1500 µl
C_3	25 µl	65 µl	50 µl
iC_4	50 µl	25 µl	65 µl
C_4	65 µl	50 µl	25 µl
iC_5	25 µl	65 µl	50 µl
C_5	50 µl	25 µl	65 µl
C_6	65 µl	50 µl	25 µl
C_7	25 µl	65 µl	50 µl
C_8	50 µl	25 µl	65 µl
C_9	65 µl	50 µl	25 µl
C_{10}	0,025 g	0,050g	0,065g

Once every fatty acid is added in each flask, NaOH solution is added carefully up to the 250 mL mark in each volumetric flask which are then sealed with the corresponding cap ad wrapped into parafilm to ensure tightness before being put into the fridge at 7°C.

3.2.2.2.3 Fatty acids extraction

Both samples and calibrating solutions undergo the same extraction process. For the extraction, glass screw top test tubes (REF : Pyrex) with plastic caps and silicone seal are used. First, approximately 1 g of NaCl is put into the test tube (for salting out) along with 1,00 mL of distilled water and 1,00 mL of the sample or calibrating solution. Afterwards, 0,40 mL of the prepared 4-MV internal standard is added in each tube. Finally, 0,2 mL of the H_2SO_4 solution, and then quickly after, 2,00 mL of pure ether (Sigma-Aldrich) are added in each test tube before closing them rapidly.

The tubes are then gently shaken for 3 minutes to complete the extraction process. To separate the water phase from the ether phase, the tubes are centrifuged for 6 minutes at 3000 rpm in the centrifuge.

3.2.2.2.4 Gas chromatography analysis

Once centrifuged, the supernatant of each centrifuged test tube is collected with a micropipette and put into a glass vial which is then sealed with a PTFE/silicone screw cap (VWR, Germany). The vials are then analysed by the gas chromatograph (Thermo Scientific, USA) with an 30m, 0,25 mmID, nonpolar column (Restek, USA), a FID detector at 240°C and a Split-Splitless injector at 230°C. The run time is 34 min and temperature starts at 40°C followed by an increase by 10°C/min during 18 minutes until it reaches 220°C where it stays for 15 minutes.

3.2.2.2.5 Calibration calculations

First, every exact concentration of each VFA present in each calibration solution is calculated using the exact weight measured during the preparation of the calibrating solutions. Every peak area obtained by the integration of the chromatograph is normalised 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 analysed VFA 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 VFA in the analysed sample while the linear regression coefficient (r^2) will determine if the calibration was well done.

3.3 CALCULATIONS

3.3.1 Theoritical COD

If COD can be measured, it is also useful to determine stoichiometrically expected COD of specific molecules.

The general formula for every organic substance that does not contain phosphorus, nitrogen or any heteroatom is :

$$C_xH_yO_z + \left(\frac{4x + y + 2z}{4}\right) O_2 \leftrightarrow xCO_2 + \frac{y}{2} H_2O$$

Hence, one mol of $C_XH_YO_Z$ reacts with $\left(\frac{4X+y-2Z}{4}\right)$ mol of O_2.

Considering that the molar mass of $C_XH_YO_Z$ is (12x+y+16z) g/mol and of O_2 is 32 g/mol, COD_{th} of molecule is calculated by the following equation :

$$COD_{th} = \frac{8 * (4x + y - 2Z)}{12x + y + 16z}$$

Calculation for VFA that were encountered during the experiment are :

Table 3-2 : COD value in gCOD/g for the VFA (from C2 to C10)

VFA	C2	C3	iC4	C4	iC5	C5	C6	C7	C8	C9	C10
COD_{th} (g_{COD}/g)	1,07	1,51	1,82	1,82	2,04	2,04	2,21	2,34	2,44	2,53	2,60

3.3.2 Electrical COD

To be able to present COD balances for the BES, the electron input of the electric current has to be taken into account in terms of COD as well. Since our system runs in constant current, calculus are simplified. First the current has to be converted in mmol electron equivalent per day (mmol e-/d)

$$1\text{mA} = 10^{(-3)}\frac{\text{C}}{\text{s}} * \frac{1\text{mole}^-}{96485\text{C}} * 86400\frac{\text{s}}{\text{d}} = 0{,}896\text{mmol e}^-/\text{day}$$

The mmol e^- can be converted into COD regarding the following equation

$$\frac{1}{4}\text{O}_2 + \text{H}^+ + 1\text{e}^- \rightarrow \frac{1}{2}\text{H}_2\text{O}$$

So giving the formula above and the molar mass of O_2 :

$$1\text{mmol of e}^- = 1/4\text{mol of O2} = 0{,}008\text{g}_{\text{COD}}$$

Finally, 1mA of electricity provides :

$$1\text{mA} = 0{,}896\frac{\text{mmole}^-}{day} * 0{,}008\frac{g_{COD}}{mmole^-} = 0{,}007168\text{g}_{\text{COD}}/\text{day}$$

4 RESULTS & DISCUSSIONS

4.1 ELECTRO-FERMENTER

4.1.1 Results

The EF experiment lasted 142 days. To get a clear view of the evolution of the experiment, the total duration was divided into 3 different periods of time.

The first period lasted from day 0 to day 49 and is called “stabilisation period” because of the process initiation of the EF. During the beginning of the stabilisation period, gas leaks in the gas collecting device made the collected gas data unusable. Therefore Figures 3 and 4 only present gas production starting from day 37 which was the first day where reliable gas data could be collected and COD balances were only made starting from day 37.

The second period was called the “Batch Production Period”. MCFAs elongation was first achieved during this period and C2 was replenished without impacting the volume of the reactor. It was an 8 days period stretched from day 50 to day 58.

Finally, from day 59 to day 142, the reactor was set into Fed-Batch mode and was therefore named : Fed-Batch Period. Each fed accounted for 50ml while the mixed liquid volume was 600 mL, the reactor was diluted by a factor 1,1 at each Fed. In total, volume was sampled and replenished 18 times during this period accounting for a HRT of :

$$\frac{\text{Mixed Liquid volume (L)}}{\frac{\text{Number of Feds * volume per Fed (L)}}{\text{Time of the Feb-Batch (t)}}} = \frac{0{,}6}{\frac{18*0{,}05}{142\text{-}59}} = 55{,}33 \text{ days}$$

4.1.1.1 Graphs

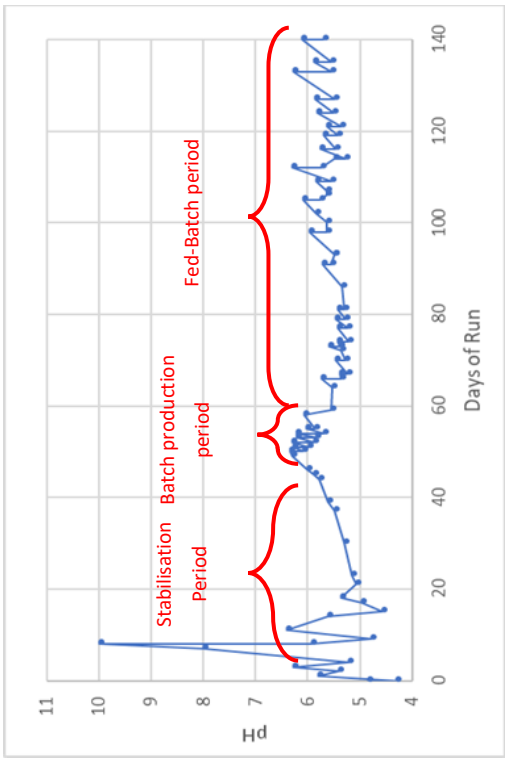

Figure 4-4 : Evolution of pH as a function of time in the EF

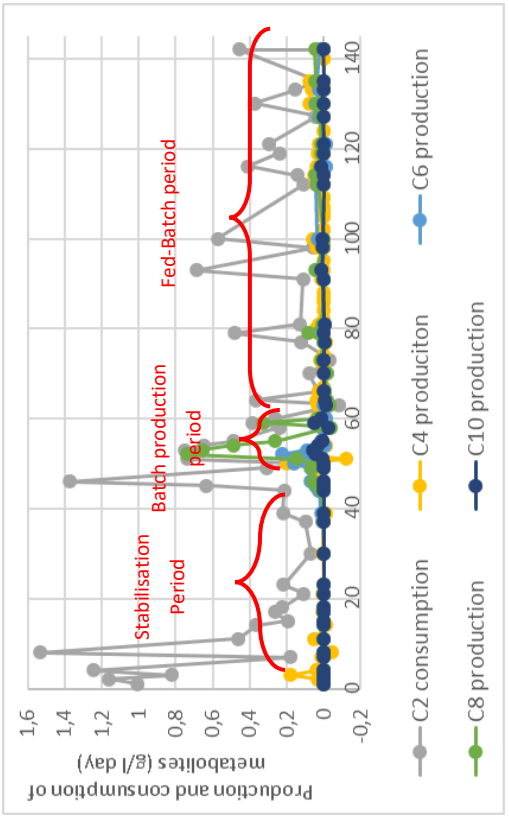

Figure 4-3 : Rate of consumption of C2 and production of the corresponding longer chain fatty acids (butyric, caprylic, caproic and capric) during the run of the EF.

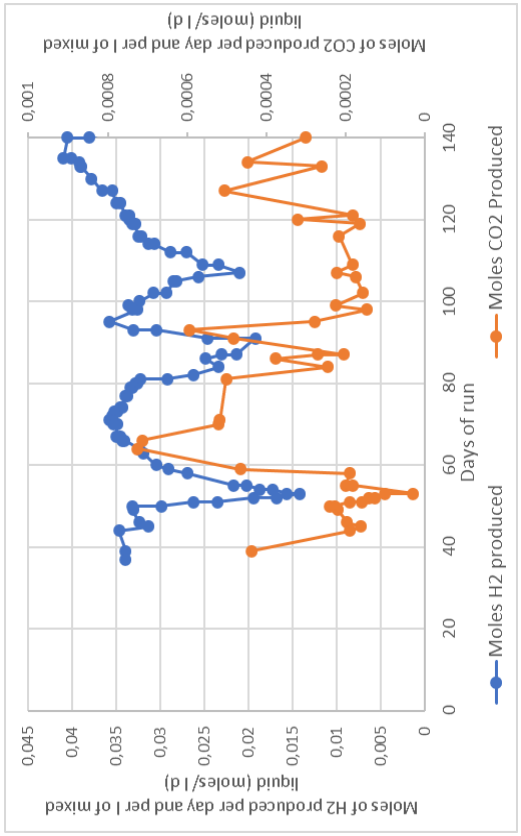

Figure 4-2 : Production of H2 and CO2 in moles per day and per L of mixed culture.

