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Microbial elongation of short chain carboxylates: investigation with environmental inoculum and complex biomass substrate

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I. Abbreviation list

BER	bio-electro-fermenter
BP	beet pulp
BSG	brewer's spent grain
BuOH	butanol
COD	chemical oxygen demand
C2	ethanoic or acetic acid
C3	propionic acid
C4	butyric acid
C5	pentanoic or valeric acid
C6	hexanoic or caproic acid
C7	heptanoic acid
C8	octanoic or caprylic acid
C9	nonanoic acid
C10	decanoic acid
EtOH	ethanol
ECFA	even chain fatty acids
FID	flame ionization detector
FreBSG	fresh brewer's spent grain
FroBSG	frozen brewer's spent grain
BSG+T	fresh brewer's spent grain (95% $w_{\text{fresh mass}}/w$) with trub (5% $w_{\text{fresh mass}}/w$)
GC	gas chromatography
HRT	hydraulic retention time
iC4	iso-butyric acid
iC5	iso-valeric acid
MCFA	medium chain fatty acids
OLR	organic loading rate
PR	pressurized bioreactor
SCFA	short chain fatty acids
SRT	solid retention time
S&MCFAs	short and medium chain fatty acid
TCD	thermal conductivity detector
UCFA	uneven chain fatty acid

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1 Introduction

The global population grows and will grow for the upcoming decades. The United Nation expects to reach a world population of 11 billion people on earth in 2100 (United Nation, 2017). This demographic increase will lead to an increase of natural resources consumption, which will be a problem since most of those resources come from non-renewable feedstocks. Fossil fuels like coal, natural gas or oil are the best examples of finite source, their respective estimated feedstock are sufficient for 134, 52 and 50 years at the current consumption rate (BP, 2018). Oil is the most used energy source for the transport sector that represent 56% of the world's oil consumption. The petrochemical industry is the other huge consumer and account for 14% of world's oil consumption. This industry enables the transformation of carbon-based molecules into fuels, fertilizers, plastics, polymers and high value chemicals. Those chemicals allowed developed countries to reach a comfortable life standard: enhancement of food production, use of better construction materials, production of pharmaceuticals, creation of packaging and other consumer goods (International Energy Agency, 2009). No one wants to go back and lower its life standards; thus, a demographic increase will inexorably lead to an increase demand for chemicals.

Another consequence of population rise will be the increase of waste production. In 2012 about 1,3 billion tons of waste were produced worldwide, this number jumped up to 2,01 billion for the year 2016. Food and green waste account for 44% of the world waste production, paper and cardboard for 17% and plastics for 14% (Kaza *et al.*, 2018). Depending of their nature, waste disposal will be different: most of plastics and polymers will be recycled or incinerated for energy recovery. Cardboard or paper can be recycled with up to 65% recycling (American forest and paper association, 2018). Food and green waste can be composted or used along with food industry by-products for cattle feeding. Another way to valorize those fermentable waste is to use them in biorefineries and produce energy or valuable chemicals for the green chemistry sector. Biorefining can be defined as *“the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)”* (International Energy Agency, 2009). This work investigates the ability to valorize fermentable biomass by producing platform chemicals, especially carboxylates, in bio fermenters.

2 State of the art

Biomass processed in biorefineries can follow pathways passing through three main intermediate platform molecules: the syngas platform, the sugar platform and the carboxylate platform. Platform molecules can be defined as *“a chemical compound whose constituent elements originate wholly from biomass (material of biological origin, excluding fossil carbon sources), and that can be utilized as a building block for the production of other chemicals.”* The most common processes used to convert biomass to platform molecules are: thermal treatment, chemical-catalytic treatment, biological treatment and extraction (Farmer et Mascall, 2015).

2.1 The syngas platform

The principle of the syngas platform is to gasify the (lignocellulosic) biomass to syngas (CO, CO₂, H₂O, H₂ and CH₄) under high temperature (>700°C). Once the biomass is transformed in those small building blocks, various pathways exist to use them for the synthesis of high value chemicals like alcohols, dimethyl ether, gasolines, olefins and hydrocarbons. It has two main advantages: i) it can use a broad variety of biomass as carbon source ii) the syngas can be converted into chemicals with high efficiency. The drawback of the process is the huge energy consumption since gasification is an endothermic process (Dahmen *et al.*, 2017).

2.2 The sugar platform

The sugar platform uses biomass to produce sugars. The first-generation sugar platform uses sugar rich substrate like sugar cane, sugar beet or starch crops from which sugars are extracted. The second generation uses lignocellulosic rich substrate like straw or wood as substrate. In this case, the biomass has to be hydrolyzed by enzymes or acids in order to obtain glucose (Holtzapple et Granda, 2009). The main advantage of the first-generation over the second generation is that the sugar is easily extracted from the raw material. The negative aspect is the direct competition with the agro-industry sector (Cherubini, 2010).

2.3 The carboxylate platform

The carboxylates platform uses fermentable biomass like green waste or low value by-products from the agro-industry (like beet-pulp, brewer's spent grain, ...) in a fermentation process to produce carboxylates intermediates (acetate, butyrate, caproate, ...) (De Groof *et al.*, 2019). The fermentable biomass undergoes four successive transformation steps by

microorganisms. As shown in Figure 1, the first step is a hydrolysis, which breaks the fermentable by-products such as proteins, carbohydrates and lipids down to soluble monomers as amino acids, sugars, long chain fatty acids and glycerol.

The hydrolysis is followed by an acidogenesis that ferments those monomers to produce short chain fatty acids (SCFA) (mostly acetate, propionate and butyrate), alcohols, lactate, H₂ and CO₂. Inorganic compounds like ammonia and reduced sulfurs are also released during the acidogenesis.

The acetogenesis continues to convert the acidogenesis products into acetate, H₂ and CO₂. The carboxylate platform process can go a step further: under specific conditions, the acetogenesis can be followed by an elongation step, which enables the elongation of SCFA into medium chain fatty acids (MCFA), as shown in Figure 1 with the red pathway (Arslan *et al.*, 2016). The elongation leads to the formation of MCFA by elongating the carbon chain of SCFA. Few mechanisms of this process are known, but the elongating reactions of acetate and butyrate are shown in Table 1.

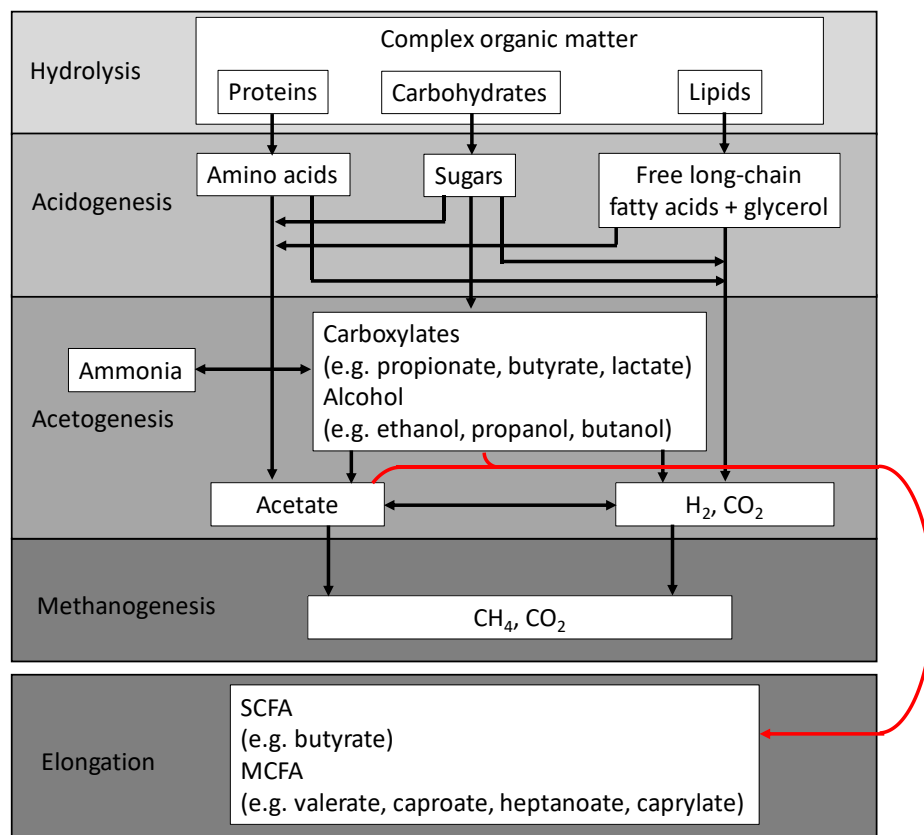


Figure 1 : Main steps of the anaerobic digestion of fermentable organic matter in mixed culture of microorganisms (adapted from Agler *et al.*, 2011 and Arslan *et al.*, 2016).

2.4 Anaerobic digestion

This process follows the same steps as the carboxylate platform until the acetogenesis (Figure 1). In anaerobic digestion, the acetogenesis is followed by a methanogenesis which transforms acetate and other small molecules into a gas mixture of CH₄ and CO₂ called biogas. The carboxylate platform tries to avoid the production of biogas since it consumes all the carboxylates produced.

2.5 Reactions occurring during a carboxylate chain elongation

Numerous reaction occurring during an anaerobic digestion are reported in the scientific literature, as shown in Table 1, but most of them are observed in single culture of microorganisms. A mixed culture is more complex, microorganisms are competing for the substrate, some rely on each other for electron transfer and the microbial community may also change over time (Hollister *et al.*, 2010). Consequences are that some reactions will only slightly occur, despite a favorable $\Delta G_r^{0'}$.

Table 1 : Biological reactions occurring during anaerobic digestion and carboxylate chain elongation.

Reaction	Microbe	Carboxylate conversion reaction	Coupled repetitions	$\Delta G_r^{0'}$ (kJ/mol _{carboxylate})
Homoacetogenesis	<i>Acetobacterium woodii</i>	$4H_2 + 2CO_2 \rightarrow acetate^- + H^+ + 2H_2O$		-86,78 ^b
Carboxylate reduction with molecular hydrogen	Observed in undefined mixed culture	$Propionate^- + H^+ + 2H_2 \rightarrow propanol + H_2O$		-7,49 ^a
		$n-butrate^- + H^+ + 2H_2 \rightarrow butanol + H_2$		-3,58 ^a
		$n-caproate^- + H^+ + 2H_2 \rightarrow hexanol + H_2$		-7,55 ^a
Aceticlastic methanogenesis	<i>Methanosaeta soehngenii</i>	$Acetate^- + H^+ \rightarrow CH_4 + CO_2$		-39,06 ^b
Chain elongation of lactate and acetate to n-butyrate	<i>Megasphaera elsdenii</i>	$Lactate^- + acetate^- + H^+ \rightarrow n-butrate^- + CO_2 + H_2O$		-59,43 ^b
Ethanol oxydation	<i>Clostridium kluyveri</i>	$Ethanol + H_2O \rightarrow acetate^- + H^+ + 2H_2$	x1	7,54
Reverse β oxydation		$Ethanol + acetate^- \rightarrow n-butrate^- + H$	x5	-191,37 ^c
Chain elongation of acetate into n-butyrate		Overall: $6 ethanol + 4 acetate^- \rightarrow 5 n-butrate^- + H^+ + 2H_2 + 4H_2O$		Total = -183,83 ^{bc}
Ethanol oxydation	<i>Clostridium kluyveri</i>	$Ethanol + H_2O \rightarrow acetate^- + H^+ + 2H_2$	x1	7,54
Reverse β oxydation		$Ethanol + n-butrate^- \rightarrow n-caproate^- + H_2O$	x5	-190,22 ^c

Chain elongation of n-butyrate into n-caproate		Overall: 6 ethanol + 5 n-butyrate ⁻ -> acetate ⁻ + 5 n-caproate ⁻ + H ⁺ + 2 H ₂ + 4 H ₂ O	Total = -184,95 ^{bc}
Hydrogenotrophic methanogenesis	<i>Methanospirillum hungatei</i>	4H ₂ + CO ₂ -> CH ₄ + 2H ₂ O	-125,84 ^b

^AΔG_R^{0'} values are taken from (Agler *et al.*, 2011)

^BΔG_R^{0'} values are taken from (Spirito *et al.*, 2014)

ΔG_R^{0'} values were calculated considering the reactants in aqueous phase with a concentration of 1M, except for H₂ and CO₂ which are gaseous at 1 atm, a pH of 7 and a temperature of 37 °C

^CΔG_R^{0'} is calculated for 5 moles of n-butyrate

2.6 Microorganisms involved in the elongation of carboxylates.

Today different microorganisms were found that can perform the elongation of carboxylates in pure culture, as listed in the Table 1 below.

Table 2 : Microorganisms able to produce MCFA by an elongation process.

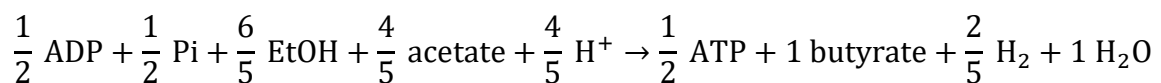
Microorganism	Origin	Reaction involved into	Electron donor	Reference
<i>Clostridium kluyveri</i>	Isolated from bovine rumen and canal mud	Elongation of acetate in caproate	Ethanol	(Barker et Taha, 1942; Weimer et Stevenson, 2012)
<i>Eubacterium pyruvativorans</i>	Isolated from sheep rumen	Elongation of propionate/n-butyrate in n-valerate/n-caproate	Amino acids, peptides, pyruvate	(Wallace <i>et al.</i> , 2003; Wallace <i>et al.</i> , 2004)
<i>Clostridium sp BS-1</i>	Isolated from Korean WWTP	Produce n-caproate from galactitol	Not analyzed	(Jeon <i>et al.</i> , 2013)
<i>Eubacterium limosum</i>	Isolated from sheep rumen	Convert CO ₂ and methanol into acetate, n-butyrate and n-caproate	Not analyzed	(Genthner <i>et al.</i> , 1981)
<i>Megasphaera elsdenii</i>	Isolated from sheep rumen	Ferment DL-lactate, glucose, fructose and sucrose into acetate, propionate, n-butyrate, n-valerate n-caproate, CO ₂ and H ₂	Not analyzed	(Elsden <i>et al.</i> , 1956; Choi <i>et al.</i> , 2013)
<i>Rhodospirillum rubrum</i>	Photosynthetic bacteria	Produce n-caproate during dark fermentation of pyruvate	Not analyzed	(Kohlmiller et Gest, 1951)

From those six bacteria, *Clostridium kluyveri* is the most studied one. Its mechanism of elongation has been identified as a reverse β oxidation (Spirito *et al.*, 2014). During this process, the carbon chain of carboxylic acid is elongated by two carbons at the time. The elongation can be repeated, adding 2 carbons at each step: acetate (C2) is elongated to n-butyrate (C4), n-butyrate (C4) to n-caproate (C6), n-caproate (C6) to n-caprylate (C8), ... Weimer and Stevenson reported that replacing acetate by propionate enhanced the production of n-valerate and n-heptanoate, suggesting that the reverse β oxidation also enables the elongation of odd chain fatty acids (Weimer et Stevenson, 2012).

2.7 Metabolic pathways of carboxylate elongation in *C. kluyveri*.

2.7.1 Elongation of acetate to n-butyrate under high substrate availability

As shown in Table 2 above and Figure 2 below, *C. kluyveri* uses ethanol as carbon and electron source, to elongate acetate in n-butyrate. The global reaction of this metabolic pathway is: 6 ethanol + 4 acetate $\rightarrow$ 5 n-butyrate + H^+ + 2 H_2 + 4 H_2O and has a $\Delta G^{0'}$ = -184,5 kJ/5 mol_{n-butyrate}. This Gibbs free energy is calculated considering 1 M of substrate and products, a H_2 partial pressure of 1 atm, a pH of 7 and a temperature of 25°C. This cycle requires the production of at least 2,5 ATP (the stoichiometry of Figure 2 is confirmed, as the synthesis of 1 mol of ATP costs 72 kJ, while 184,5 kJ are available: 2,5 x 72 = 180 kJ) (Angenent *et al.*, 2016). Table 3 below enable a better overview of the carbon and electron balance of the cycle and shows the global equation of the elongation of acetate to butyrate with ATP production. In order to compare the energy ratio between this condition and the one that follows, the equation is balanced for the production of 1 butyrate as shown below:



This cycle enables the production of 0,5 ATP/butyrate.

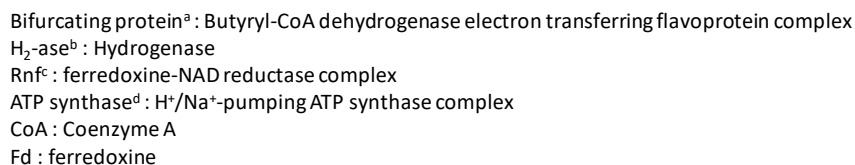


Table 3 : Carbon, electron, and substance balance of the elongation process in high substrate availability.

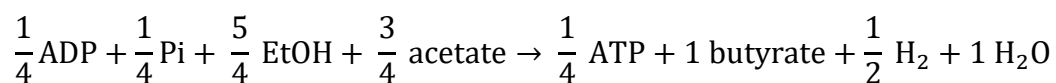
19

2.7.2 Elongation of n-butyrate to n-caproate under high substrate availability

Further elongation pathway with high substrate concentration and high H₂ partial pressure has not been enlightened yet despite it has been shown to occur (Schoberth et Gottschalk, 1969). Angenent et al tried out a model based on the pathway above to explain the production of n-caproate with hydrogen, acetic acid and ethanol but found themselves in a deadlock due to poorly exergonic reactions. However, this model only considered n-caproate as final product and Angenent et al suggested that an alternative pathway, with further elongation of carboxylates should exist that enable the production of n-caproate, n-caprylate or even longer fatty acids (Angenent *et al.*, 2016). This has been verified experimentally since production of n-caproate and n-caprylate from acetate and ethanol has been observed under high H₂ partial pressure (1 atm) (Arslan *et al.*, 2012).

2.7.3 Elongation of acetate to n-butyrate under low substrate availability.

However, high substrate concentration (> 1M) is quite exceptional in anaerobic fermenters and this is why the metabolic pathway of elongation of *C. kluyveri* has also been studied at low substrate and product concentration (1 mM) (other conditions remain the same: H₂ partial pressure of 1 atm, a pH of 7 and a temperature of 25°C) (Figure 3) (Angenent *et al.*, 2016). With those conditions, too few ATP are produced and the metabolic pathway shifts. The global reaction becomes: 5 ethanol + 3 acetate → 4 n-butyrate + 1 H⁺ + 2 H₂ + 3 H₂O with ΔG' = -77 kJ/4 mol_{n-butyrate}. This energy still enables the production of 1 ATP (1 mol of ATP cost 72 kJ/mol while 77kJ are available). Table 4 below enable a better overview of the carbon and electron balance of the cycle and shows the global equation of the elongation of acetate to butyrate with ATP production. In order to compare the energy with the high substrate availability condition, the equation is balanced for the production of 1 butyrate:



Elongation still occurs, but only 0,25 ATP/butyrate is produced, which is less than in the conditions with high substrate availability.

Those 3 observations led to the conclusion that high H₂ partial pressure could have no negative impact on the elongation process since *C. kluyveri* offers multiple alternatives for ATP synthesis (Angenent *et al.*, 2016).

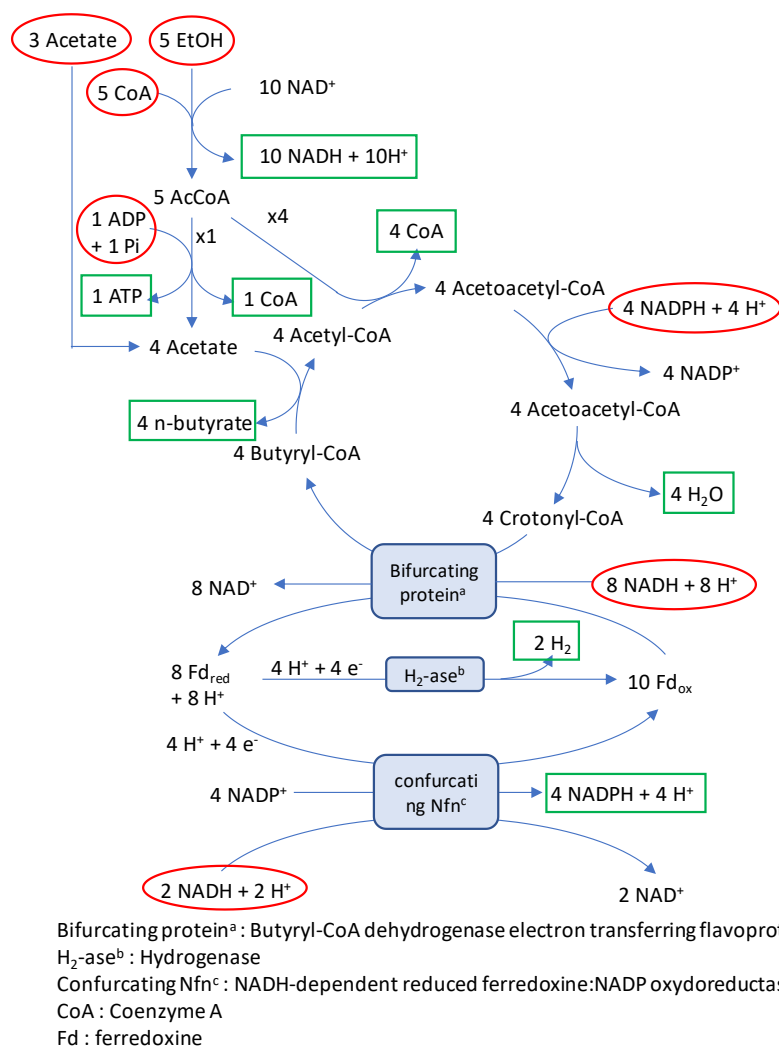


Figure 3: Elongation pathway of *C. kluyveri* under high H₂ partial pressure and low substrate conditions (Angenent et al., 2016). Red circle shows the inputs and green squares the output of the cycle.

Table 4 : Carbon, electron, and substance balance of the elongation process in low substrate availability.

IN			OUT		
Reactants	e ⁻	Carbons	Products	e ⁻	Carbons
1 ADP + 1 Pi			1 ATP		
5 CoA			(1+4) CoA		
5 EtOH + 3 acetate	5x4 + 3x0 = 20	5x2 + 3x2 = 16	4 H ₂ O		
(8+2) NADH+H ⁺	(8+2) x2 = 20		4 butyrate	4x4 = 16	4x4 = 16
4 NADPH + 4 H ⁺	4x2 = 8		10 NADH+H ⁺	10x2 = 20	
			4 NADPH + 4 H ⁺	4x2 = 8	
			2 H ₂	2x2 = 4	
Total	48	16	Total	48	16
1 ADP + 1 Pi + 5 EtOH + 3 acetate			1 ATP + 4 butyrate + 2 H ₂ + 4 H ₂ O		

2.8 Parameters influencing the elongation process

2.8.1 Substrate composition

Substrate composition is a key parameter for the fermentation process and determines which products will eventually be obtained. It can be synthetic and directly added to the reactor or more complex but still biodegradable. The intermediates required for the elongation process can be obtained by the fermentation of complex substrate (De Groof *et al.*, 2019). Three major types of substrates were studied in the literature: carbohydrates, lipids and proteins. Protein rich substrates tend to produce more odd carbon numbered fatty acids during the fermentation, with up to 78%¹ of propionate and 20-30% of n-valerate (Min *et al.*, 2005; Arslan *et al.*, 2016). In order to produce even carbon number chain carboxylates, protein rich substrate should be avoided. Carbohydrates rich substrates produce mainly n-butyrate, up to 60% with an operating pH between 5 and 6 and 70% with pH below 5. However, a pH > 6 or long batch time may lead to an important production of acetate at the expense of n-butyrate. Carbohydrate rich substrates leads to poor propionate production with a maximum of 30% (Arslan *et al.*, 2016). Lipid rich substrates have low S&MCFA production mainly due to their low hydrolysis (Arslan *et al.*, 2012). Odd number SCFA are more likely to produce odd number MCFA as n-valerate and n-heptanoate as exposed by Weimer et al (Weimer et Stevenson, 2012).

For complex substrate, hydrolysis rate is the limiting step of the fermentation; poorly hydrolysable substrate will need longer residence time in the reactor in order to be digested (Angenent *et al.*, 2016).

Various starting substrate concentrations are used in the studies and it is expected that the higher the substrate concentration, the higher the total volatile fatty acid (TVFA) production. Arslan *et al.* (2016) observed that the production capped at a maximum TVFA concentration of 20 g/L for substrate concentration higher than 40 g_{COD}/L.

Addition of ethanol in ethanol based elongation or lactate in lactate based elongation enhances the fermentation towards elongation (De Groof *et al.*, 2019). High ethanol to acetate ratio leads to more specific n-caprylate formation. Spirito *et al.* (2018) observed the highest specificity toward n-caprylate formation at a ratio ethanol-acetate of 275 g_{COD}/g_{COD}.

¹ Percentages are expressed in g_{fatty acid}/g_{total volatil fatty acid}

Elongation has also been observed in a reactor with *C. kluyveri*, acetate, molecular hydrogen and without ethanol addition. However, ethanol as intermediate was still observed and the n-caprylate production rate was lower than in a reactor with acetate and ethanol (Steinbusch *et al.*, 2008).

2.8.2 Gas composition

Different gas phases were tested in elongating anaerobic reactors: N₂, H₂, CO₂ or a mix. Based on multiple studies, Arslan *et al.* (2013) concluded that continuous N₂ sparging or flushing had no impact on S&MCFA production. However, intermediate N₂ sparging or flushing creates a disequilibrium of the dissolved gasses in the liquid phase and generates a stress on the microbial biomass that decreases the carboxylates yield. CO₂ addition has been reported to enhance the n-butyrate fraction in the TVFA (Arslan *et al.*, 2013). Some studies report that high H₂ partial pressure enhance the production of longer chain fatty acids (Arslan *et al.*, 2016). However, the production of n-caproate doesn't necessarily requires high H₂ partial pressure (Ding *et al.*, 2010).

2.8.3 pH

The pH is one of the most important parameters in the fermentation and elongation process. It determines which metabolic pathway are thermodynamically possible, which microorganism are able to survive and can also ease the hydrolysis of the substrate. The optimal pH for hydrolysis is between 5 and 7 (Aehle *et al.*, 2007). The optimal pH for the growth of some species of microorganisms is also known: a neutral pH is optimal for acetoclastic methanogens and *C. kluyveri* has been known to growth optimally between pH 6,4 and 7,4 (Weimer et Stevenson, 2012). However, high elongation rate has been reported between pH 5,25 and 5,5, while the highest production rate of n-caproate has been reported (8,17 g_{L_{reactor}}⁻¹d⁻¹) at pH 7 by Steinbush et al (Steinbusch *et al.*, 2010; Vasudevan *et al.*, 2014; Ge *et al.*, 2015). In the literature, the majority of the conditions used for elongation were in a pH ranging between 5 and 6 (Arslan *et al.*, 2016).

It has to be noted that acidifying acidogenesis can occur, which will lead to a decrease in pH. In those cases, addition of alkalinity is needed to maintain a constant pH.

2.8.4 Temperature

Temperatures for elongating reactors were tested between 25 and 55°C, but no clear trend has been enlightened (Arslan *et al.*, 2016). However, De Groof *et al.* (2019) reduced the temperature range between 30 and 45°C. The ideal temperature for the growth of *C. kluyveri* is 39°C (Wallace *et al.*, 2003).

2.8.5 Hydraulic retention time (HRT) in fed-batch or continuous reactors

In the studies reviewed, the (semi)-continuous systems were continuously stirred, meaning that the HRT is equal to the solid retention time (SRT). The SRT is the mean duration that the solid substrate remains in the reactor. Most reactors operated at an HRT of 3-50 hours, but low hydrolysis rate substrate can require longer HRT's (Angenent *et al.*, 2016; Arslan *et al.*, 2016). Longer HRT also tend to produce more reduced products as n-butyrate and n-caproate (Angenent et Kleerebezem, 2011). However, at HRT > 30 hours, acetoclastic methanogens can develop and have to be inhibited (Mes *et al.*, 2003).