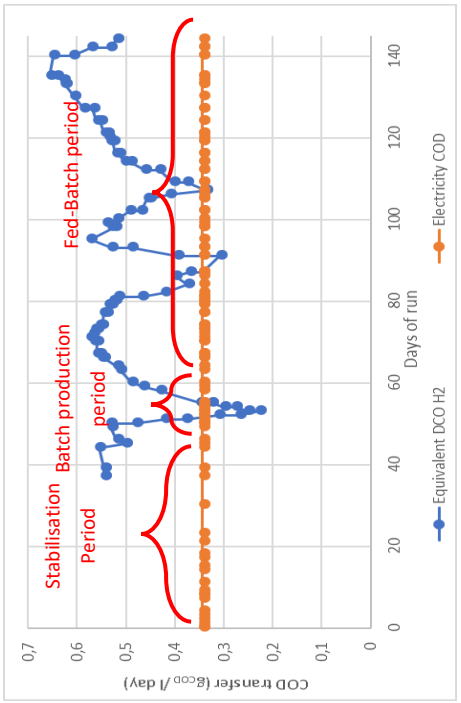

Figure 4-1 : EF H2 production and Electron transfer variation during the run in gCOD/L of mixed liquor per day.

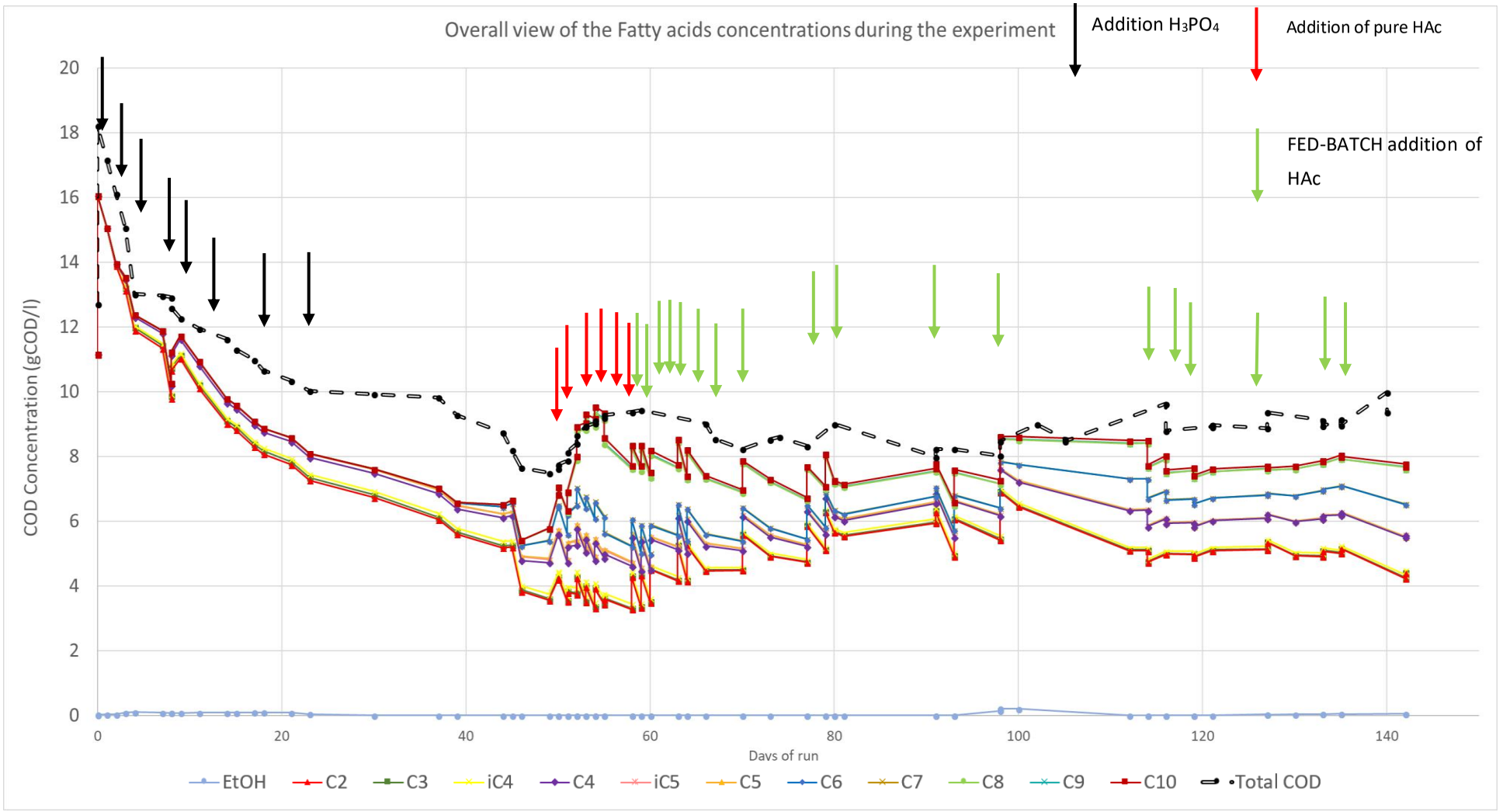

Figure 4-5: Evolution of the metabolites concentrations as a function of time. Fed-batch started on day 60. Metabolites are presented as cumulated contribution by order of carbon number: C2 on top of EtOH, C3 on top of C2, C4 on top of C3, etc. H3PO4 addition accounts for 6,4mmol LML-1. Addition of pure HAc accounts for 0,4 gCOD LML-1. FED batch addictions accounts for 0,4 gCOD LML-1 and a fed volume of 50 mL.

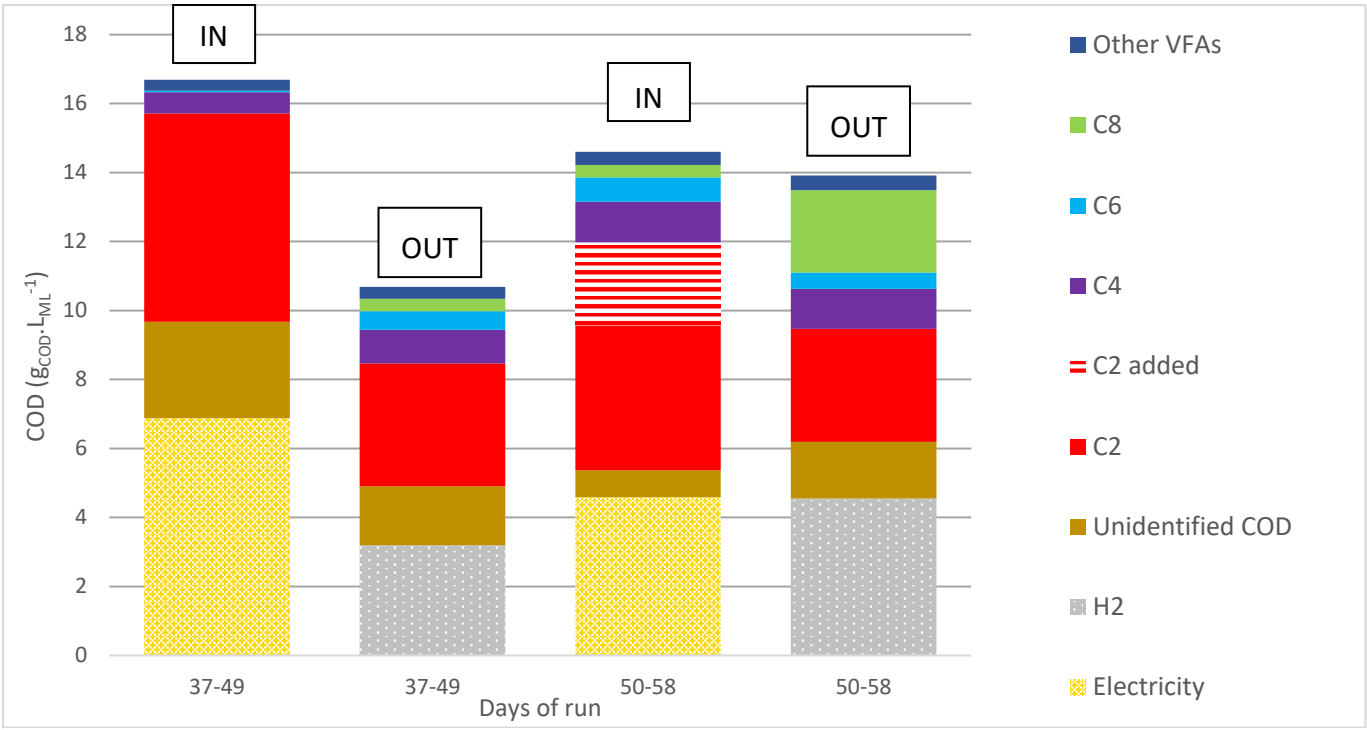

Figure 4-6 : Two left bars: IN: state at day 37 and total inputs between day 37 and day 49. OUT : state at day 49 and total outputs between day 37 and day 49 (end of the stabilisation period). Two right bars: IN: state at day 50 and total inputs between day 50 and day 58. OUT : state at day 58 and total outputs between day 50 and day 58 (beginning and end of the Batch production period).

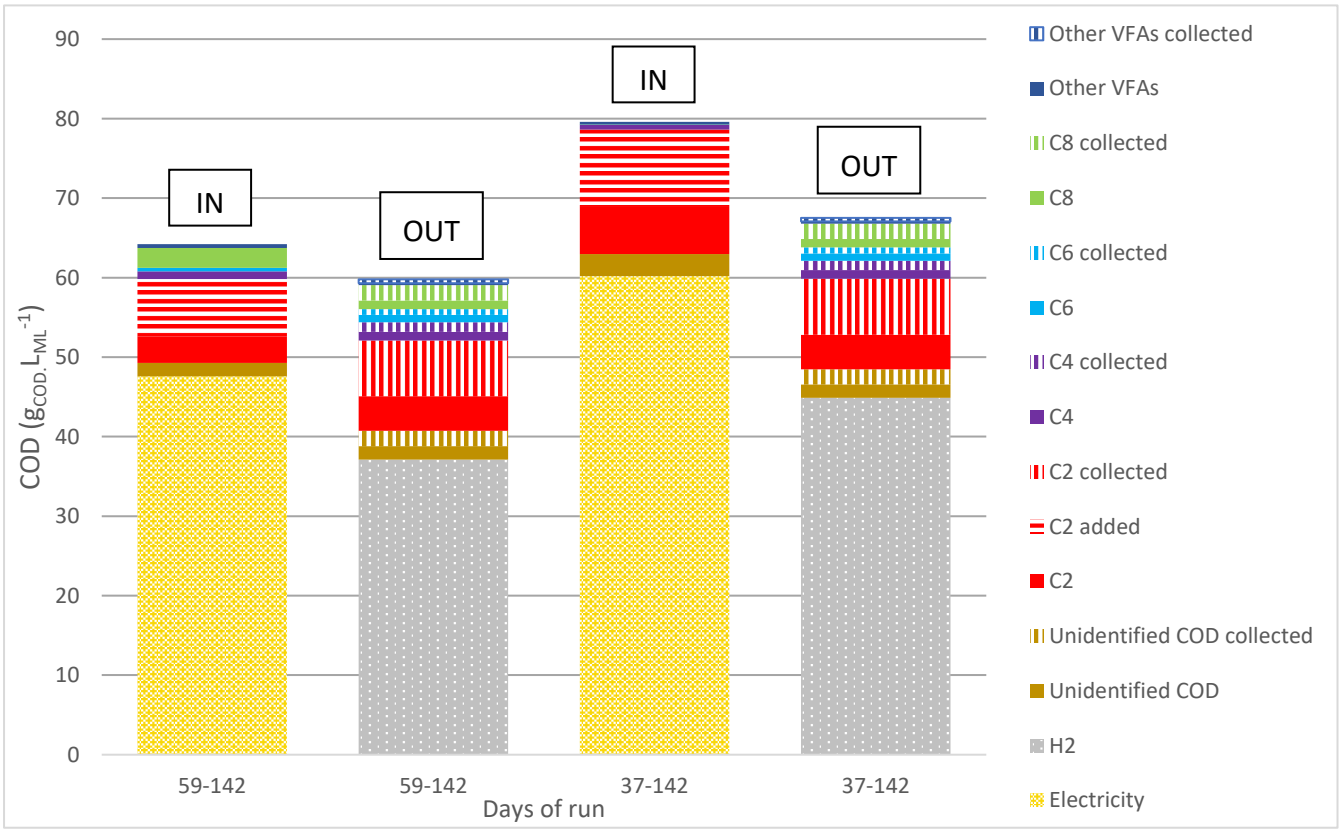

Figure 4-7 : Two left bars: IN: state at day 59 and total inputs between day 59 and 142. OUT: final state and total outputs from day 59 to day 142 (end of the Fed-Batch period). Two right bars : overall balance during the experiment: IN: state at day 37 and total inputs between day 37 and day 142, OUT: state at the day 142 and total outputs between day 37 and day 142.

The COD removed from the reactor due to sampling in the fed-batch period is presented as " Samples" or "Collected".

4.1.1.2 The stabilisation period (day 37 to 49)

As shown in Figure 1, pH was unsteady during stabilisation period and stabilised only after day 20. Since C2 levels were already high at the beginning of the experiment (16 on day 0), an addition of 200µl H_3PO_4 was used 8 times to lower the pH.

Figure 5 shows a continuous decrease in C2 concentrations from 16 $g_{COD}.L_{ML}^{-1}$ at the start to 4 $g_{COD}.L_{ML}^{-1}$ at the end of the stabilisation period.

The C4 was the first S-MCFA to show its concentration to increase during this period. It reached a maximum concentration of 0,98 $g_{COD}.L_{ML}^{-1}$ on day 54. C6 and C8 concentrations started to increase on the very end of the stabilisation period (day 44) and reached concentrations of 0,54 and 0,35 $g_{COD}.L_{ML}^{-1}$ respectively on day 49. Other S-MCFAs were only present at low concentrations (<0,15 $g_{COD}.L_{ML}^{-1}$) during the stabilisation period.

The massive decrease in C2 concentrations on figure 5 is reflected on figure 6 by the high difference between the IN and OUT concentrations of C2 (from 6 to 3,56 $g_{COD}.L_{ML}^{-1}$). However, the balance shown in figure 6 corresponds to the period between day 37 and 49, while figure 5 shows that the C2 concentration had already dropped from 16 to 6 $g_{COD}.L_{ML}^{-1}$ by the time the experiment reached the 37th day.

Unidentified COD dropped as well between day 37 and 49 by 1 $g_{COD}.L_{ML}^{-1}$.

Finally, COD recovery rate for the stabilisation period of the total OUTPUT of COD on the total INPUT of COD is 64%. However, this is only a part of the stabilisation period and recovery rate may be even lower since the major drop of 10 $g_{COD}.L_{ML}^{-1}$ in C2 before day 37 was not taken into account.

4.1.1.3 The Batch Production period (day 50 to day 58)

The Batch Production Period is characterised by a sawtooth like pH curve (cfr. Figure 1) due to 6 additions of C2 which lowered the pH at each addition.

During this period, the C2 consumption rate presented on figure 2 peaked and while the effect of C2 consumption may be overlooked in the sawtooth concentration curve of figure 5, figure 6 highlighted a strong decrease of 3,32 $g_{COD}.L_{ML}^{-1}$ in C2 having a 6,6 $g_{COD}.L_{ML}^{-1}$ INPUT (initial concentration + additions) and a 3,28 $g_{COD}.L_{ML}^{-1}$ OUPUT.

Regarding the S-MCFAs, during this period, Figure 5 shows an increase in total S-MCFAs concentrations. Figure 2 showed a strong increase in C8 production rate almost simultaneous with the C2 consumption peak. The resulting C8 concentration increase accounts for the major part of the total S-MCFAs concentrations increase shown on figure 5 by the global curve

increase during this period. In addition, figure 6 highlights the major difference between IN and OUT concentrations of C8 accounting for a net increase of 2,02 $g_{COD}.L_{ML}^{-1}$.

Other fatty acids concentrations were rather stable during this period.

The COD recovery rate in the Batch Production Period of the total OUTPUT of COD on the total INPUT of COD is 95%.

In the meantime, a major drop in H_2 and CO_2 production rates can also be observed on figure 3 and 4.

4.1.1.4 The Fed-Batch Period (day 59 to day 142)

During this phase of the experiment, the reactor was put into fed-batch mode. Each batch accounted for a 50 mL while the volume of liquid mixture at this point of the experiment was 600ml.

During the Fed-Batch period the pH curve kept a sawtooth pattern like during the Batch Production Period and oscillated between 5,2 and 6,2.

While C2 consumption peaks up to 0,65 $g_{COD}.L_{ML}^{-1}.d^{-1}$ are present during the Fed-Batch period on figure 2, the S-MCFAs production rate was lower than 0,2$g_{COD}.L_{ML}^{-1}$. However the overall balance in C2 between day 59 and day 142 (Figure 7) showed a higher OUTPUT than INPUT.

As shown on figure 5, the other S-MCFAs concentrations stayed rather stable or even decreased due to the dilution at each feed episode. However, the S-MCFAs kept on being produced by the system, as shown in figure 7, which gives a better understanding of the S-MCFAs variations between day 59 and 142. Table 1 is a summary of the values of INPUT and OUTPUT concentrations presented in figure 7.

Table 4-1 : S-MCFA balance for the Fed-Batch Period (from day 59 to day 142)

S-MCFA	Category	IN (d59)	OUT (d142)
C2	Concentration gCOD.LML-1	3,33	4,35
	Added gCOD.LML-1	7,2	
	Collected gCOD.LML-1		7,02
	OUT-IN	0,84	
C4	Concentration gCOD.LML-1	1,00	1,08
	Collected gCOD.LML-1		1,20
	OUT-IN gCOD.LML-1	1,28	
C6	Concentration gCOD.LML-1	0,42	0,92
	Collected gCOD.LML-1		0,73
	OUT-IN gCOD.LML-1	1,23	
C8	Concentration gCOD.LML-1	2,51	1,06
	Collected gCOD.LML-1		1,94
	OUT-IN gCOD.LML-1	0,49	

The COD recovery rate for the Fed-Batch period between the inputs and outputs is 93%.

Finally, two decreases in H_2 and CO_2 gas production, similarly to the one during the Batch Production Period, occurred around day 90 and day 110.

4.1.1.5 Global results from the experiment (day 37 to 142)

Figure 7 shows the global COD balance between day 37 and 142. The overall COD recovery rate of the total OUTPUT of COD on the total INPUT of COD is 84%. The graph also shows that the majority of the COD was brought into the system through electricity (76% of the total INPUT COD) and majority left the system under the form of hydrogen (66% of the total OUTPUT COD).

No methane, neither oxygen have been found at any time during the experiment in the headspace.

4.1.2 Discussion

4.1.2.1 pH

The pH variation during the stabilisation period was quite problematic since 8 additions of H_3PO_4 were needed on top of the already added buffer before the pH stabilised. The acetic acid was gradually consumed during this period, thus lowering the buffering strength which could explain the difficulty to keep a correct pH. Nevertheless, even before C2 concentrations dropped below $10 g_{COD}.L_{ML}^{-1}$, pH already peaked meaning that even at high concentration, the buffer wasn't sufficient. Such strong pH increases could harm the microorganisms present in the fermentation broth or could even damage the reactor and are therefore a problematic issue.

The constant pH increase could be due the water hydrolysis. In the EF system an electrical circuit is formed by electrons going from the anode to the cathode through the power supply and protons going from the anolyte to the catholyte by passing the cation exchange membrane. Since the power supply was set in "constant current" mode it was delivering at any time of the experiment 0,04 A to the system by adapting the voltage. Therefore, a constant flux of electrons was provided to the cathode, no matter the state of the fermentation broth. One of the ways for the fermentation broth to eliminate these electrons is the production of H_2 gas from protons, hence releasing constantly protons in the form of gaseous molecular hydrogen. If the proton flux coming from the anolyte through the membrane was not sufficient to replenish the eliminated protons in the fermentation broth, a proton depletion could occur and cause a pH increase.

However, if a proton shortage caused by the forced production of H_2 could explain why pH kept on increasing at the beginning of experiment, it does not provide an explanation for the fact that the system was unable to counterbalance the effect by transferring protons from the anolyte to the catholyte. Since the only driving force for protons to pass through the membrane is concentration gradient, the proton concentration decrease in the catholyte should indeed have been counterbalanced with a higher transfer from the much more concentrated anolyte.

Furthermore, an explanation also has to be found for the fact that this phenomenon only occurred at the beginning of the experiment and stopped at day 10 while the power supply and other parameters remained unchanged.

An explanation could be a transition period through which the membrane has to get before it can ensure proper proton transfer. This could explain the fact that at the beginning, proton replenishment from the anolyte failed to counterbalance H_2 production and that the phenomenon was limited in time. To confirm this explanation, further research has to be done on the proton transfer through a cation exchange membrane in the beginning phase of an EF experiment. However, no other published paper ever mentioned such problems with EF experiments.

An alternative explanation can be given by the initial presence of a bicarbonate/carbonic acid buffer in the fermentation broth brought by the activate sludge used for inoculating the reactor. Since a bicarbonate/carbonic acid buffer has a pKa of 6,37 and that pH was most of the time lower than 6 during this period, it can be assumed that majority of the protons arriving from the anolyte through the membrane were consumed for the protonation of bicarbonates. Hence, protons kept on leaving the system through the release of H_2 gas whilst the arriving protons were consumed by the protonation of bicarbonate. Hence, the acetic acid/acetate buffer could not counterbalance this phenomenon. Around day 20, the buffer capacity of the bicarbonate/carbonic acid buffer was eventually overtaken and pH stopped increasing strongly since the arriving protons were not consumed by the bicarbonate/carbonic acid buffer.

4.1.2.2 Gas production

Carbon could exit the reactor either in the form of solutes or suspended particles in the fermentation broth samples which have been taken into account in the calculations Carbon could also leave the reactor via the gaseous phase. The end products of both aerobic and anaerobic metabolism of carbon, CO_2 and CH_4 respectively, are indeed both gaseous at atmospheric pressure. However, no traces of methane were found at any moment of the experiment. On the contrary, CO_2, albeit in rather small amounts, was always detected during the gas analysis as shown on figure 4, bringing two elements in light.

First, although the system was meant to be anaerobic and that at no point during the experiment traces of O_2 were found in the head space of the cathodic compartment, molecular oxygen could have been present during the experiment in the reactors headspace.