2.8.6 Organic loading rate (OLR) in fed-batch or continuous reactors.

The organic loading rate is the feed stream of the reactor in $\text{g}_{\text{COD added}} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$. OLR can be increased in two ways: by increasing the substrate concentration in the reactor or by reducing the HRT. By increasing the substrate concentration, the fermentation products become more concentrated and more reduced (Arslan *et al.*, 2016). On the other hand, by decreasing the retention time, the substrate will be less hydrolyzed, leading to a less effective process (Arslan *et al.*, 2016). Most studied reactor had an OLR between 5 and 50 $\text{g}_{\text{COD}} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$ but some studies went up to > 200 $\text{g}_{\text{COD}} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$ (Kyazze *et al.*, 2006; Chen et Lin, 2003). Generally, increasing the OLR increases the productivity of SCFA but again a maximum at 36 $\text{g}_{\text{TVFA}} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$ is observed around an OLR > 100 $\text{g}_{\text{COD}} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$. The origin of this maximum of production could be the substrate overload (SO). SO can have two different impacts: an acidification and/or a carboxylates accumulation. Both will lead to a growth inhibition of the microorganisms which will cause the leaching of the elongating bacteria (Leite *et al.*, 2015; Arslan *et al.*, 2016).

2.8.7 Acetoclastic methanogenesis inhibition

For an optimal elongation process, methanogenesis has to be inhibited since it is in competition for substrate through the acetoclastic methanogenesis as shown in Table 1. Several methods were found to inhibit the methanogenesis process for short or longer term

in anaerobic reactors. Chemicals like 2-bromoethanesulfonate, iodoform, chloroform, ethylene, ethane and methyl chloride are known to have inhibitory effects. They target and block specific enzymes involved in the methanogenesis pathway. However they are, expensive and toxic for the environment (Valdez-Vazquez et Poggi-Varaldo, 2009). A heat shock pretreatment of the inoculum and the substrate can partially or totally inactivate the methanogens microorganisms: methane production is stopped after a heat shock at 60°C during 5 min., but can eventually recover. Methanogenesis is irreversibly inactivated after a heat shock at 70°C during 5 or 15 min (Mei *et al.*, 2016). An HRT below 30 hours avoids the accumulation of acetoclastic methanogens and let elongating bacteria outcompete them (growth rate of *C. kluyveri* = 0,1 h⁻¹ and growth rate of acetoclastic methanogens = 0,02 h⁻¹) (Mes *et al.*, 2003; Angenent *et al.*, 2016). Acidic pH, below 5,5, can also inhibit methanogenesis for long term operating reactors. High carboxylates concentration can also have an inhibitory effect as described in the section below (Agler *et al.*, 2014).

2.8.8 Product toxicity

Carboxylates can have a toxic effect on microorganisms by interacting and destabilizing the membrane. Their toxicity depends of their hydrophobicity. The inhibitory concentrations of even chain fatty acids are shown in Table 5 below.

Table 5: Even chain fatty acid physical properties and toxicity.

Substance	Carbon chain length	Maximum solubility	pKa	Inhibition of elongation	Reference
Carboxylates	NA	NA	NA	Around 20 g/L	(Arslan <i>et al.</i> , 2016)
Acetate	C2	Soluble	4,75	36 g/L at pH 7	(Fu et Holtzapple, 2011)
Propionic acid	C3	Soluble	4,87	1 g/L at pH 5	(Inanc <i>et al.</i> , 1999)
Undissociated n-butyric acid	C4	Soluble	4,82	4,4 g/L at pH 4,8	(van den Heuvel <i>et al.</i> , 1992)
Undissociated n-caproate	C6	10,82 g/L	4,88	0,87 g/L	(Angenent <i>et al.</i> , 2016)
n-caprylate	C8	0,68 g/L	4,89	NA	(Angenent <i>et al.</i> , 2016)

2.9 Bio-electro-fermentation

Figure 4a shows a simplified view of a bio-electro-reactor (BER). In this kind of reactor, electricity is used as reducing equivalent source for the production of chemicals by

microorganisms. The microorganisms and the substrate are contained in the catholyte compartment, which is separated by a proton permeable membrane from the anolyte compartment where protons are produced by oxidation. The mechanism of electron exchange from the cathode to the microorganisms for the elongation of carboxylates is not fully understood. Some hypothesis however are reported in the scientific literature as shown in Figure 4b below (Choi et Sang, 2016).

The BER has three advantages over classic anaerobic fermenters: 1) The direct use of electrons as reducing equivalent enables a better control of the microbial biomass and less competitiveness since only some microbial species are able to use them. 2) Electrons are produced in situ and are thus better distributed in the liquid phase than if a gas like H_2 is sparged. 3) Better process control thanks to the continuous steering of the electrons (Schievano *et al.*, 2016).

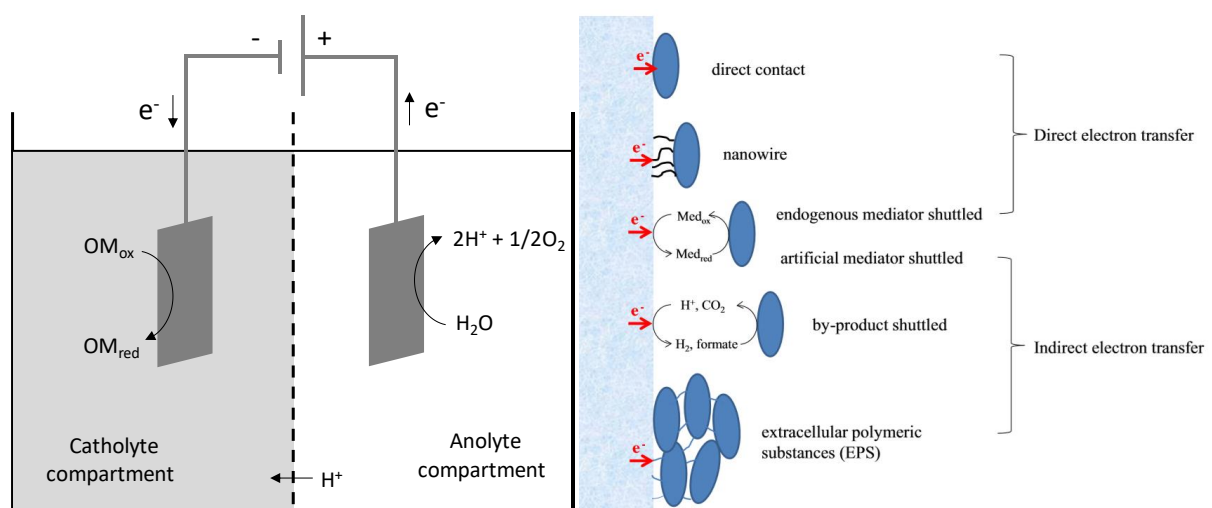


Figure 4a: Left: Principle of electro-biosynthesis. An oxidation process (like water hydrolysis) in the anolyte compartment produce electrons, which are consumed in the catholyte compartment to reduce oxidized organic matter (OM_{ox}) into more reduced organic matter (OM_{red}). This reduction is catalyzed by microorganisms. A proton permeable membrane enables the circulation of protons from the anolyte to the catholyte compartment to keep the ionic balance. Adapted from (Schievano *et al.*, 2016).

Figure 4b: Right: Mechanisms of electron transfer from the cathode to microorganisms. Electron transfer can occur i) by direct contact between the electrode and the bacteria ii) a pilus can be used as link between the cathode and microorganism iii) an endogenous intermediate or iv) chemical intermediate like H_2 can be used as electron transporter between the electrode and the bacteria and finally v) multiple microorganisms can be implied, one species takes the electron from the cathode and is able to transfer it to other microorganisms that will use it to produce reduced chemicals. Adapted from (Choi et Sang, 2016).

Raes *et al* already tested the elongation of acetate under CO_2 atmosphere in a BER with a pH of 5 and a temperature of $30^\circ C$. Two different current were applied: 5 mA and 15 mA. The highest production rate of n-butyrate was 4,5 times higher ($0,49 \pm 0,06$ g/L.d) in the BER at 15 mA compared to the 5 mA, despite a current only 3 times higher. In the 15 mA, acetate was

consumed, H₂ produced, n-caproate was also produced ($0,07 \pm 0,04$ g/L.d) but no ethanol was detected. Higher applied current enabled higher production rate, concentration and more reduced compounds. The authors also tested the two scenarios of elongation: i) electricity wasn't provided and ii) no microorganisms were present. In both cases, no significant elongation could be observed, meaning that the elongation was caused by the microorganism that used the electrons from the applied current as reducing agents (Raes *et al.*, 2017).

3 Aims

Different parameters and operating processes were described in the section above, however most of them were observed under very specific conditions. The aim of this work is to transpose the process described under simple condition (simple substrate, simple strain of bacteria, ...) to complex condition representative of real life (complex substrate, mixed culture...).

Since a mixed culture is used, the first step is to master the elongation process and avoid other unwilled competitive reactions like methanogenesis. In order to do so, parameters (pH, gas phase composition, reducing equivalent source, substrate, HRT, OLR, ...) are investigated to observe their impact on the elongation of carboxylates and their inhibitory effect on the methanogenesis. This enable to enlighten the key parameters that enhance the elongation towards the production of caproate, caprylate or even longer chain carboxylates.

4 Materials and methods

4.1 Solutions preparation and description

4.1.1 Medium stock solutions

Three acetate buffers were prepared using solid sodium acetate (Sigma-Aldrich chimie GmbH) and acetic acid (Sigma-Aldrich, USA) at a concentration of 6 g of acetic acid/L in a 1L graduated flask. The moles of each compound used to make 1 L of solution are reported in Table 6.

Table 6 : Targeted concentrations of acetic acid and sodium acetate in order to get the buffer solutions with the desired pH

	Theoretical pH	n _{potassium acetate} (mol/L)	n _{acetic acid} (mol/L)
Buffer 1	5,5	0,0151	0,0849
Buffer 2	6,5	0,0017	0,0983
Buffer 3	7	0,0006	0,0994

The results obtained in Table 6 are based on the equations below.

$$K_a = \frac{[H^+][CH_3COO^-]}{[CH_3COOH]} \quad [CH_3COOH] + [CH_3COO^-] = 0,1 \text{ mol/L}$$

$$\Rightarrow [CH_3COOH] = \frac{0,1 \frac{[H^+]}{K_a}}{1 + \frac{[H^+]}{K_a}}$$

Three different “mineral solutions”, were prepared and their respective composition is shown in Table 7 below.

Table 7 : Composition of the 3 oligo-element solution.

	Substance	Concentration (g/L)	Concentration (mol/L)
Mineral solution 1	NH ₄ H ₂ PO ₄	216	1,88
Mineral solution 2	MgCl ₂ .6H ₂ O	7,6	3,74.10 ⁻²
	CaCl ₂ .2H ₂ O	1,6	1,09.10 ⁻²
	KCl	0,9	1,21.10 ⁻²
Mineral solution 3	H ₃ BO ₃	0,032	5,18.10 ⁻⁴
	FeCl ₂ .4H ₂ O	0,158	7,95.10 ⁻⁴
	ZnSO ₄	0,011	6,81.10 ⁻⁵
	MnCl ₂	0,0041	3,26.10 ⁻⁵
	CoCl ₂	0,02	1,54.10 ⁻⁴
	CuCl ₂	0,0009	6,69.10 ⁻⁶
	NiCl ₂	0,002	1,54.10 ⁻⁵
	Na ₂ MoO ₄	0,0032	1,55.10 ⁻⁵
	Na ₂ Se ₂ O ₃	0,0013	5,16.10 ⁻⁶

4.1.2 Solid substrates composition

The compositions of the two substrates used in this study are given in Table 8 below.

Table 8: Brewer's spent grain and beet pulp composition. Adapted from Perimenis et al., 2016; Henry et al., 2019; Mathias et al., 2014.

Substance	Brewer's spent grain composition (% of total solid)	Beet pulp composition (% of total solid)	Trub composition (% of total solid)
Hemicellulose	33,4	32,1 ± 0,9	-
Proteins	25,1	9,6 ± 0,1	50-70
Cellulose	17,9	20,6 ± 0,2	4-8
Starch	7	< dl	
Pectin	< dl	11,2 ± 0,1	
Lignin	4,8	2,7 ± 0,3	-
Free Sugars	0,8	0,1 ± 0,1	-
polyphenols	-	-	5-10
Hops bitter substances	-	-	10-20
Ash	4,1	5,1 ± 0,1	3-5
Total	93,1	81 ± 2	72-100

Brewer's spent grain was used either as fresh BSG (FreBSG), frozen BSG (FroBSG) or fresh BSG (95% of fresh mass) + trub (5% of fresh mass) (BSG+T). The substrate whether it was BP or BSG was used without any pretreatment. The major part of trub and brewer spent grain were frozen for better preservation.

4.1.3 Inoculum

Activated sludge from the wastewater treatment plant in Mont-saint-Guibert is used as inoculum for the fermentation broth. It undergoes no pretreatment and is homogenized before being collected and transferred into the reactors

4.2 Pressurized bioreactor (PR)

4.2.1 Bioreactor design

Each PR consists in a 1L Scott bottle. The GL 45 cap of each bottle is made of a stainless-steel headpiece with a 2-holes nozzle. The headpiece is pressed on a nylon seal by a pierced screw cap. On the outside of the bottle, the nozzle of the headpiece is connected to two flexible PVC tubes that are sealed with a two-way Luer lock valve for gas sampling tube and a Mohr pinchcock for the liquid sampling tube. On the reactor side of the cap, a flexible PVC tube is

sealed on the liquid sampling nozzle; this tube is long enough to reach the bottom of the reactor and is used as a liquid sampling tube. The sealing of the PVC tubes over the nozzles is done using a short piece of silicone tube 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.

When indicated 2L Scott bottle were used to increase the volume of the head space and for better mixing and gas exchange in the solution. The configuration was similar to the one-liter reactors.