Secondly, aerobic microorganisms were present in the fermentation broth and were able to activate their metabolism as soon as traces of oxygen were available using it to oxidise any type of organic matter into CO_2. However, as said before, CO_2 was only found in small amounts since the maximum production ever to be measured reached only 0,00075 moles.L_{ML}^{-1} d (=0,033g.L_{ML}^{-1} d). CO_2 is also a biproduct of anaerobic fermentations processes.

Finally, H_2 production may have been underestimated due to its diffusion through the plastic elements of the reactor but especially through the membrane. The cap, the gas collecting tubes, syringes and the cylinder holding the membrane were indeed all made out of acrylic or silicone, H_2 permeable materials while the membrane, due to its small thickness, was probably even more permeable to H_2. However, since the diffusion of H_2 through these materials is slow, the error should have been minimised with the reduced time between two measurements. Nevertheless, since the total production of H_2 by the EF was a sum of all the productions during the experiment, the error may have been amplified.

4.1.2.3 C2 consumption

While acetic acid is used along with potassium acetate to buffer the initial fermentation broth, the main role of C2 is to provide a carbon source for the production of S-MCFAs through elongation. This is illustrated on figure 2 by the simultaneous peaks of C2 consumption and C8 production on days 52 and 60. This is also reflected on figure 6 with the great C2 decrease happened at the same moment C8 strongly increased. However C2 consumption cannot always be explained by its metabolization into longer S-MCFAs. During the Fed-Batch period, C2 consumption peaked several times without having a longer chain S-MCFAs production growth. Moreover, during the stabilisation period, more than 10g_{COD}.L_{ML}^{-1} of C2 left the liquid phase of the fermenter without any production of S-MCFAs.

The C2 could have been lost in the form of CO_2, like explained in the section above. However, even if all released CO_2 was produced from acetic acid, it would only account for a small fraction of the C2 loss. As a matter of comparison, the maximum production rate of CO_2 (0,0075 moles.L_{ML}^{-1}.d) would correspond to a daily consumption of acetic acid of 0,0225 g.L_{ML}^{-1}.d^{-1} (=0,024 g_{COD}.L_{ML}^{-1}.d). While the average concentration decrease during the stabilisation period was of 0,24g_{COD}.L_{ML}^{-1}.d^{-1} (=0,22 g_{COD}.L_{ML}^{-1}.d).

C2 could also have left the reactor in its gaseous form. Acetic acid is indeed a volatile compound with a vapor pressure of 2,7 kPa at 30°C ("Vapor Pressure Calculation by Antoine Equation (Acetic acid)," n.d.)) when present under its protonated form. Acetic acid has a pKa of 4,76 and since pH was kept around 5,5 during the most of the experiment, a significant amount of C2 was present in its protonated form.

$$\mathrm{pH} = \mathrm{pKa} + log\left(\frac{base}{acid}\right)$$

$$\frac{base}{acid} = 10^{(pH-pKa)}$$

So 15% of the C2 present in the mixed liquid was present in its protonated form.

In addition, through Henry's law, presented below, the concentration in the liquid and the gas partial pressure above the liquid are proportional.

$$H^{cp} = \frac{c_a}{p}$$

Hence, at the beginning of the experiment, during the stabilisation period, the high concentrations (up to 16 g_{COD}.L_{ML}^{-1}) may have increased the effects of the C2 transition to the gas phase. Moreover, the loss of C2 through gas would go unnoticed since the gas chromatograph used to analyse gas composition only detects H_2, O_2, N_2, CO_2, CH_4 and water vapour. This could explain the high C2 losses that are not recovered in the balance, especially during the stabilisation period were concentrations were higher. However, further analysis of the gases present in the EF headspace need to be carried out to confirm this hypothesis.

Finally, the cathodic compartment of the reactor presented several heterogeneities. Biomass was floating on the surface or attached to the emerged parts of the cathode, membrane or reactor walls. Microbial growth in these areas is a net loss in COD in the liquid mixture since elements are taken up by the biomass out of the fermentation broth and are not measured anymore in the liquid analysis.

Another interesting point to look at is the high loss of C2 through sampling during the Fed-Batch Period. Figure 4-19 shows indeed that the collected C2 through sampling was almost equal to the addition of C2 during the Fed-Batch Period, questioning the volume and frequency of the Fed.

4.1.2.4 C4 production

Butyric acid was already produced early during the experiment 0,41 $g_{COD}.L_{ML}^{-1}$ at day 9. However, butyric acid is found as a product of many biological process beside S-MCFAs elongation. It is for instance one of the main products of the acidogenesis phase of the anaerobic digestion at acidic and neutral pH (Horiuchi et al., 2002). However, since the fermentation broth was made out of sludge from a WWT plant, the biodegradable biomass available for anaerobic digestion is limited. Furthermore, only minor decreases in unidentified COD were observed.

The role of butyrate as a precursor for the elongation of longer S-MCFAs seems to be enlightened by the drops in concentration during the periods were longer S-MCFAs concentrations increased. The most striking example is during the days 57 to 60, were a massive production of C8 and an overall decrease in C4 concentrations happened simultaneously.

Maximum observed concentration of C4 was 1,36 $g_{COD}.L_{ML}^{-1}$ (0,76 $g.L_{ML}^{-1}$) and found on day 53 of the experiment. The highest production rate for C4 was found on day 50 with a production of 0,2032 $g_{COD}.L_{ML}^{-1}.d^{-1}$ (0,11 $g.L_{ML}^{-1}.d$). These results are lower than the ones achieved in a similar study where Van Eerten-Jansen et al. (2013) found a maximum concentration of 0,263 $g.L_{ML}^{-1}$. However, Van Eerten-Jansen et al. (2013), in this experiment, the production of longer S-MCFAs was targeted, C4 being an intermediate rather than a final product. Other studies using different kinds of electron providers like ethanol show much higher concentrations. For example, when yeast-fermentation beer (unprocessed fermentation broth with undistilled, dilute ethanol from the corn kernel-to-ethanol industry) was used as a substrate, C4 concentrations up to 11,6 $g_{COD}.L_{ML}^{-1}$. Finally, another type of EF coupled with the use of an electron mediator under the form of neutral red achieved a maximum butyrate productivity of 0,31 and 0.28 $g.L_{ML}^{-1}.h^{-1}$ (7,44$g.L_{ML}^{-1}$ d and 6,72$g.L_{ML}^{-1}$) respectively for a reactor without and with pH control (Choi et al., 2012).

4.1.2.5 C6 production

C6 can be a targeted product since it is the first MCFA and has already very interesting properties. Maximum concentration obtained during the experiment was 1,2 $gCOD.L_{ML}^{-1}$ (=0,54 $g.L_{ML}^{-1}$) while maximum production rate was reached on day 52 with 0,22 $gCOD.L_{ML}^{-1}.d^{-1}$ (0,1 $g.L_{ML}^{-1}.d^{-1}$). In another EF experiment without electron mediator, C6 concentrations reached only 0,036 $g.L_{ML}^{-1}$ (=0,08 $gCOD.L_{ML}^{-1}$) (Van Eerten-Jansen et al., 2013). As a comparison, Ge et al. (2015) obtained with a fermentation from yest-fermentation beer (thus with ethanol as an electron donor) a maximum production of 3.38 ± 0.42 $g.L_{ML}^{-1}$ d (7.52 ± 0.94 $gCOD.L_{ML}^{-1}$ d^{-1}) thanks to the in-line continuous extraction system that was implemented into the system. Such high production rates however were only reached after 500 days of run and several modifications in the way the reactor operates.

Hence, electricity as an electron provider turned out to be more effective than in sort like experiment but not as the best results published about elongation where ethanol was used as an electron donor.

4.1.2.6 C8 production

C8 was the most important product of the experiment. C8 concentration reached its maximum on day 55 with 2,96 $g_{COD}.L_{ML}^{-1}$ (=1,21 $g.L_{ML}^{-1}$) while the highest production rate was found on day 52 with 0,75 $g_{COD}.L_{ML}^{-1}$ (=0,31 $g.L_{ML}^{-1}$). No other EF experiment ever published showed such concentrations and production rates. However, other EF experiments, like Van Eerten-Jansen et al. (2013) ran for only 20 days and their maximum concentration of 0,739$g.L_{ML}^{-1}$ was already reached after 4 days when no traces of C8 were detected in our EF experiment.

A relation between a decrease in C6 and an increase in C8 concentration has not been enlightened as between C4 and C8. However, if C8 is produced through β-reverse oxidation by the elongation of C6 as is suspected, C6 should be consumed in the periods were C8 was produced. Yet no decrease in C6 has been reported, and even an important increase in C6 production is showed on figure 2, simultaneously to the C8 production on day 57. An explanation for this could be the conditions promoting C8 formation are also favourable for the C6 synthesis and both happen at the same time. Hence, C6 production could exceed the consumption needed for C8 production, making the overall concentration of C8 increase.

4.2 NITROGEN REACTORS

4.2.1 Results

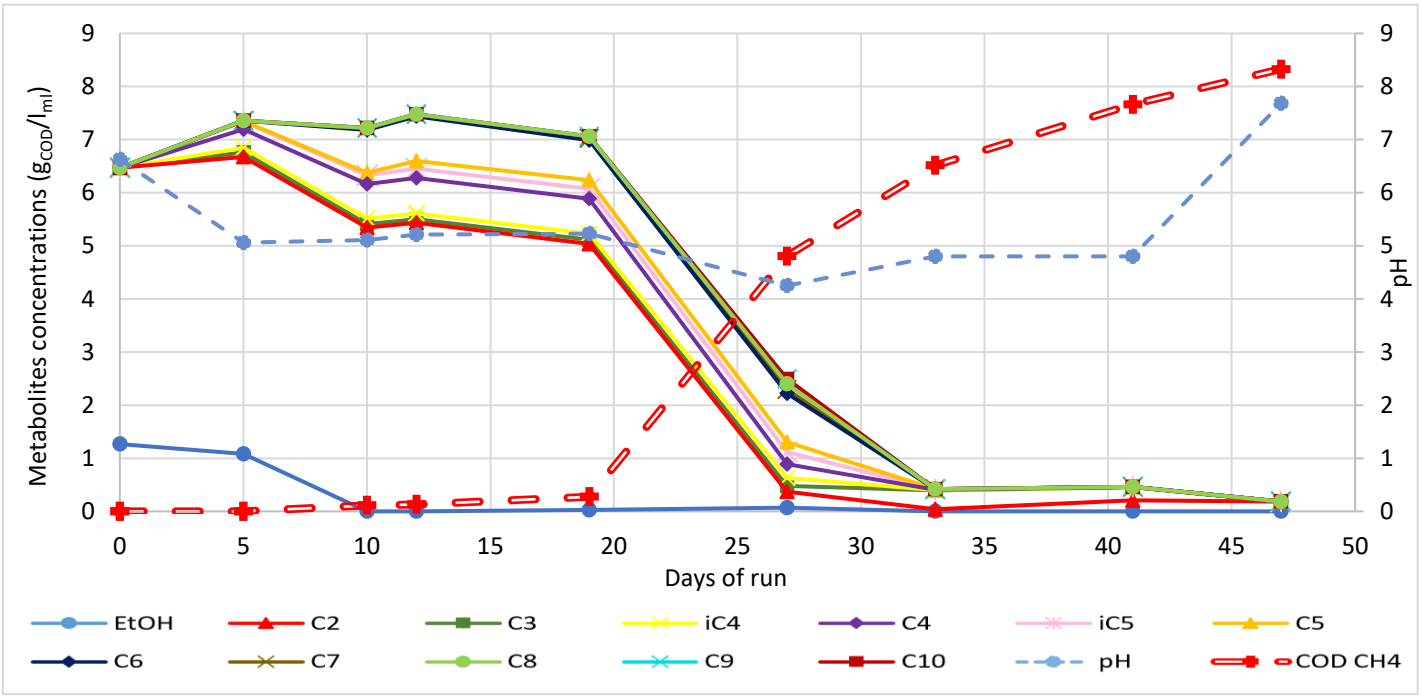

Figure 4-8 : Evolution of pH and S-MCFAs concentration as well as time cumulated CH4 production (gCOD/ l of mixed liquor) during the experiment in the N2 batch reactor n°1. Metabolites are presented as cumulated contribution by order of carbon number: C2 on top of EtOH, C3 on top of C2, C4 on top of C3, etc.

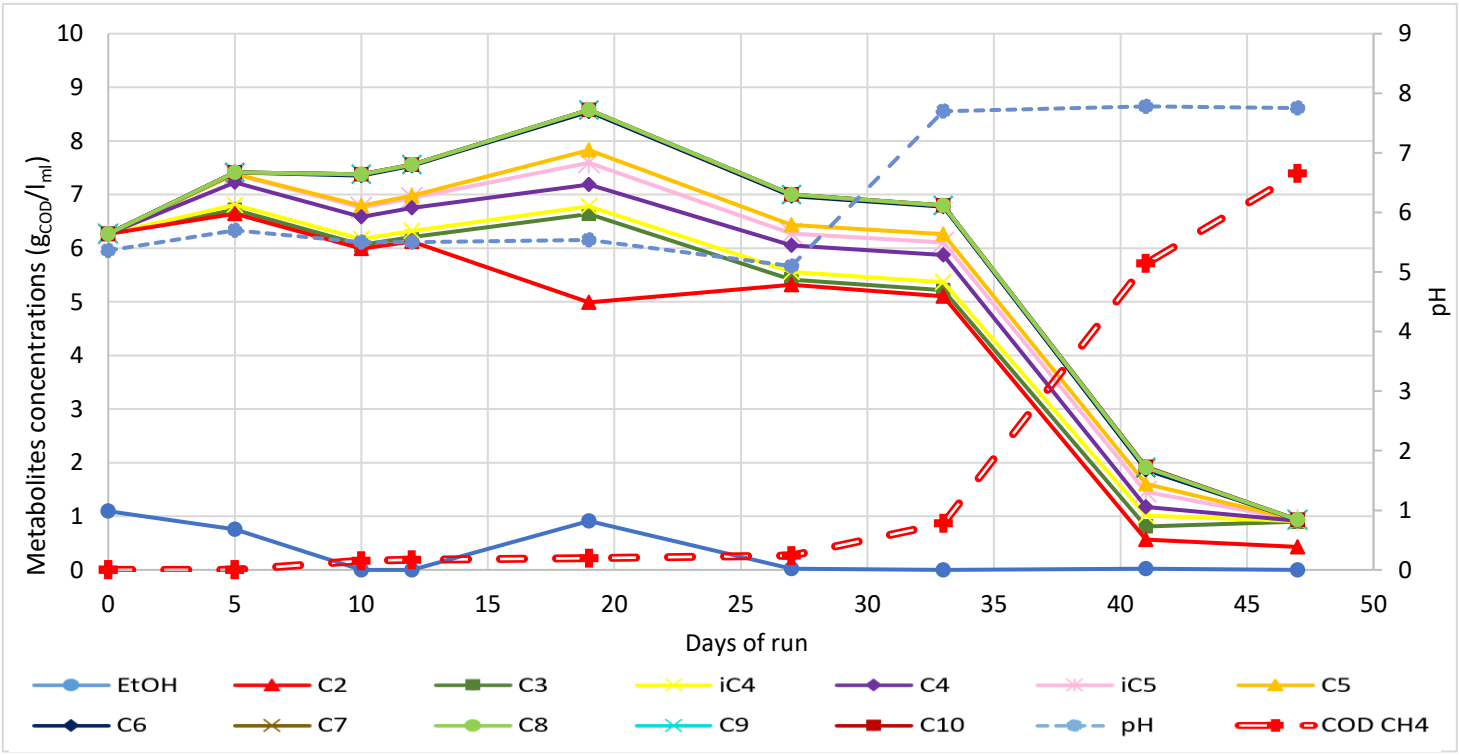

Figure 4-9 : Evolution of pH and S-MCFAs concentration as well as time cumulated CH4 production (gCOD/ l of mixed liquor) during the experiment in the N2 batch reactor n°2. Metabolites are presented as cumulated contribution by order of carbon number: C2 on EtOH, C3 on top of C2, C4 on top of C3, etc,…

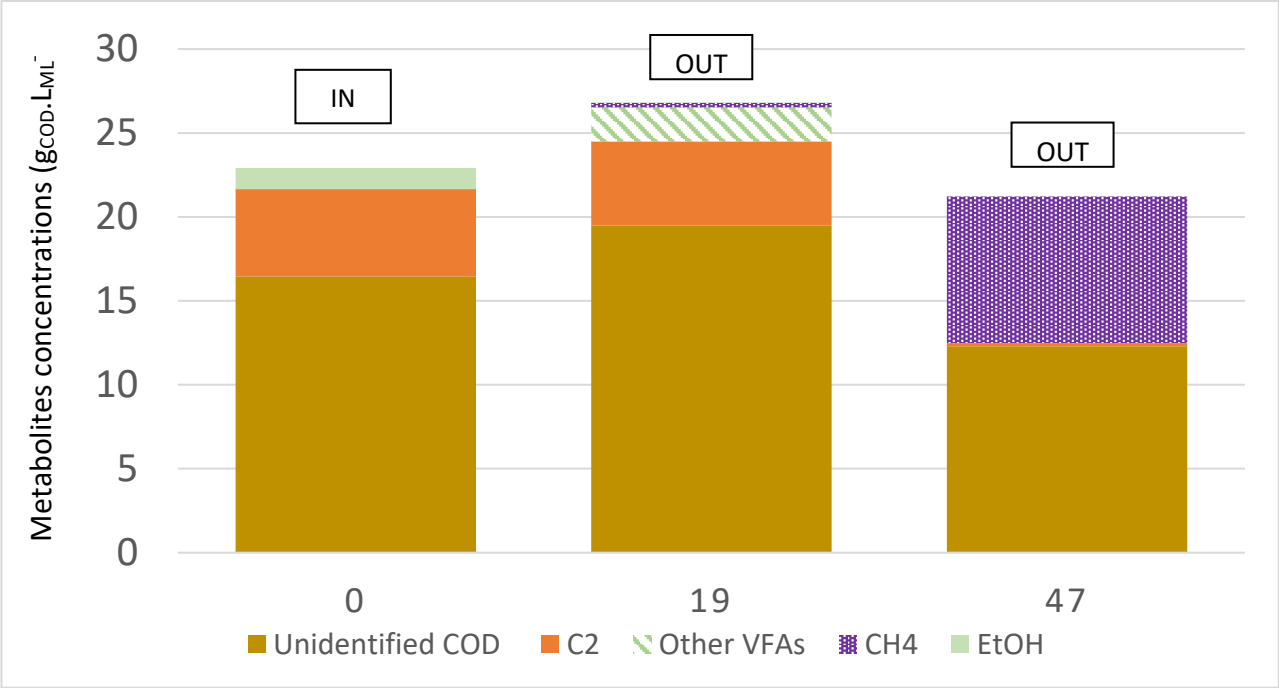

Figure 4-10 : COD Balance between day 0, 19 and 47 corresponding to the beginning of the experiment, after the S-MCFAs production period and at the end of the experiment, for nitrogen reactor N2A.

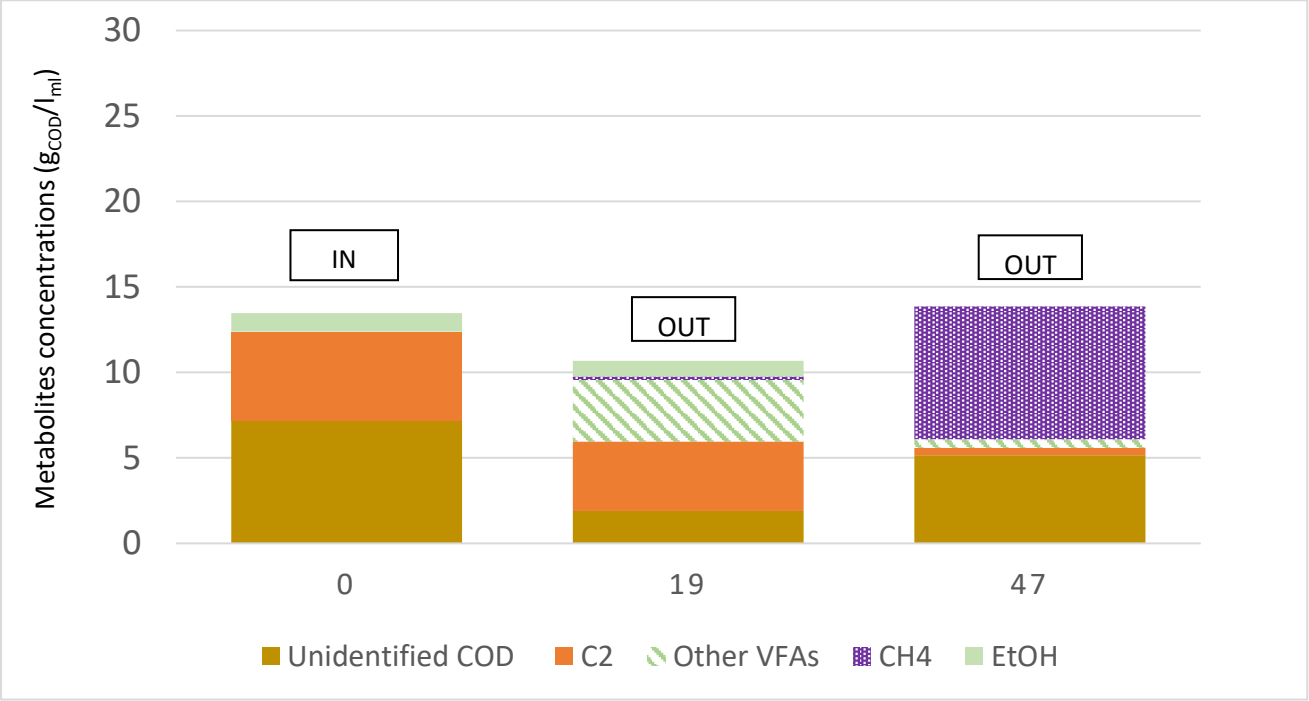

Figure 4-12 : COD Balance between day 0, 19 and 47 corresponding to the beginning of the experiment, after the S-MCFAs production period and at the end of the experiment) for nitrogen reactor N2B.