4.2.2 Fermentation start-up of the PR

The fermentation broth of each reactor is summarized in the Table 9 of section 4.4. Once the different solutions and substrates has been introduced in the reactors, hydrogen or nitrogen is flushed in the PR by connecting the gas supply line to the liquid sampling tube and by opening the gas sampling two-way valve. After 10 minutes of continuous gas flow a gas sample is taken and analyzed to be sure that no air remains. The PR can then be pressurized. 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 an absolute pressure of 1,3 bar and a first gas sample can be taken for analysis. The reactors are put on an orbital shaker at 120 rpm (Kühner Shaker Lab-Shaker) to ensure good mixing in a heated chamber at 35°C.

4.2.3 Fermentation management and sampling of the PR

4.2.3.1 Reactors in batch mode

Thrice a week, pressure in the reactor is measured with the manometer. Gas samples are taken with 60 mL polypropylene syringes (BD Plastipack, USA) equipped with a three-way valve. Liquid samples are taken with a 60 mL polypropylene syringe (BD Plastipack, USA) that is previously weighed and attributed to one specific reactor.

1) First the initial pressure in the reactor is measured. If some reactors are over pressurized and are beyond the measuring limit of the manometer, they are depressurized by removing a known volume of gas (typically 60 ml) and the pressure is measured again afterwards. This operation is repeated until the pressure is under the maximum pressure limit of the

manometer. The unknown initial pressure can then be calculated with the equations shown in section 4.6.1.

- 2) A gas sample is collected (± 20 mL) for gas analysis.
- 3) The pressure is measured again after the gas sampling.
- 4) A liquid sample is collected (± 15 mL) for liquid analysis.
- 5) The syringe with the liquid sample is weighed.
- 6) The liquid sample is transferred into a Pyrex Falcon tube of 15 mL (Greiner Bio-One Cellstar® tubes) and centrifuged at 500 rpm for 10 min in a centrifuge (ThermoScientific Heraeus Megafuge 40 centrifuge).
- 7) 10 mL of the supernatant of the Pyrex tube is transferred in a Falcon tube for further analysis and the pellet remains in the centrifugated tube.
- 8) the pH of each reactor is measured with a pH meter (WTW pH-Electrode SenTix® 41, WTW Multiline P4 universal meter) by measuring the pH of the supernatant transferred in the Falcon tube. Mixed liquor with a pH difference of more than half a unit compared to the initial targeted pH are titrated: 1 mL of the supernatant in the falcon is taken with a P20 micropipette (VWR ergonomic high performance) and titrated with KOH 1M if the pH is too acid or H₂SO₄ 1M if pH is too alkaline.
- 9) The amount of KOH 1M or H₂SO₄ 1M needed in each reactor to adjust the pH is calculated and added in the corresponding Pyrex tube with the remaining pellet.
- 10) A total volume of KOH 1M or H₂SO₄ 1M + distilled water or acetate buffer is adjusted to 10 mL and added to the corresponding tube to compensate the 10 mL sample taken for analysis.
- 11) The content of each Pyrex tube is transferred in the corresponding syringe and the filled syringe is weighted.
- 12) Each suspension is reinjected in its corresponding reactor.
- 13) The headspace pressure is measured.
- 14) The H₂ reactors are pressurized up to 1,3 bars with H₂.

15) The headspace pressure is measured.

4.2.3.2 *Reactors in fed-batch mode*

Most of the time, monitoring remains the same as for batch reactors. However, some steps change when addition of substrate or liquid feed occurs.

1) -> 9) see reactor in batch mode.

10) In fed-batch mode, the amount of acetic acid or water added in each reactor is higher than the volume used for analysis. This allows a progressive increase of mixed liquor volume and a dilution of S&MCFAs to avoid any toxic effects on the microorganisms. Beet pulp is also added in the reactor in order to give additional substrate for the microorganism.

11) to 15) remains the same as for the batch reactors.

4.2.3.3 *Reactors in semi-continuous mode*

Monitoring of reactors in semi continuous mode follows the same procedure as for the batch reactor, except when feeding occurs. The management of substrate and liquid addition in the reactors is described below.

1) -> 3) see reactor in batch mode.

4) A larger volume (approximately 60 mL) of supernatant is collected.

5) The syringe with the sample is weighed.

6) The samples are transferred in a 50 mL Falcon tubes and centrifugated at 500 rpm for 10 min.

7) 50 mL of supernatant are transferred in 50 mL Falcon tubes for further analysis while the pellet remains in the centrifugated Falcon tube.

8) -> 9) see reactor in batch mode.

10) A total volume of KOH 1M or H₂SO₄ 1M + distilled water or acetate buffer is adjusted to 50 mL and added to the corresponding tube to compensate the 50 mL sample taken for analysis. BP, BSG or BSG+T is also added in the tube containing the pellet in order to give additional substrate for the microorganism. BSG and BSG+T are grinded with (Retsch Emax

High Energy Ball Mill Emax) before injection in the tube containing the pellet to facilitate the transfer into the reactor.

11) to 15) see reactor in batch mode.

4.2.4 End of fermentation

When it is decided that the reactors have to be stopped, the same operations steps are done, regardless of the reactor operating mode.

1) -> 3) see reactor in batch mode.

4) The reactor is opened under a hood.

5) The reactor with its content is weighed.

6) The content of the reactor is transferred into the 0,75-liters bucket previously tared of the centrifuge (ThermoScientific Heraeus Megafuge 40 centrifuge).

7) The 0,75-liter bucket is weighed and then centrifugated at 500 rpm during 10 min.

8) 50 mL of the supernatant is transferred to a 50 mL Falcon tube for liquid analysis. The remaining supernatant is thrown away.

9) The bucket with the remaining pellet is weighed.

10) The pellet is transferred into a 50 mL Falcon tube.

11) The bucked is weighed.

12) The falcon tube containing the pellet is stored in a freezer at -20°C.

Both supernatant and pellet are characterized for fresh, dry, volatile matter and COD.

4.3 Bio-electro-reactor (BER)

4.3.1 Bioreactor design

As shown in Figure 5 below, the bio-electro-reactor (BER) is a 2 L glass vessel. A handmade acrylic cap is pressed by a metallic ring on a silicone seal at the top of the reactor. The cap has a 5,5 cm hole in its center. In this central hole, a 5 cm inner diameter, 10 cm long acrylic tube is inserted and glued with hard PVC glue (Pattex, Germany). Around the center hole, 4 holes were made, one for the reference electrode, one for the cathode connection to the power supply, one for flushing the headspace and liquid sampling and one for the gas outlet.

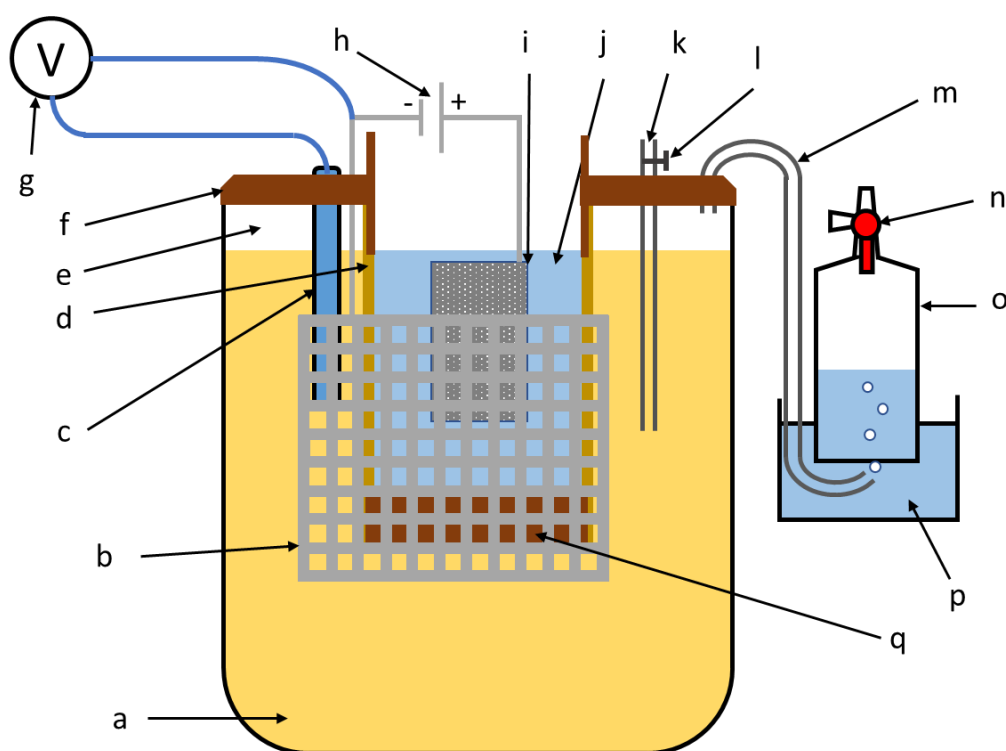


Figure 5 : The bio-electro fermenter. a) mixed liquor in the cathode compartment. b) non-woven stainless-steel cathode c) reference electrode Ag/AgCl (+197mV vs NHE, SI Analytics GmbH, Germany) d) cation exchange membrane CMI7000T e) headspace f) acrylic cap g) voltmeter (Wiss-Techn. Werkstätten Empfindlichkeit) h) power supply (RND LAB DC power supply RND320-KD3005D i) platinum-iridium oxide coated anode (Zmanode, China) j) anode compartment containing K_2SO_4 0,1 M k) liquid sampling pipe l) Mohr pinchcock m) gas outlet pipe n) three-way valve o) syringe without it's piston p) bucket with water q) acrylic cap

The vessel is separated into the anodic and cathodic compartments by a tubular cation exchange membrane CMI7000T (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.

4.3.1.1 *Anode compartment*

The inner compartment separated by the membrane is the anode compartment. A platinum-iridium oxide coated anode (Zmanode, China) is submersed in 450 mL of 0,1M K₂SO₄ (VWR, Germany) aqueous anolyte solution.

4.3.1.2 *Cathode compartment*

The outer compartment is the cathode compartment. The cathode is a non-woven stainless-steel plate of 45cm x 10cm. It folds around the membrane into an accordion shaped circle (to maximize specific surface). It is connected via the electrical connectors in the cap. The catholyte consists of 600 mL of fermentation broth described below.

Two 5ml polystyrene serological pipettes (Greiner Bio-One, Austria) are put through the holes in the cap. One is cut just behind the narrowing part of the pipette to have a constant diameter along the 20 cm tube that is left over. This tube goes down into the fermentation broth and is used for liquid sampling. The second serological pipette is cut to only reach the headspace (± 6 cm) and is used for gas sampling. The upper ends of those two serological pipettes, which are located outside of the reactor, are connected to a flexible PVC tube. The liquid sampling tube is sealed with a Mohr pinchcock. The other extremity of the gas outlet tube plunges in a water bucket under a vertical syringe cylinder and closed with a three-way valve. This syringe will collect the gas produced in the EF. Both pipettes are sealed with a plastic cable gland, which is itself sealed to the cap of the reactor.

An Ag/AgCl reference electrode (+197mV vs NHE, SI Analytics GmbH, Germany) is also submerged in the fermentation broth.

A constant potential 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 connectors on the cap of the vessel while the anode is connected via a wire through the anode compartment plug.

4.3.2 *Fermentation start-up of the BER*

The solutions, substrate and inoculum used in the BER are described in section 4.1. and the composition of the fermentation broth, contained in the catholyte, is described in Table 9 of section 4.4. Once the different solutions and substrates have been introduced in the BER, the reactor is sealed with the acrylic cap to which is fixed the cation exchange membrane. A

solution of K_2SO_4 0,1 M is poured in the anolyte compartment until the membrane is submerged. Nitrogen is flushed in the BER by connecting the gas supply line to the liquid sampling tube. After 10 minutes of continuous gas flow, a gas sample is taken and analyzed to be sure that no air remains. The power supply in constant potential mode is then switched on and a gas sample is taken for analysis. The reactor is then placed on a rotary shaker at 120 rpm in a 35°C heat chamber.

4.3.3 Monitoring and sampling of the BER

Thrice a week, the gas flow and the potential difference between the cathode and the reference electrode is measured. The gas is sampled and analyzed. The liquid is also sampled in order to measure the pH and for liquid analysis. As for the PR, a syringe (BD Plastipack, USA) is assigned to each bio-electro fermenter for the liquid sampling.

4.3.3.1 Electrical measurements

Thrice a week, the constant voltage applied and the resulting current at the 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 (Wiss-Techn. Werkstätten Empfindlichkeit).

4.3.3.2 Cyclic voltammetry

Once a week, a cyclic voltammetry measurement is performed on the BER in order to determine if the electron transfer between the cathode and the catholyte evolves during the experiment.

- 1) The voltmeter is connected to the reference electrode (c) and to the cathode (b) to measure the cathode potential as shown in Figure 5.
- 2) The applied potential at the power supply is switched to 0 and then increased until the current starts to increase.
- 3) The applied potential, the current measured at the power supply and the cathode potential measured with the voltmeter (g) are recorded.
- 4) The applied potential is then increased by steps of 0,1 V and the 3 values recorded. The potential at the cathode and current could take some time to stabilize, thus a time laps of 1

min between the applied potential increase and recording of the values is arbitrary fixed. This may cause some inaccuracy but saves a lot of time.

5) Step 4) is repeated until the applied potential reaches the arbitrary value of 3 V.

6) The applied potential is decreased by steps of 0,1 V and all the same parameters are recorded.

7) step 6) is repeated until the measured current reaches 0.

4.3.3.3 Gas collection and gas flow measurement system

The gas collected in the vertical syringe cylinder shown in Figure 5 and Figure 6 is first pumped up with a 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 flow of fresh gas coming from the cathodic compartment can be calculated by the time needed by the water to drop from a given level to a lower one.

$$\text{Gas flow (mL/s)} = \frac{\text{volume of water displaced by the gas (mL)}}{\text{time (s)}}$$

Once 15 mL of gas are present in the collecting syringe, the gas is pumped by the 10 mL polypropylene syringe and analyzed.