Residual ethanol was present at the start in both reactors at concentrations of 1,18 ± 0,085 $g_{COD}.L_{ML}^{-1}$ and was consumed within 10 days. Reactor N2B showed again traces of ethanol around day 19 up to 0,91 $g_{COD}.L_{ML}^{-1}$.

In both nitrogen PR, C2 levels started from 5,188 ± 0,018 $g_{COD}.L_{ML}^{-1}$ and remained steady before suddenly dropping down to almost 0. However, both reactors did not see their C2 concentrations fall simultaneously. N2A concentrations dropped indeed on day 19 while the same drop occurred in reactor N2B only 14 days later on day 33.

C4 concentrations already increasing at day 5 in both reactors. They however stabilised quickly between 0,4 an 0,6 $g_{COD}.L_{ML}^{-1}$ until day 19 and 33 respectively for reactor H2A and H2B where concentrations dropped dramatically .

C6 levels rose to reach maximum levels on day 27 with 0,921 $g_{COD}.L_{ML}^{-1}$ and 0,57 $g_{COD}.L_{ML}^{-1}$ on day 10, respectively for reactor N2A and N2B. C6 concentrations then dropped to 0 in both reactors after day 33.

C8 production was only marginally present during the experiment in nitrogen PR. A maximum concentration of 0,1 $g_{COD}.L_{ML}^{-1}$ on day 27 was observed in reactor N2A before fading away at the end of the experiment like the other S-MCFAs. No significant traces of C8 were found in reactor N2B during the experiment.

No C10 was found in any nitrogen PR during the experiment.

Besides the studied even carbon numbered S-MCFAs, C3 was also found during the experiment with concentrations reaching 0,415 ± 0.059 $g_{COD}.L_{ML}^{-1}$ before decreasing down to 0 like the other S-MCFAs.

Methane production started simultaneously with the strong decrease in S-MCFAs COD, thus at day 19 for N2A and day 33 for N2B. Methane concentration in the headspace reached 8,33 $g_{COD}.L_{ML}^{-1}$ and 7,39 $g_{COD}.L_{ML}^{-1}$ on day 47.

Unidentified COD evolution changed significantly between both reactors. In reactor N2A unidentified COD went from 16,45 to 27,9 to drop back to 12,48 $g_{COD}.L_{ML}^{-1}$ respectively on day 0, 33 and 47. In reactor N2B on the contrary, unidentified COD dropped first from 7,2 to 1,67 $g_{COD}.L_{ML}^{-1}$ between day 0 and 33 to increase again up to 5,16 $g_{COD}.L_{ML}^{-1}$ on day 47.

4.2.2 Discussion

4.2.2.1 Unidentified COD

One can notice that the reactor N2A started with more than twice as much unidentified COD than reactor N2B. Only an experimental error could explain such a difference. Since both 200ml fermentation broths present in each reactor were pre-treated together inside the same 1l Schott bottle along with the two fermentation broths for the hydrogen reactors. Only after the whole pre-treatment, the 1l fermentation broth was poured into each reactor, this to prevent any heterogeneity between the different reactors. If the values of the unidentified COD in the hydrogen reactors are compared with one another at day 0, 17,7 and 9,13 $g_{COD}.L_{ML}^{-1}$ for reactor H2A and H2B respectively, one can notice that they are very different too.

On the other hand, C2 concentrations in both reactors at the beginning of the experiment were similar (5,2 and 5,17 $g_{COD}.L_{ML}^{-1}$) confirming the homogeneity of both reactors since the acetic acid/acetate buffer was added before the two fermentation broths were divided. C2 concentrations observed at day 0 in the hydrogen reactors, 5 and 5,2 $g_{COD}.L_{ML}^{-1}$ for reactor H2A and H2B respectively, are also similar to one another and to the concentrations found in the nitrogen reactors.

Since solutes seem to be equally spread between all four reactors, a dilution after the separation into each reactor could not have happened. The problem can also not be blamed on an analytical error since different independent measurements on different days confirmed the first measurements.

An explanation for the unidentified COD differences can be suspend solids. Since the pre-treated fermentation broth was poured out of the bottle into each bottle, each soluble compound was divided equally. However, solid matter present in the fermentation broth would split in an uncontrolled way between each bottle. This problem could have been solved by centrifugation of the fermentation broth before pouring it into the reactors. This, however, brings a new problem since the aim of the activated sludge is to provide an undefined microorganism community to perform the fermentation and that centrifugation may eliminate the microorganisms of the broth as well. Visual observations during the fermentation however confirmed this hypothesis since dried suspended matter attached to the inside wall of the reactors has been observed several times in all of the PR. Further experimentation needs to be done to confirm this hypothesis and, if it were true, to solve the problem of solid matter in the fermentation broth.

4.2.2.2 Ethanol

Both reactors were launched with the unwanted presence of ethanol which was left over from the cleaning process. The presence of ethanol was quite problematic since the purpose of the nitrogen reactors was to be a control experiment with no external source of electron donors (besides the unidentified COD) to compare with the results of the hydrogen reactors. However, the results were still interesting since both reactors evolved likewise and that ethanol was only present in small amounts (1,26 and 1,1 $g_{COD}.L_{ML}^{-1}$ for reactor N2A and N2B respectively) compared to, for example, the electricity COD provided to the EF or the H_2 COD provided consumed by the hydrogen reactors.

4.2.2.3 S-MCFAs

Both reactors showed a short production period for S-MCFAs showing both the highest total concentrations (all S-MCFAs except C2) 3,59 and 2 $g_{COD}.L_{ML}^{-1}$ for the N2A and N2B reactors respectively. The S-MCFAs production mostly concerned C4 and C8. While plenty of different bio-synthesis routes could explain the production of C4, the C8 concentration increase is probably due to β-reverse elongation with the remaining ethanol playing the role of electron donor.

Ethanol consumption in both reactors was consumed on the same period the majority of S-MCFAs were produced. This could enlighten the fact that ethanol was used as an electron donor for the elongation of S-MCFAs and could explain why it stopped shortly after the beginning of the elongation.

All S-MCFAs present in the fermentation broth were consumed, probably by the methanogenesis, starting from day 19 for N2A and 27 for the N2B.

4.2.2.4 Methanogenesis

Methanogenesis occurred on day 19 and 27 and caused the progressive disappearance of the major part of the S-MCFAs present in the fermentation broth. Methane levels reached 8,74 and 7,77 $g_{COD}.L_{ML}^{-1}$ in reactor N2A and N2B respectively. Figure 10 and 11 demonstrates that methane on the last day of the experiment accounts for almost the entire loss in S-MCFAs (C2 included) and a part of the unidentified COD for reactor N2A.

The fact that both control reactors went through methanogenesis while it was avoided in the EF and the hydrogen reactors (see section 4.3.2.4) is encouraging. It shows that both EF and H2 reactors were able to block methanogenesis successfully starting from the same pre-treated fermentation broth containing no methanogenesis inhibitors.

4.3 HYDROGEN REACTORS

4.3.1 Results

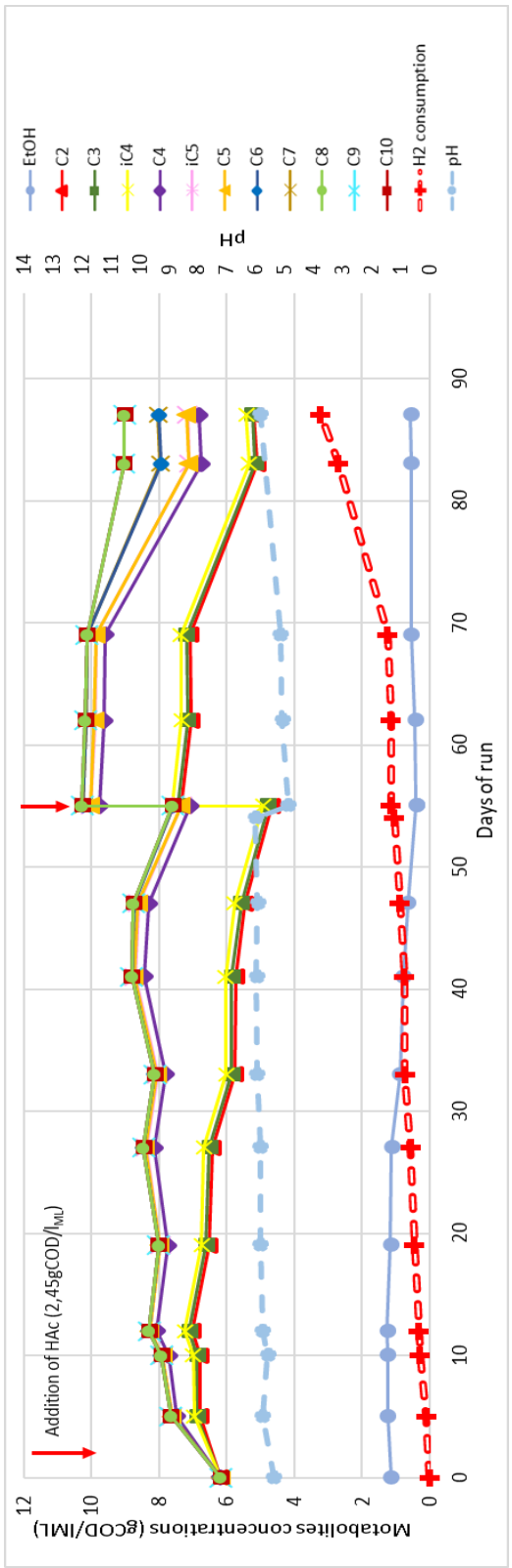

Figure 4-13 : Evolution of pH and S&MCFA concentrations as well as time cumulated H2 consumption (gCOD/ l of mixed liquid) during the experiment in the hydrogen Batch reactor N2A. Metabolites are presented as cumulated contribution by order of carbon number: C2 on top

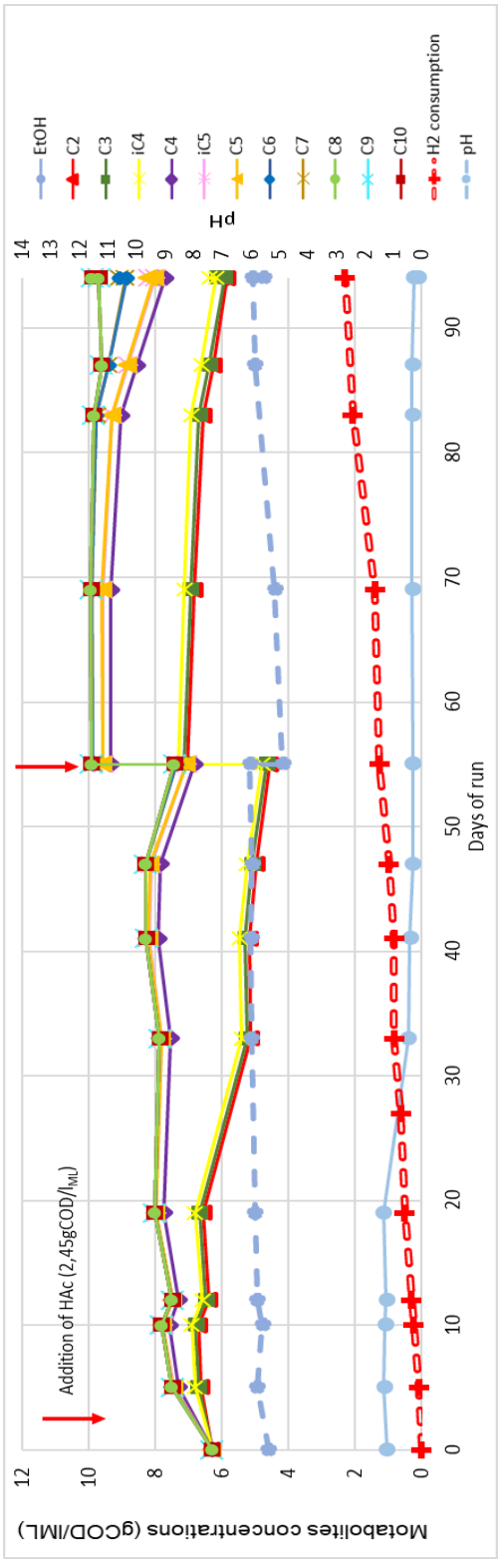

Figure 4-14 : Evolution of pH and S&MCFA concentrations as well as time cumulated H2 consumption (gCOD/ l of mixed liquid) during the experiment in the hydrogen batch reactor N2A. Metabolites are presented as cumulated contribution by order of carbon number : C2 on top.

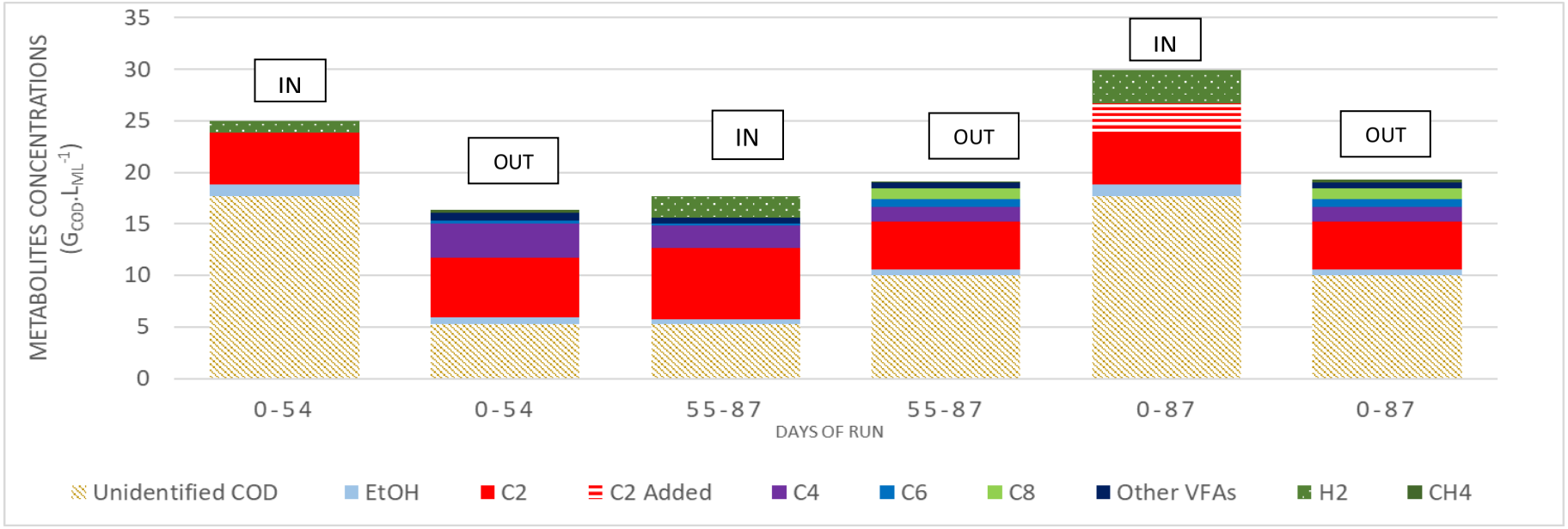

Figure 4-15 : COD Balance between day 0,41, 55 and 87 (The beginning of the experiment, in the middle of the S-MCFAs production period and at the end of the experiment) for nitrogen reactor H2A.

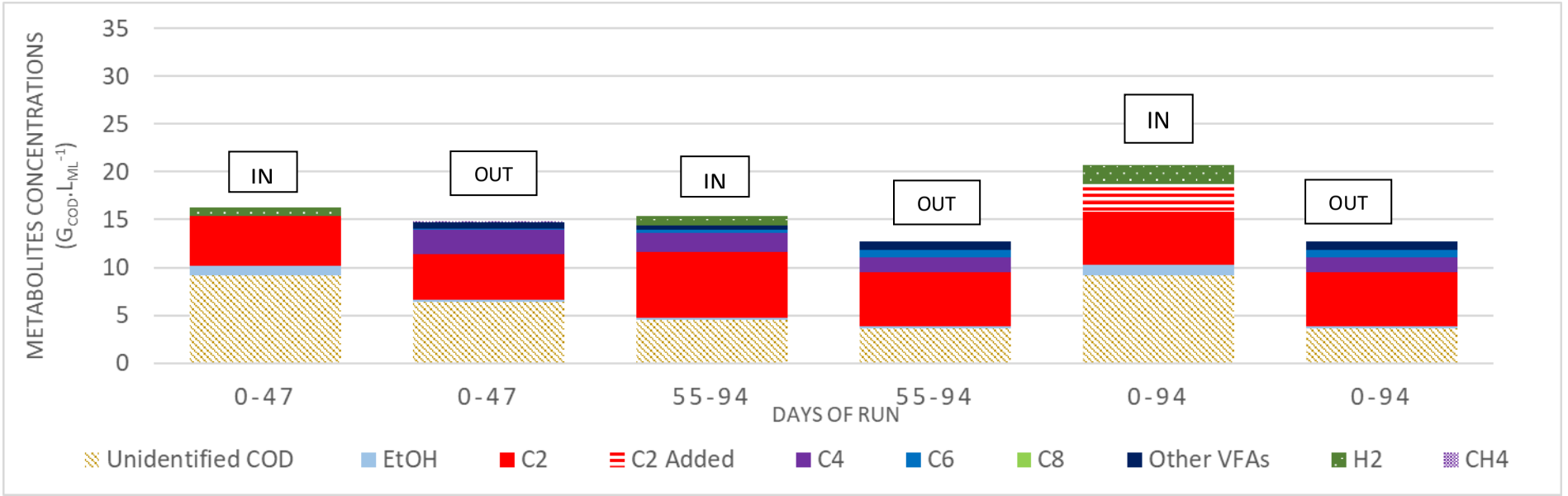

Figure 4-16 : COD Balance between day 0, 94 and 119 (The beginning of the experiment, in the middle of the S-MCFAs production period and at the end of the experiment) for nitrogen reactor H2B.

Hydrogen PR reactors experiments lasted 87 and 94 days respectively for reactor N2A and N2B. Both experiments were divided into two periods. The H2A experiment was divided into a first period from day 0 to day 54 and a second period from day 55 to day 87. While H2B experiment was divided in a first period from day 0 to 47 and a second one from day 55 to day 94. This division of both experiments is due to the addition, on day 55, of a 50 mL replenishment of acetic acid/acetate buffer to raise the C2 concentrations in each reactor by 2,45 $g_{COD}.L_{ML}^{-1}$. Volume of the mixed liquid due to previous samplings was then 100 mL. Hence, the addition of the replenishment caused a dilution of the fermentation broth by 1,5.

Residual ethanol from the cleaning process was initially present in both reactors at a concentration of 1,1 ± 0,04 $g_{COD}.L_{ML}^{-1}$. Ethanol concentrations dropped slowly in both reactors, mainly during the 50 first days before stabilising below 0,4 $g_{COD}.L_{ML}^{-1}$.

C2, after slightly increasing the first 10 days from 5,2 to 5,6 $g_{COD}.L_{ML}^{-1}$ saw its concentration drop to reach 4,69 $g_{COD}.L_{ML}^{-1}$ on day 47. After the replenishment, concentrations went from 6,885 ± 0,065 $g_{COD}.L_{ML}^{-1}$ to 4,62 $g_{COD}.L_{ML}^{-1}$ and 5,98 $g_{COD}.L_{ML}^{-1}$ respectively for reactor H2A and H2B.

Concerning other S-MCFAs, C4 concentrations grew at a constant rate in both reactors reaching a concentration of 2,57 ± 0,025 $g_{COD}.L_{ML}^{-1}$ on day 47. After the C2 replenishment, C4 levels went from 2,15 ± 0,06 $g_{COD}.L_{ML}^{-1}$ to 1,335 ± 0,2 $g_{COD}.L_{ML}^{-1}$.

A minor increase in C6 concentrations was also observed in both reactors was observed before the C2 replenishment reaching 0,21 and 0,40 $g_{COD}.L_{ML}^{-1}$ respectively for reactor H2A and H2B. After the dilution, C6 levels kept on rising up to 0,82 and 0,87 $g_{COD}.L_{ML}^{-1}$ respectively for H2A at day 87 and H2B at day 94.

While no C8 was observed in both reactors before the day of the dilution, concentrations increased dramatically at the end in the last 10 days of each experiment reaching 0,89 ± 0,09 $g_{COD}.L_{ML}^{-1}$.

Besides the studied even carbon number S-MCFAs, iC4 and iC5 respectively reached 0,21 ± 0,02 $g_{COD}.L_{ML}^{-1}$ and 0,345 ± 0.025 $g_{COD}.L_{ML}^{-1}$ on day 55 and stabilised after the dilution.

No other S-MCFAs were detected during this period.

Unidentified COD went from 17,7 to 10 gCOD.L_{ML}^{-1} in reactor H2A while it dropped from 9,13 to 3,62 gCOD.L_{ML}^{-1} and from 9,13 to 3,62 gCOD.L_{ML}^{-1} in reactor H2B.

Finally, gas analysis showed an overall consumption in H_2 3,22 gCOD.L_{ML}^{-1} in the H2A and 2,07 gCOD.L_{ML}^{-1} in H2B over the whole experiment. CH_4 traces were barely detectable all along the experiment in both reactors and no traces of CO_2 were found during the experiment. As showed on figure 13 and 14, H_2 consumption was rather low before the dilution and rose after the dilution until the end of the experiment.

4.3.2 Discussion

4.3.2.1 Unidentified COD

As explained in the unidentified COD section for the nitrogen reactors, initial unidentified COD was different between the two hydrogen reactors as well. It however gradually decreased during the experiment in both reactors, 17,7 to 10 $g_{COD}.L_{ML}^{-1}$ for the H2A reactor and 9,6 to 3,62 $g_{COD}.L_{ML}^{-1}$ for reactor H2B. The decrease could be partially explained by the dilution but if this was the only factor, unidentified COD would rather be around 11,8 and 6,4 gCOD.L_{ML}^{-1} respectively for reactor N2A and N2B.