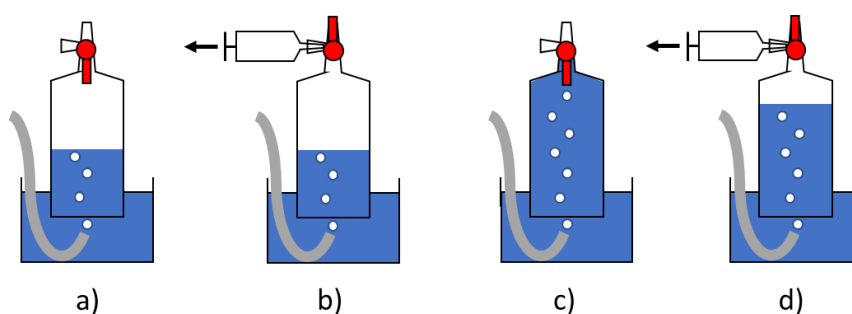


Figure 6 : Gas collection from the electro-fermenter, the gray pipe is connected to the gas outlet of the catholyte compartment of the electro fermenter. a) the gas in the vertical syringe is no longer representative of the head space in the bio-electro fermenter. b) the gas is removed using a second syringe. c) fresh gas accumulates in the vertical syringe. d) the fresh gas is collected for gas analysis.

4.3.3.4 Liquid sampling

See step 4) to 12) for the monitoring of reactors in batch mode in section 4.2.3.1.

4.3.4 End of fermentation

Current, applied potential and potential between the cathode and the reference electrode are measured before the potential at the power supply is turned off. The other steps remain the same as for the shutdown of pressurized reactors described in section 4.2.4.

4.4 Summary of the different condition in each experiment

The composition of all the reactors in the three experiments are shown in Table 9 below.

Table 9 : Starting composition of the reactors in the different experiments

Experiment	Name of the reactors	Operating mode	Gas phase	Buffer pH	Volume of acetate buffer (mL/L _{ML})	Substrate type	Mass of substrate (g _{COD} /L _{ML})	Volume of inoculum (mL/L _{ML})	Volume of "Mineral solution 1" (mL/L _{ML})	Volume of "Mineral solution 2" (mL/L _{ML})	Volume of "Mineral solution 3" (mL/L _{ML})
1	BER	Batch	N ₂	5,5	800	BP	38,8	126	16,3	16,3	3,3
	1L PR		H ₂	5,5	782 ± 4	BP	38,4 ± 0,4	134 ± 3	15,8 ± 0,2	15,8 ± 0,2	3,15 ± 0,03
	1L PR		H ₂	6,5	785 ± 5	BP	38,8 ± 0,2	132,8 ± 0,8	15,857 ± 0,006	15,857 ± 0,006	3,171 ± 0,001
2	2L PR	Fed-batch	H ₂	6,5	797 ± 1	BP	38,30 ± 0,07	129 ± 2	15,96 ± 0,03	15,96 ± 0,03	3,192 ± 0,006
3	FreBSG	Semi-continuous	H ₂	7	798,3 ± 0,2	Fresh BSG	13,28 ± 0,08	127,73 ± 0,03	15,967 ± 0,004	15,967 ± 0,004	3,1933 ± 0,0008
	BSG+T		H ₂	7	796,9 ± 0,1	Fresh BSG + T	14,21 ± 0,06	127,51 ± 0,02	15,941 ± 0,002	15,941 ± 0,002	3,1878 ± 0,0005
	FroBSG		H ₂	7	797,9 ± 0,2	Frozen BSG	13,81 ± 0,07	127,66 ± 0,02	15,958 ± 0,003	15,958 ± 0,003	3,1916 ± 0,0006
	Without acetate		H ₂	7	- ²	Frozen BSG	13,7 ± 0,2	127,72 ± 0,07	15,965 ± 0,009	15,965 ± 0,009	3,193 ± 0,002
	BSGN ₂		N ₂	7	798,34 ± 0,07	Frozen BSG	13,4 ± 0,2	127,77 ± 0,01	15,972 ± 0,001	15,972 ± 0,001	3,1943 ± 0,0002
	No substrate		H ₂	7	830	-	-	133	16,6	16,6	3,3
	BPH ₂		H ₂	7	798,62 ± 0,02	BP	39,34 ± 0,03	127,780 ± 0,004	15,9725 ± 0,0004	15,9725 ± 0,0004	3,19450 ± 0,00008
	BPN ₂		N ₂	7	798,66 ± 0,03	BP	39,29 ± 0,04	127,786 ± 0,005	15,9733 ± 0,0006	15,9733 ± 0,0006	3,1947 ± 0,0001

² Acetate buffer was replaced by 798,2 ± 0,5 mL/L_{ML} of distilled water.

4.5 Analytical methods

4.5.1 Gas composition

4.5.1.1 *Compact GC characteristics*

The samples are analyzed by gas chromatography (GC) with thermal conductivity detector (TCD) (Compact GC, Global Analyzer Solutions, Interscience, The Netherlands). This gas analyzer can detect H₂, N₂, O₂, CO₂, and CH₄. The GC includes two channels (the front and back channel) both composed of an injection loop of 25 µL and a thermal conductivity detector (TCD). The front channel is equipped with an RI-QBond column (10 m length, 0,32 mm ID, Restek, France) in a 60°C oven and uses Helium as carrier gas at a flow rate of 1 mL/min. The front channel can only separate and quantify CO₂. The back channel is equipped with two columns placed in series in an oven at 70°C with Argon as a carrier gas at a flow rate of 1 mL/min. The first column is a RI-QBond column (2 m length, 0,32 mm ID, Restek, France) and the second is a Molsieve5A column (7 m length, 0,32 mm ID, Restek, France). A 3-ways valve is placed between the two columns. It is switched just after H₂, O₂, N₂ and CH₄ enter in the second column and while CO₂ remains in the first column. CO₂ is consequently backflushed out of the GC. As the other gases continue to progress in the second column, they are separated and quantified by the TCD of the back channel. Retention times of H₂, O₂, N₂ and CH₄ were 21,7, 27,7, 35,0 and 51,0 seconds respectively.

4.5.1.2 *Sample injection*

The gas sample contained in the syringe is gently injected through an injection valve into the GC and passes then successively through each injection loop before leaving the GC through an exit valve. Chromatograms are then automatically integrated; the peak areas are converted to gas concentration using experimentally calibrated conversion factors. The gas composition is given as percentage of the different gases (H₂, O₂, N₂, CH₄ and CO₂) analyzed by the Compact GC.

4.5.1.3 *Calibration of the Compact GC*

Gas analyzer are calibrated with five gas samples: four artificial 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 (ratio between gas

concentration and peak area on the chromatogram) for each gas component. The detection limit of the GC is 0,1%V/V.

4.5.2 Fresh matter, total solid and volatile matter

- 1) The crucibles are put in the oven at 550°C for 2 hours.
- 2) The crucibles are transferred from the oven in the desiccator to remain dry and to cool down.
- 3) Once the crucibles are at room temperature, they are tared. A fresh sample is then introduced in each crucible.
- 4) Each crucible is weighed with its content and then placed in the oven at 105°C for 8h.
- 5) The crucibles are transferred from the oven to the desiccator until they reach room temperature.
- 6) The cooled crucibles with their content are weighed and then placed in the oven at 550°C for 2h.
- 7) The crucibles are transferred from the oven to the desiccator until they reach room temperature.
- 8) Each cooled crucible is weighed with its content.

Each sample is analyzed three time.

The fresh matter (FM) is the mass of sample transferred in the crucible before it's introduced in the oven at 105°C.

The total solid (TS) expressed in g_{TS}/g_{FM} is the mass of sample that remains in the crucible after the crucible stayed 8 hours in the oven at 105°C and has cooled down to room temperature.

The ashes are the mass of sample expressed in g_{ashes}/g_{FM} that remains in the crucible after the crucible stayed 2 hours at 550°C and has cooled down to room temperature.

The volatile matter expressed in g_{VM}/g_{FM} is the difference between the TS and the ashes.

4.5.3 COD measurement

The oxidizable materials under the conditions of the test are oxidized in an acid medium by an excess of potassium dichromate solution in the presence of mercury sulfate, which forms a complex with chlorides, and of silver sulphate, which serves as a catalyst. The remaining dichromate can be measured either by colorimetry or by titration with iron ammonium sulfate solution using iron orthophenanthroline as indicator.

4.5.3.1 Liquid homogeneous sample

COD measurement kits (Spectroquant, Merck KGaA, Germany) for samples between 500 and 10 000 mg_{COD}/L were used. First, 1 mL of distilled water is introduced into each tube followed by 1 mL 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 liter of solution introduced in the tube with a confidence interval of ± 5 mg_{COD}/L. The obtained value is then multiplied by 2 to get the COD concentration present in the initial sample. The total soluble COD in the reactors tend to increase over time, thus the volume of sample put in the kits is progressively reduced and the volume of distilled water adapted to reach a total added volume of 2 mL in the kits.

4.5.3.2 Solid samples

4.5.3.2.1 Preparation of the needed reagents

- 1) **Distilled water** without any organic matter
- 2) **Solid mercury sulfate** (HgSO₄)
- 3) **Concentrated sulfuric acid** (d = 1,83 - 1,84)
- 4) **Sulfuric solution of silver sulfate**

Commercial solution of 6,6 g/L of silver sulfate in concentrated sulfuric acid (AVS TITRINORM® for COD measurement).

- 5) **Potassium dichromate solution 0,25 N**

12,259 g of dry K₂Cr₂O₇ are dissolved in 1L of distilled water.

- 6) **Indicator solution**

1,48 g of 1-10 orthophenanthroline monohydrated and 0,7 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ are dissolved in 100 mL of distilled water.

7) **Iron sulfate and ammonium solution 0,25 N (Mohr salt)**

196 g of $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ are dissolved in 1,6 L of water. 40 mL of concentrated H_2SO_4 are transferred in the solution before distilled water is added until the volume of solution reaches 2L. This solution is standardized before every use with the potassium dichromate solution. To do so, distilled water is added to 20 mL of the dichromate solution, until the total volume of solution reaches 250 mL and 20 mL of concentrated H_2SO_4 is then added to the solution. Once the solution has cooled off, 12 droplets of the indicator are added to the solution. The solution is then titrated with the Mohr salt solution. The normality ratio between the Mohr salt solution and the potassium dichromate is then given as

$$f = m/n$$

with m the volume (mL) of potassium dichromate 0,25 N solution introduced and n the volume (mL) of Mohr salt used for titration.

4.5.3.2.2 COD measurement

An amount equivalent to 30 mg of volatile matter of the sample is introduced into a 500 mL spherical flask. 50 mL of $\text{K}_2\text{Cr}_2\text{O}_7$ solution and 0,4 g of HgSO_4 are then poured in the flask. 66 mL of sulfuric solution of silver sulfate are transferred in the 500 mL flask while gently shaking to make sure the water and concentrated sulfuric acid are well mixed and that only one liquid phase remains in the flask. The flask is connected to a 30 cm condenser and boiled at reflux during 30 min once boiling has begun.

Once the 30 min are over, the condenser is washed with about 184 ml of distilled water and let to cool down. 12 drops of the indicator are transferred into the solution. The excess of potassium dichromate is titrated with iron-ammonium sulphate solution until the solution turns from blue-green to red-brown.

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

The COD ($\text{g}_{\text{COD}}/\text{g}$) of the sample is then calculated thanks to the equation below:

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

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

4.5.4 Carboxylate and alcohol metabolites analysis

4.5.4.1 Reagents

A solution of 8 g/L of NaOH is prepared to dilute the fatty acids of the calibration solutions.

An internal standard solution is prepared by diluting 100,0 µl of 4-methylvalerate (4-MV) (Sigma Aldrich, USA), a carboxylic acid which is not present naturally in the fermentation broth, in 100,0 mL of distilled water.

A 1 g/L of H₂SO₄ solution is prepared by diluting 55 µl of H₂SO₄ 97% (ChemLab, Belgium) in a 100 mL flask with distilled water. The flask must be filled with 40 mL of water before adding the acid to prevent any excess heat emission.

4.5.4.2 Analytes extraction

Metabolites are extracted from the mixed liquor in ether. Both samples and calibrating solutions undergo the same extraction process. For the extraction, glass screw top Pyrex test tubes with plastic caps and silicone seal are used. First, approximately 1 g of NaCl is 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, 0,400 mL of the prepared 4-MV internal standard is added in each tube. Finally, 0,200 mL of the H₂SO₄ solution, and then quickly 2,000 mL of pure ether (Sigma-Aldrich) are added into each test tube before closing them rapidly.

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

4.5.4.3 Gas chromatography

Once centrifuged, the supernatant of each test tube is collected with a micropipette and transferred into a glass vial which is then sealed with a PTFE/silicone screw cap (VWR, Germany). The ether extracts are then analyzed by gas chromatography (Thermo Scientific, USA). The samples are injected with an autosampler in a split-splitless injector in split mode

maintained at 230°C. The separation is performed by a 30 m, 0,25 mm ID, nonpolar column (Restek, USA). The detection is performed by a flame ionization detector (FID) at 240°C. The run time is 34 min. The oven temperature is set at 40°C and increase by 10°C/min for 18 minutes until it reaches 220°C. It remains at this temperature for 15 minutes. Chromatograms are then automatically integrated; each peak area is normalized by the peak area of the internal standard and finally converted in concentrations using the corresponding calibration factor.

4.5.4.4 Calibration

Three calibrating solution were prepared by randomizing the targeted concentration of pure compounds in each solution. Every peak area obtained by the integration of the chromatograph is normalized by the peak area of the internal standard. This ratio can then be plotted as a function of the calculated concentrations of each fatty acid. A calibration line is then plotted for each analyte and the slope of a calibration line crossing the origin is determined by linear regression. The calculated slope is used to determine the concentration of each analyte in the analyzed sample.

4.6 Calculation

4.6.1 Calculation of the initial pressure in the reactor.

Equations for the calculation of the initial pressure in the reactor.

$$P_{\text{initial}} V_{\text{HS}} = P_1 (V_{\text{HS}} + V_{\text{syringe}}) \quad (1)$$

$$P_{\text{initial}} = P_1 + P_1 \frac{V_{\text{syringe}}}{V_{\text{HS}}} \quad (2)$$

With P_{initial} the initial pressure, V_{HS} the volume of head space in the reactor, P_1 the pressure after 1 depressurization, V_{syringe} the volume of gas in the syringe taken from the reactor.