The role of Unidentified COD seems unclear since in both reactors, unidentified COD difference seems to account for a big part of the lack of recovery in the COD balance.

In addition, both reactors show an unidentified COD decrease before the C2 addition. However, after the addition an increase in unidentified COD has been noticed in reactor H2A (almost double). This could be explained by the heterogeneity of the fermentation broth. During the experiment, solids particles indeed were stuck onto the interior wall of the reactor due to agitation and dried like in the nitrogen PR. The dried particles could be resuspended or not depending on how the bottle was manipulated just before the sampling. Furthermore, this enlightens once more the fact that suspended particles are problematic for the total COD measurements.

4.3.2.2 S-MCFAs

C2 has been consumed in both cases and its role as a substrate for the elongation of S-MCFAs seems to be confirmed, especially when the overall consumption of C2 during the experiment is compared to the production of C4, C6 and C8. For the H2A reactor for example, 2,86 $g_{COD}.L_{ML}^{-1}$ of C2 were consumed during the whole experiment while 3,2 $g_{COD}.L_{ML}^{-1}$ of total S-MCFAs were produced. For the H2B reactor, 2,09 $g_{COD}.L_{ML}^{-1}$ of C2 were consumed while a total sum of 3,16 $g_{COD}.L_{ML}^{-1}$ of C4, C6 and C8 had been produced at the end of the experiment.

C4 maximum concentration obtained during the experiment were and 3,24 and 2,85 $g_{COD}.L_{ML}^{-1}$ respectively for reactor H2A and H2B and were both found on day 54. Compared to the when compared to the EF, the maximum was observed on roughly the same day (day 53 for the EF) but concentration was much higher, since the EF only showed a maximum concentration of 1,36$g_{COD}.L_{ML}^{-1}$.

C8 however was produced in a smaller extent. The maximum concentrations were both found on day 94 with 1,33 and 0,8 $g_{COD}.L_{ML}^{-1}$ for reactor H2A and H2B respectively, which is much lower than the 2,96 $g_{COD}.L_{ML}^{-1}$ measured on day 55 in the EF. This could show that EF are be more effective for the elongation of C8 resulting also in lower C4 concentrations since C4 is consumed to produce C8. However, the comparison needs further research and experiments need to be repeated before such a statement can be made.

4.3.2.3 Ethanol

Since the aim of the experiment was to test the influence of H_2 on the elongation of S-MCFAs, the presence of ethanol in the initial fermentation could have been problematic. However, the rather small amounts of ethanol were initially present 1,1 ± 0,04 $g_{COD}.L_{ML}^{-1}$ and their partial consumption can't explain entirely the S-MCFAs elongation. 0,58 and 0,87 $g_{COD}.L_{ML}^{-1}$ were consumed during the experiment respectively by the H2A and H2B reactor during the experiment. As said in the state of the art, if acetic acid and ethanol are used for elongation, for every mole of butyrate produced, 4/5 moles of C2 and 6/5 moles of ethanol are needed. and moles of butyrate could have been produced in H2A and H2B respectively from ethanol, accounting for 0,80 and 1,21 $gCOD.L_{ML}^{-1}$. This calculation only is limited by the fact that it considers that the entire consumption of ethanol was used for C4 production and that C4 was not consumed for further elongation of S-MCFAs. Hence, an additional source of reducing equivalents is needed to explain the rest of the production of C4 and of other S-MCFAs.

4.3.2.4 Methanogenesis

Methanogenesis seems to be largely inhibited since only traces of methane were detected. Since the hydrogen reactors were incubated in the same conditions as the nitrogen reactors (apart from the different unidentified COD), the fact that the first ones were able to inhibit methanogenesis while the second ones were unable to, enlightens a link between S-MCFAs elongation and methanogens inhibition. Elongation products could lead to the inhibition but this is not the case of C4 since it was also present in the nitrogen PR. However, C8 was only present in the EF and hydrogen PR, and could have led to methanogenesis inhibition. This hypothesis was confirmed in an experiment where C8 was found to be slightly inhibitory. (Koster and Cramer, 1987)

4.3.2.5 Role of H_2

While ethanol can possibly have taken part into the S-MCFAs elongation in terms of reducing equivalents, it is clear that H_2 has had a role in S-MCFAs elongation when results are compared between H2 and N2 reactors. However, no evident link can be made between a high H_2 consumption rate and a high S-MCFAs production rate like for the EF since both were stable all over the experimental period for both reactors. It is however clear that H_2 played a role since C8 was synthesised in both H2 reactors and in none of the N2 reactors. The link between H_2 and elongation may however not be direct. The assimilation of molecular H_2 was indeed found to use ethanol as an intermediate chemical. (Agler et al., 2011) This intermediate ethanol could have then be used as an electron by the microorganisms to elongate S-MCFAs. Further study is needed to enlighten how molecular hydrogen is used for S-MCFAs and if this mechanism is involved in electro-elongation.

5 CONCLUSION

The aim of this work was to test the capacity of the electro-fermentation technology to elongate S-MCFAs with an acetic acid substrate and electricity in order to produce in a cheap, sustainable way the platform chemicals of tomorrow : the carboxylates. While other studies focused mainly on the production of butyric and caproic acid, the targeted product for this experiment was caprylic acid because of its better energy content, useful for the production of efficient biofuels, and its higher hydrophobicity, enabling product separation from the fermentation broth.

Hence an electro-fermenter was build and ran for 142 days under a constant current of 0,04A.

The second aim of the work was to investigate the possible role of molecular hydrogen, which is produced within the fermentation broth by water electrolysis, on the elongation of S-MCFAs.

Maximum concentrations of the targeted S-MCFAs during the EF experiment were : 0,74, 0,54 and 1,2 $g.L_{ML}^{-1}$ (= 1,36, 1,2 and 2,96 $g_{COD}.L_{ML}^{-1}$) respectively for C4, C6 and C8 and were observed around day 55 of the experiment.

The hydrogen pressurised reactors produced 1,77 ±0,007, 0,37±0,002 and 0,44±0,15 $g.L_{ML}^{-1}$ (2,9 ±0,45, 0,82 ±0,005 and 1,06±0,38 $g_{COD}.L_{ML}^{-1}$) of respectively C4, C6 and C8 observed around day 55 as well.

C8 concentrations as the ones obtained in this work were, to our knowledge, never reported before with bio-electro fermenters. However, elongation only occurred after 45 days in the electro-fermenters.

The second type of reactor enlightened that molecular hydrogen could be used by the microorganisms to elongate S-MCFAs effectively. However the direct supply of electrons through the cathode was found to be more efficient for the production of C8.

Methanogenesis inhibition is also a crucial factor for the elongation of S-MCFAs since it could compete and overrun the targeted process. In this work, methanogenesis was avoided by the heat pre-treatment of the broth at 70°C for 2 hours and by keeping the pH below 6. Nevertheless ,control reactors, fed with the same fermentation broth as the previous reactors and pressurised with nitrogen both went through methanogenesis without clear evidence of S-MCFAs elongation. This could bring into light a link between S-MCFAs elongation and an inhibitory effect on methanogenesis. C8 was indeed already reported as an inhibitor for

methanogens, meaning the process could maintain itself without the need of an additional methanogenesis inhibitor.

A major drawback of this study however, is the lack of control over the bio-electro elongation process resulting in a lack of reproducibility. EF were indeed launched several times without obtaining any results, thus making the achievement of duplicates impossible. Nevertheless, results obtained with the EF are still very encouraging and bio-electro-fermentation could surely play a future role in the sustainable production of carboxylate platform chemicals, one of the key alternatives to oil-based chemistry.

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Bio-electro-elongation of small and medium chain fatty acids
Submitted by Christophe Lycops

Abstract

With the need to rethink our fossil carbon-based societies, numerous alternatives emerge all around the globe. Among the exploited fossil resources, fossil oil is the most widespread energy source for transportation, with a growing demand at least until 2040. It also underlies the majority of the current chemicals. To replace sustainably fossil oil as a raw material in these sectors, one of the most promising answers are bio sourced chemicals produced by Biorefineries. By integrating the model of the oil refineries and petrochemical industry into a bio-based extraction and transformation industry, biorefineries are able to produce both bio- based fuels and bio-based platform chemicals to replace fossil-fuel derivatives. These platform chemicals serve as the chemical building blocks to produce a wide range of more complex chemicals. One of these chemical platforms is the carboxylate platform. Carboxylates, especially short and medium chain fatty acids (S-MCFAs, from 2 to 10 carbons in the chain), have a very wide range of applications and can be produced from biowaste streams, making them the perfect candidate for a sustainable bio-based chemistry.

In this work, the aim was to test the ability of a bio-electro-fermenter (EF) inoculated with a natural microbial community to produce in an inexpensive way S-MCFAs with acetic acid as a feed, and electricity as an electron donor. The anaerobic microbial pathway producing S- MCFAs is supposed to be the β-reverse oxidation cycle which elongates S-MCFAs by the addition of an acetyl-coA, adding two carbons to the chain. The second aim of the work was to test the influence of in situ produced hydrogen through water electrolysis on the elongation of S-MCFAs.

The EF was built with a stainless steel cathode submerged in the buffered fermentation broth and a platinum-iridium coated anode submerged in a 0.1 M K2SO4 solution. Both compartments were separated by a cation exchange membrane and a constant current of 0,04A was applied to the system. To test the influence of molecular hydrogen, another type of reactor was created where the same type of fermentation broth was exposed to dihydrogen (or dinitrogen for the control reactor) in a pressurised reactor.

Maximum concentrations of the targeted S-MCFAs during the EF experiment were : 0,74, 0,54 and 1,2 $g.L_{ML}^{-1}$ (= 1,36, 1,2 and 2,96 $g_{COD}.L_{ML}^{-1}$) respectively for C4, C6 and C8 carboxylates. They were observed around day 55 of the incubation. To our knowledge, such high levels of C8 were never reported with bio-electro-fermentation processes before. Control reactors with pressurised with N_2 went through methanogenesis while methanogenesis was inhibited in the EF and H_2 reactors where elongation occurred. The H_2 reactors showed concentrations up to 1,77 ±0,007, 0,37±0,002 and 0,44±0,15 $g.L_{ML}^{-1}$ (2,9 ±0,45, 0,82 ±0,005 and 1,06±0,38 $g_{COD}.L_{ML}^{-1}$) of respectively C4, C6 and C8 observed around day 55 as well. Hence, H_2 instead of electrons was able to play the role of an electron donor but MCFAs were produced at lower levels than in the EF.

While these results are very encouraging, a lot of improvements have still to be made, especially regarding to the reproducibility of the results of the EF. The long pre-production period should also be investigated in order to be shortened and to make S-MCFAs bio-electro-synthesis an economically and sustainable alternative solution to fossil-fuel based chemistry. On the long term, this type of system could be further implemented into a broader process were the acetic acid will be produced in situ by the combination of anaerobic digestion and inhibition of the methanogenesis.