If a second depressurization is needed with the same V_{syringe} :

$$P_1 = P_2 + P_2 \frac{V_{\text{syringe}}}{V_{\text{HS}}} \quad (3)$$

$$(3) \rightarrow (2) \quad P_{\text{initial}} = P_2 + 2P_2 \frac{V_{\text{syringe}}}{V_{\text{HS}}} + P_2 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^2 \quad (4)$$

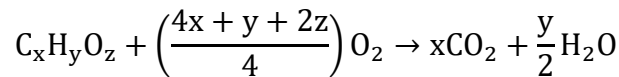
The initial pressures after 3 and 4 depressurizations can be determined with the equation (5) and (6) respectively if V_{syringe} is always the same:

$$P_{\text{initial}} = P_3 + 3P_3 \frac{V_{\text{syringe}}}{V_{\text{HS}}} + 3P_3 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^2 + P_3 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^3 \quad (5)$$

$$P_{\text{initial}} = P_4 + 4P_4 \frac{V_{\text{syringe}}}{V_{\text{HS}}} + 6P_4 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^2 + 4P_4 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^3 + P_4 \left(\frac{V_{\text{syringe}}}{V_{\text{HS}}} \right)^4 \quad (6)$$

4.6.2 Theoretical COD

To determine the stoichiometrically COD, the general formula of every organic substance that does not contain phosphorus, nitrogen or any heteroatom is:



Hence, one mol of $C_xH_yO_z$ reacts with $\left(\frac{4x+y+2z}{4} \right)$ mol 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 the molecule is calculated by the following equation:

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

COD of carboxylates and alcohols that were monitored during the experiment are shown in Table 10 below.

Table 10 : COD value in g_{COD}/g for carboxylates (from C2 to C10) and alcohols.

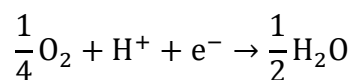
substances	C2	C3	iC4	C4	iC5	C5	C6	C7	C8	C9	C10	EtOH	BuOH
COD_{th} (g_{COD}/g)	1,07	1,51	1,82	1,82	2,04	2,04	2,21	2,34	2,44	2,53	2,60	2,09	3,08

4.6.3 Electrical COD

The electrons provided by the electric current to the BER correspond to a feed of COD. The current is converted in mmol electron equivalent per day ($mmol_{e^-}/day$):

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

The $mmol e^-$ are converted into COD considering the following equation



Giving the formula above and the molar mass of O_2 :

$$1 \text{ mmol}_{e^{-}} = \frac{1}{4} \text{ mmol}_{O_2} = 0,008 \text{ g}_{\text{COD}}$$

Finally, 1 mA of electricity provides:

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

4.6.4 Cumulated production

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

On day “t”

On day “t”, the mixed liquor of the reactor has a total volume: $V_{t_ML_start}$ (L). A liquid sample is then collected to analyze the liquid phase composition. This sample has a volume of V_{t_sample} (L). The sample is analyzed, and each metabolites concentration is determined: $C_{t_metabolite_start}$ ($\text{g}_{\text{COD}}/\text{L}_{\text{ML}}$). KOH or H_2SO_4 1 M, water, buffer and substrate are added to the reactor. $V_{t_returned}$ (L) is the volume of liquor returned to the reactor. The final volume of mixed liquor in the reactor $V_{t_ML_end}$ ($\text{g}_{\text{COD}}/\text{L}_{\text{ML}}$) is calculated as follow:

$$V_{t_ML_end} = V_{t_ML_start} - V_{t_sample} + V_{t_returned}$$

The concentration of the metabolites in the reactor after monitoring $C_{t_metabolite_end}$ ($\text{g}_{\text{COD}}/\text{L}_{\text{ML}}$) can then be calculated:

$$C_{t_metabolite_end} = C_{t_metabolite_start} * \frac{V_{t_ML_start} - V_{t_sample}}{V_{t_ML_end}}$$

On day “t+1”

On day “t+1” the mixed liquor of the reactor has a total volume: $V_{t+1_ML_start}$ (L). A liquid sample is taken to analyze the liquid phase composition. This sample has a volume of V_{t+1_sample} (L). The sample is analyzed, and each metabolites concentration is determined: $C_{t+1_metabolite_start}$ ($\text{g}_{\text{COD}}/\text{L}_{\text{ML}}$).

The net cumulated production at day t+1 (Prod_{t+1}) ($\text{g}_{\text{COD}}/\text{L}_{\text{ML}}$) is calculated as follow:

$$\text{Prod}_{t+1} = \text{Prod}_t + \frac{C_{t+1_metabolite_start} * V_{t+1_ML_start} - C_{t_metabolite_end} * V_{t_ML_end}}{V_{t+1_ML_start}}$$

Since the net cumulated production is an amount of COD normalized to the volume of the reactor, a positive slope means that the metabolite is produced, while a negative slope indicates a consumption of the metabolite.

5 Results

5.1 Experiment 1

In this experiment, 2 parameters are tested: the pH and the reducing equivalent supply (EF vs pressurized reactor). Operating conditions are as follow: acetic acid 5 g/L_{LM}, BP 40 g_{COD}/L_{ML}, H₂ or cathode as electron source and pH of 5,5 or 6,5 in pressurized reactors and 5,5 in the EF.

5.1.1 Bio-electro-reactor (BER)

Figure 7 below show the evolution of the gas concentration in the BER.

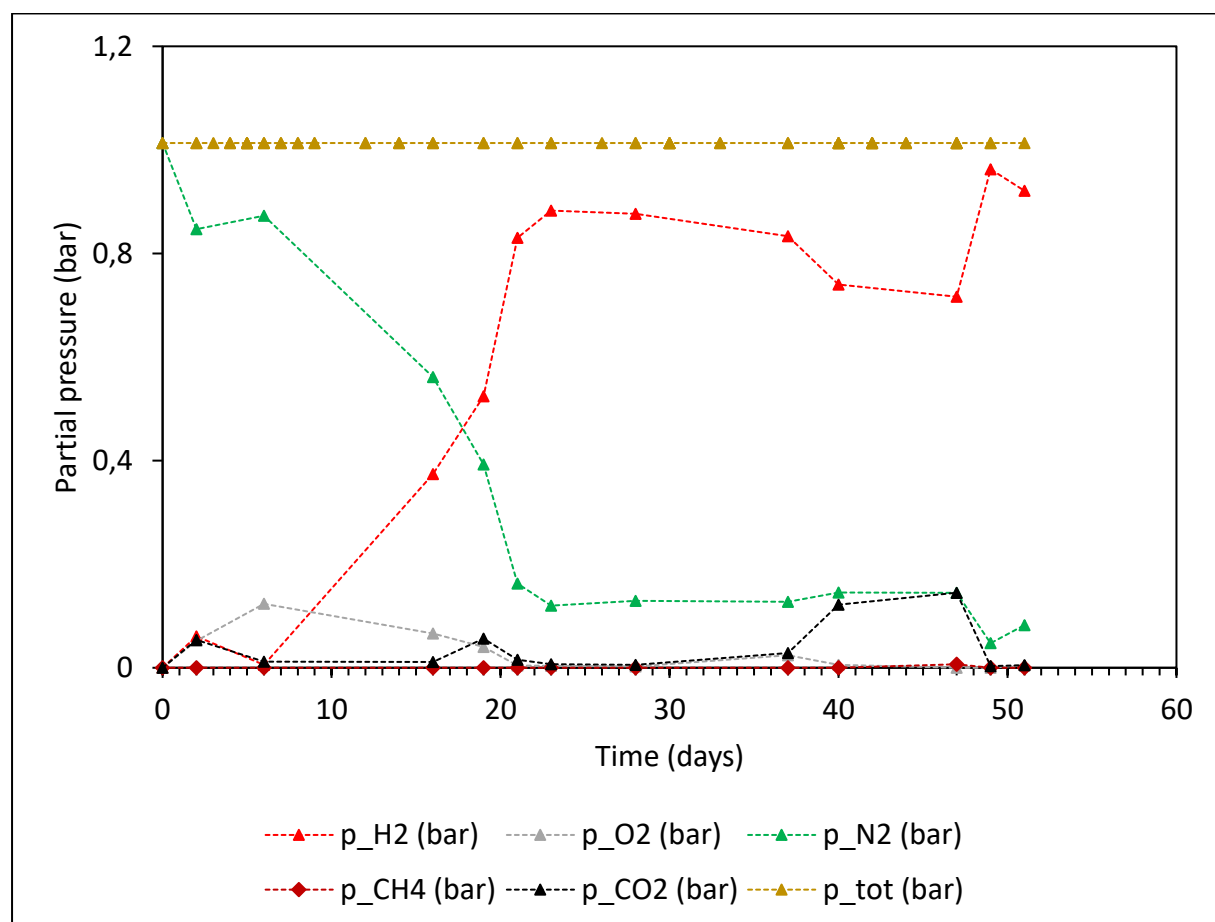


Figure 7 : Evolution of the headspace gas composition over time in the BER.

Figure 8 below shows the evolution of applied potential, pH and cathode potential in the EF.

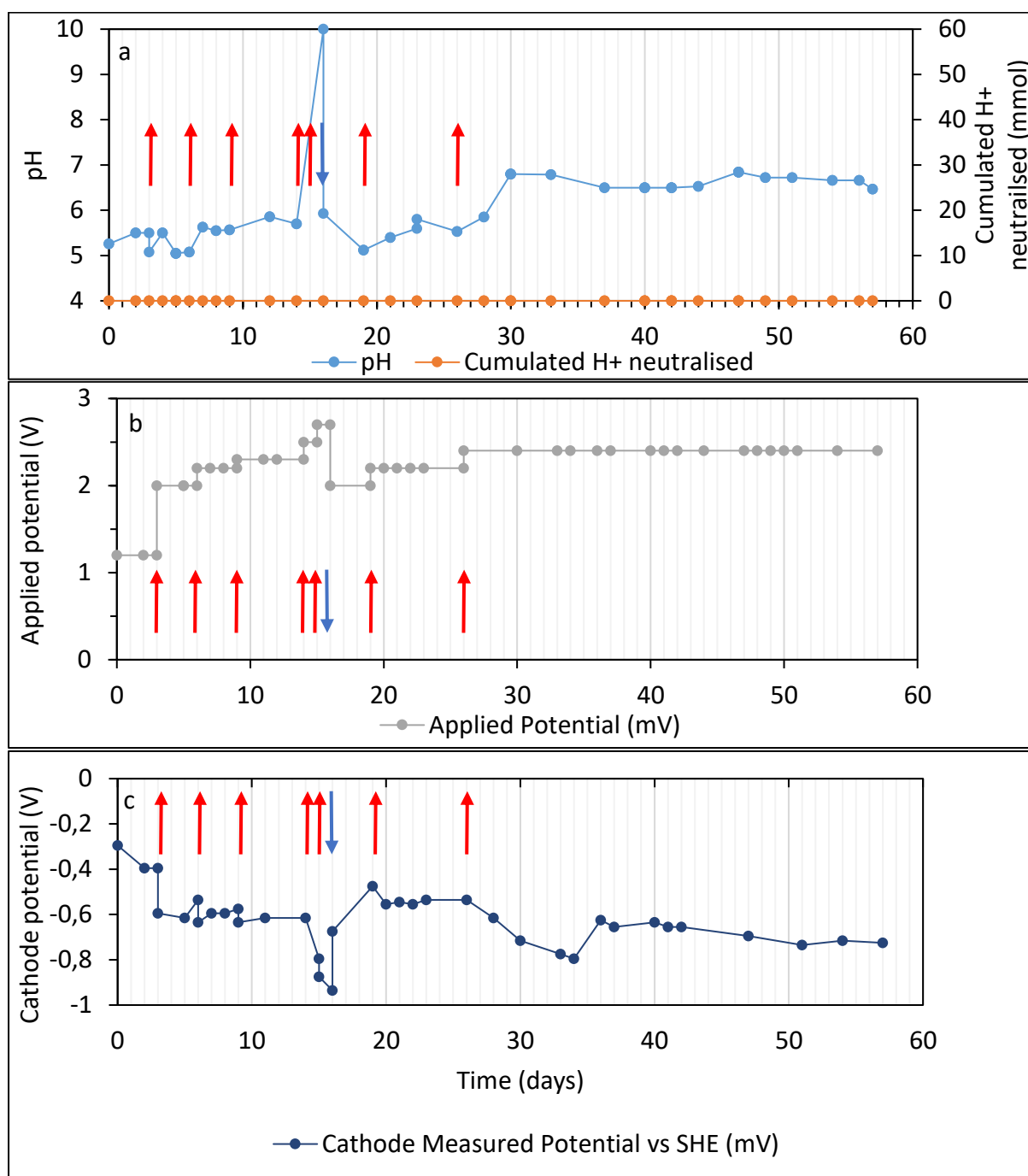


Figure 8 : a) Evolution of pH and the requirements for neutralization as a function of time in the BER b) potential applied by the power supply to the electrodes. c) potential at the cathode (stainless steel) versus the reference electrode (NHE). The red arrows represent an increase of the potential applied at the power supply and the blue arrows a decrease of the applied potential at the power supply.

Figure 9 shows the cyclic voltammetry performed at different times in the BER

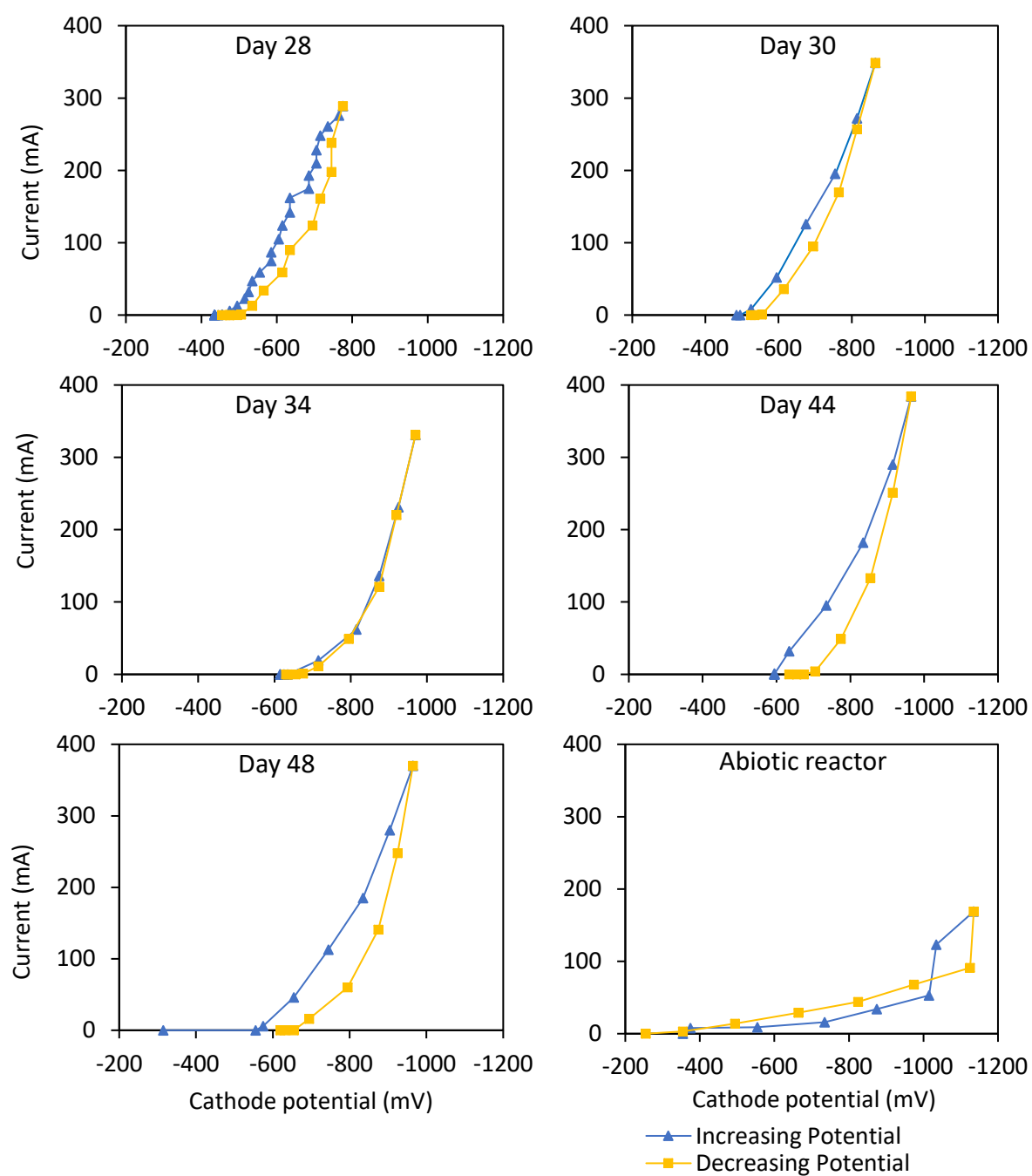


Figure 9: Cyclic voltammetry of the current as a function of the cathode potential. Blue curve indicates an increasing potential difference applied to the electrodes and yellow curve a decreasing potential applied to the electrodes.

Figure 10 shows the reducing equivalents fed to the reactor as electron and the H_2 leaving the BER. The gap between the two curves shows the net reducing equivalent “absorbed” by the mixed liquor and potentially converted to organic metabolites.

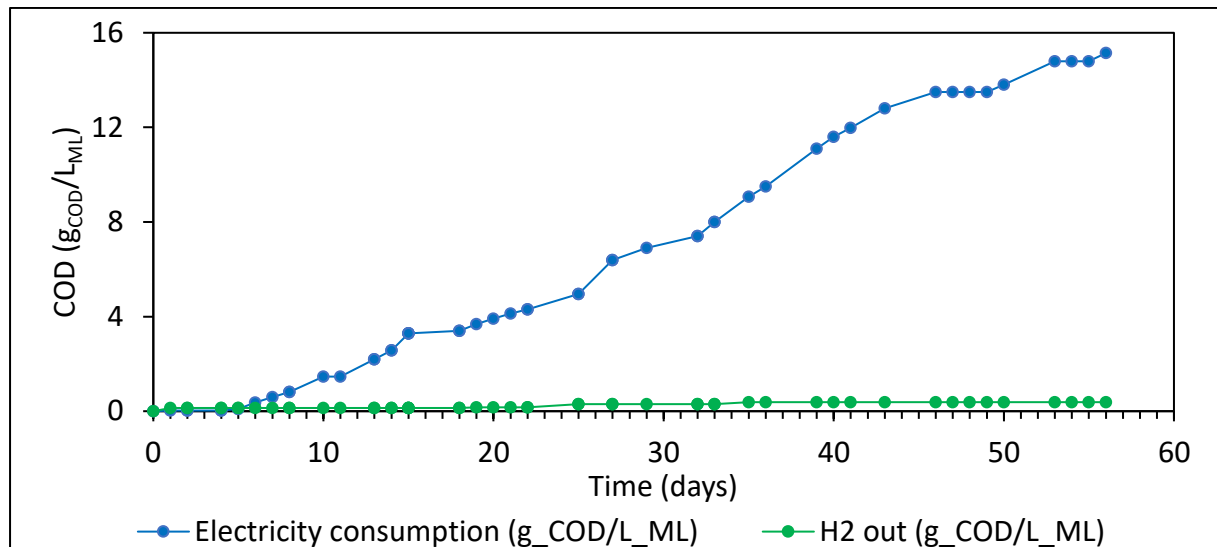


Figure 10 : Cumulated H_2 leaving the reactor (green curve) and cumulated electron fed by the cathode (blue curve) over time in grams of COD per liters of mixed liquor.

Figure 11 shows an overview of the metabolite pools during the fermentation of BP in the BER.

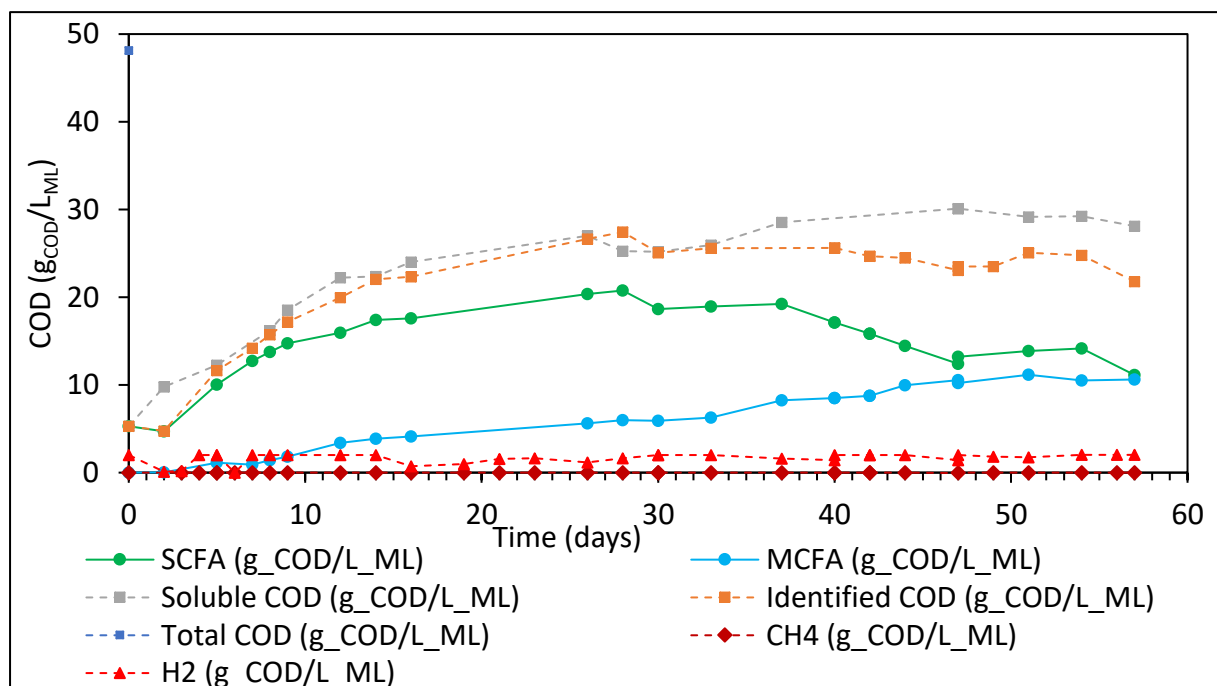


Figure 11 : Overview of the metabolite pools during the fermentation of BP in the BER.

Figure 12 below shows the evolution of the metabolite concentration over time in the BER.

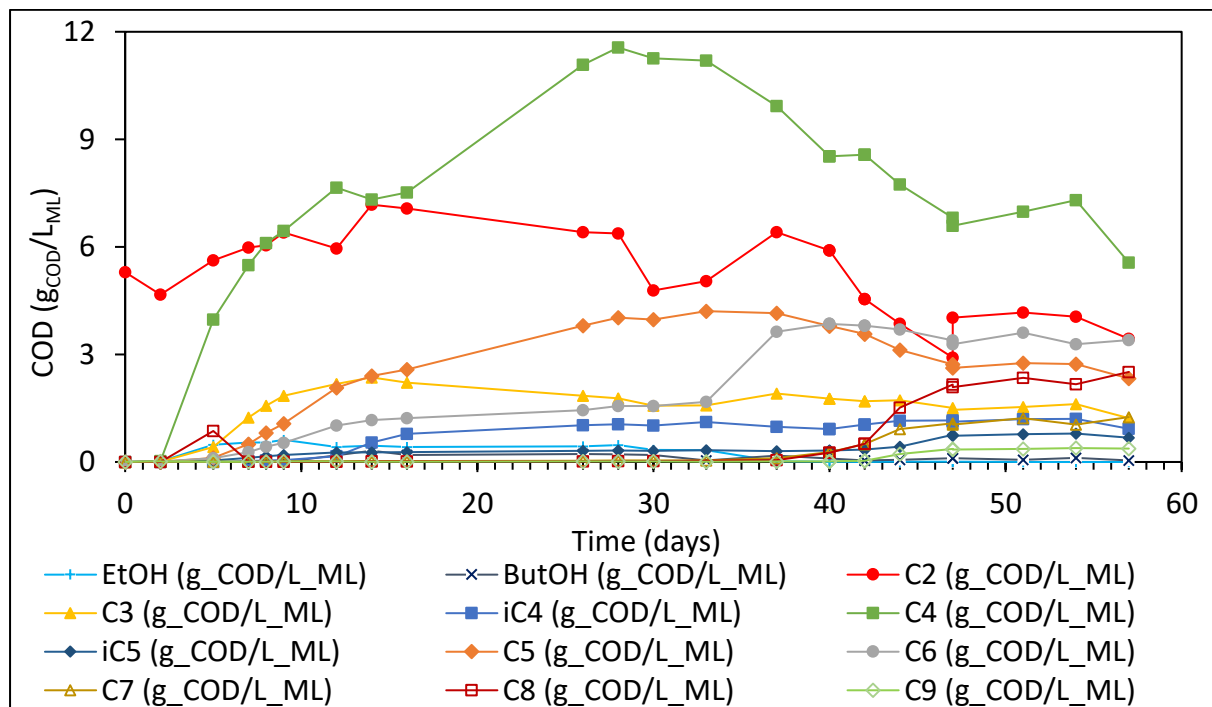


Figure 12 : Evolution of the metabolite concentrations over time in the BER.

Figure 13 shows the net cumulated metabolite production evolution over time in the BER.

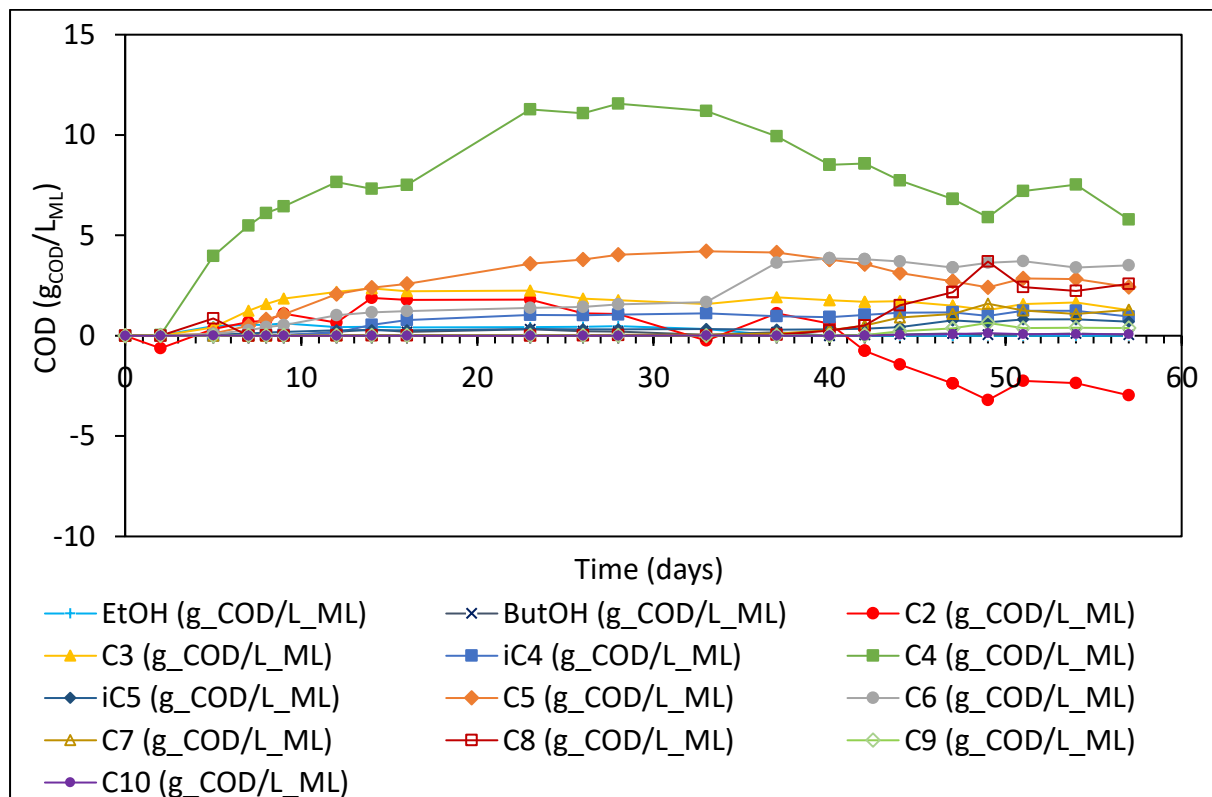


Figure 13 : Net cumulated metabolite production evolution over time in the BER.

Figure 14 shows the COD balance of the EF.

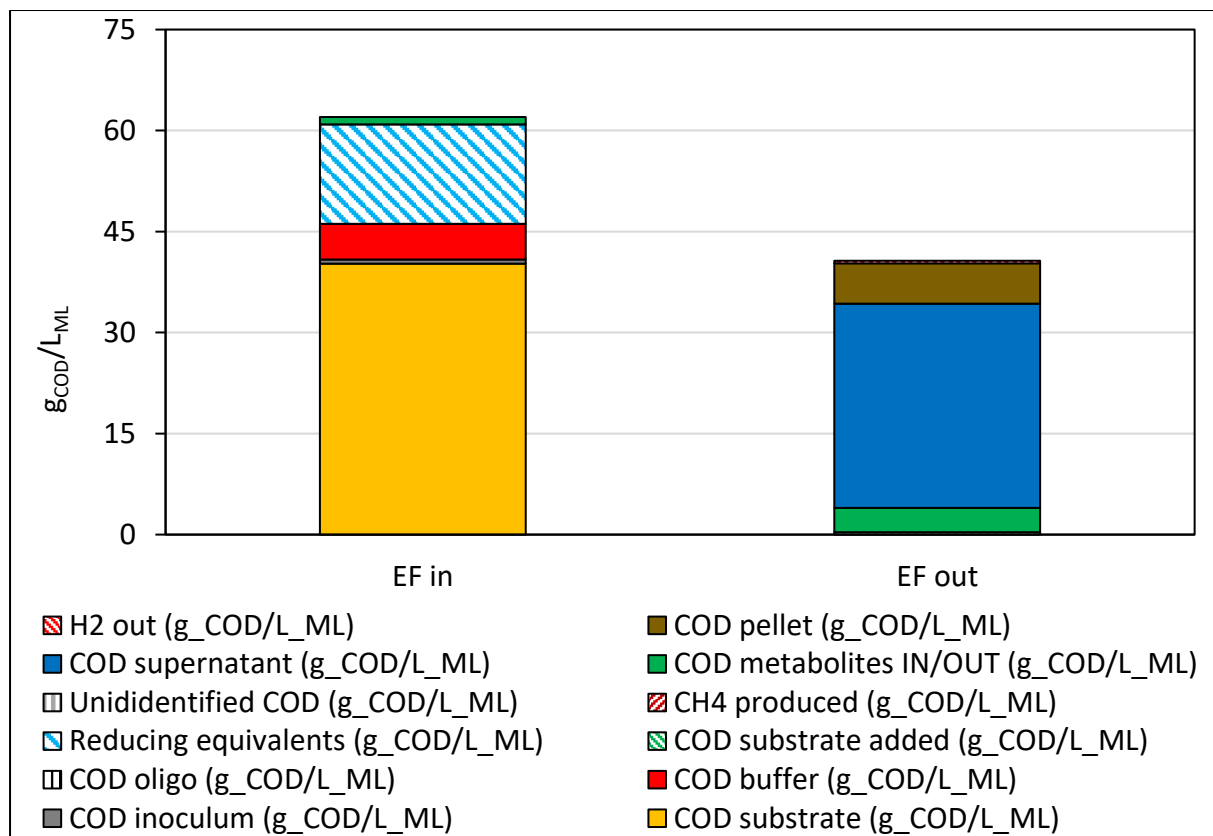


Figure 14 : COD balance of the EF.

As shown in Figure 7, H_2 partial pressure rose on day 6, the cathode potential was then -635 mV. C_6 concentration started to increase on the same day and its concentration rose until day 14 where it stabilized around 1,2 g_{COD}/L_{ML} (Figure 12).

The applied potential was increased between day 14 and 16 from 2300 to 2700 mV (Figure 8b) and resulted in a pH rise from 5,7 to 9,8 (Figure 8a). The potential was then lowered to 2000 mV on day 16 and pH decreased to 5,9. The cathode potential reached a peak of -935 mV on day 16 before going back to -675 mV on the same day (Figure 8c). The applied potential was once more increased to 2400 mV on day 26, pH increased from 5,5 to 6,8 on day 30 and cathode potential went from -535 to -715 on day 34.

As can be seen on Figure 11, SCFA and MCFA accumulated until day 28 and reached a concentration of 20,8 g_{COD}/L_{ML} and 6 g_{COD}/L_{ML} respectively. Between day 28 and 30, pH rose from 5,9 to 6,8 (Figure 8a). SCFA concentration started to decrease while MCFA concentration continued to rise until the end to reach a final concentration of 11,1 g_{COD}/L_{ML} and 10,6 g_{COD}/L_{ML} respectively (Figure 11).

Between day 33 and 36, C6 concentration rose again from 1,7 to 3,6 g_{COD}/L_{ML} (Figure 12). The pH remained constant at 6,5 (Figure 8a) and the hydrogen partial pressure remained above 0,7 bar during this time laps (Figure 7).

Between day 37 and 47, CO₂ was observed in the gas phase with a partial pressure of 0,13 bar (Figure 7) and pH remained constant around 6,5 during this period (Figure 8a). C2 and C4 concentration decreased, C6 concentration remained stable, while C8 concentration rose from 0 to 2,1 g_{COD}/L_{ML} (Figure 12).

As shown on Figure 10, H₂ produced by the cathodic compartment was much less than the electrons fed to the cathode. However, the COD balance (Figure 14) shows that all the COD fed to the reactor is not recovered in the products of the reactor.

5.1.2 One-liter pressurized bioreactor (1 L PR)

Figure 15 below shows the evolution of the headspace gas composition over time in the 1L PR.

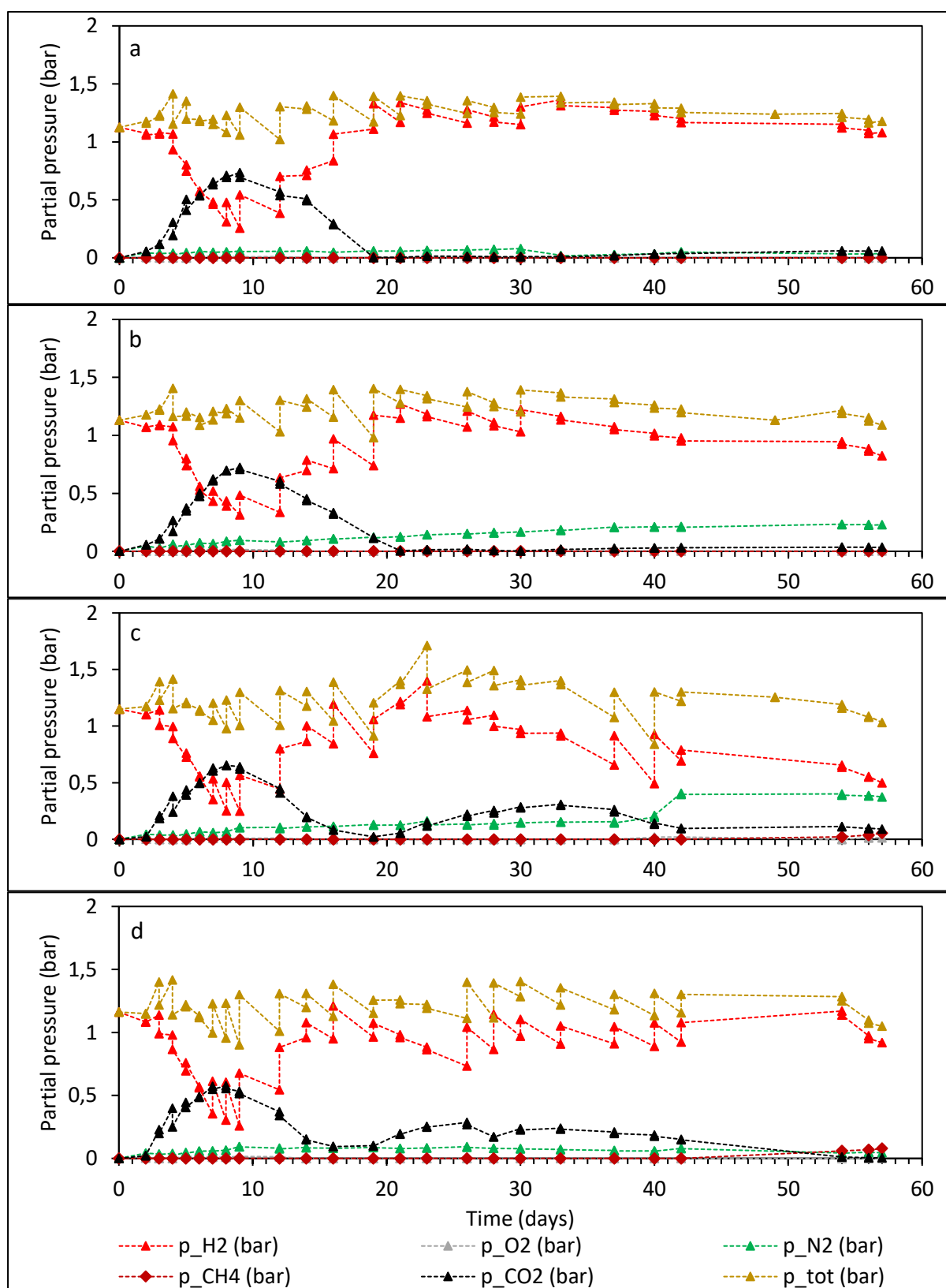


Figure 15 : Evolution of the headspace gas composition over time in the 1 L PR. a) & b) shows the gas monitoring of the two reactors at pH 5,5 and c) & d) of the two reactors at pH 6,5

Figure 16 below shows the evolution of pH and the requirements for neutralization as a function of time in the 1 L PR.

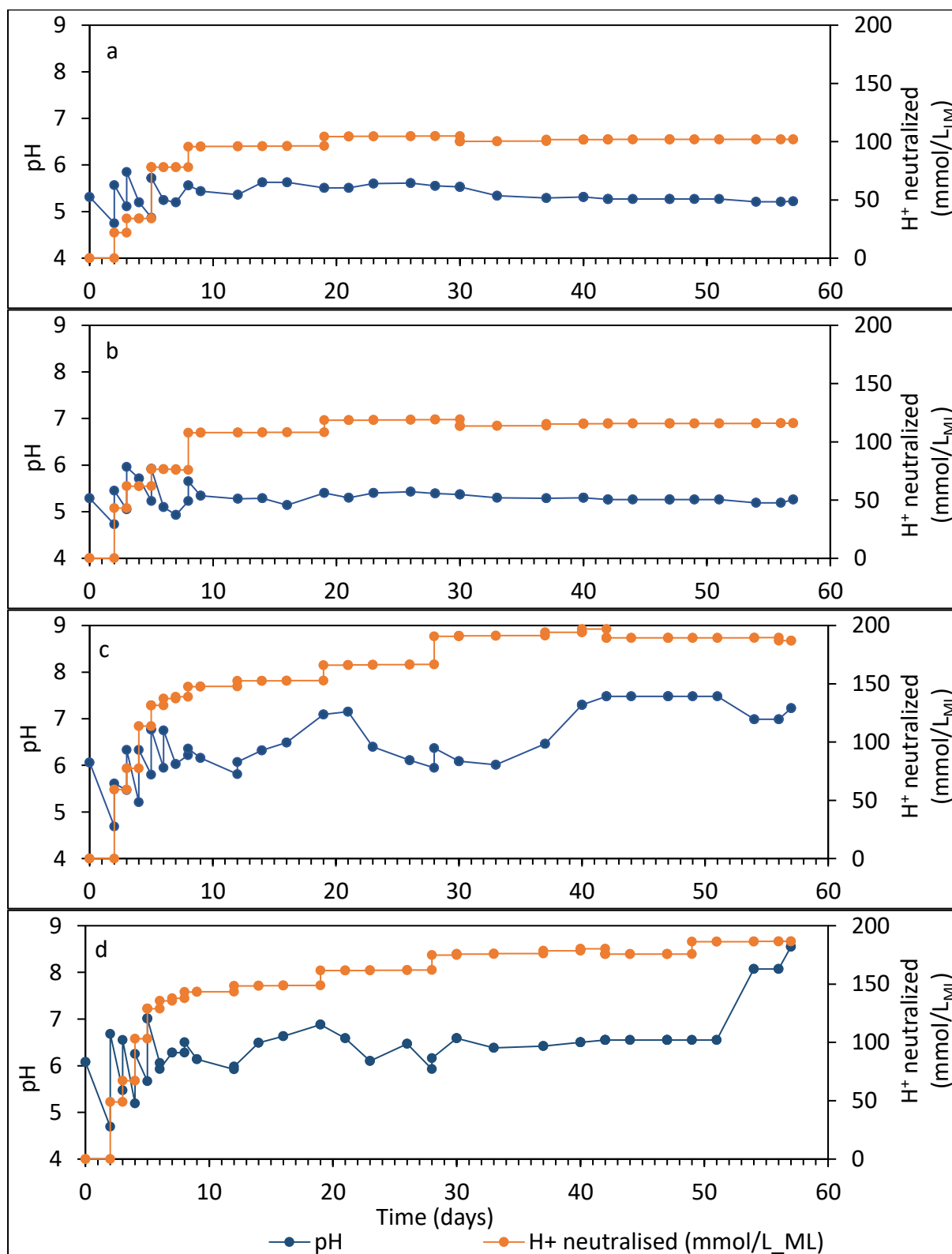


Figure 16 : Evolution of pH and the requirements for neutralization as a function of time in the 1 L PR. a) & b) refers to the reactor 1 and 2 at pH 5,5 and c) & d) to reactor 3 and 4 at pH 6,5.

Figure 17 below shows an overview of the metabolite pools during the fermentation of BP in 1 L PR.

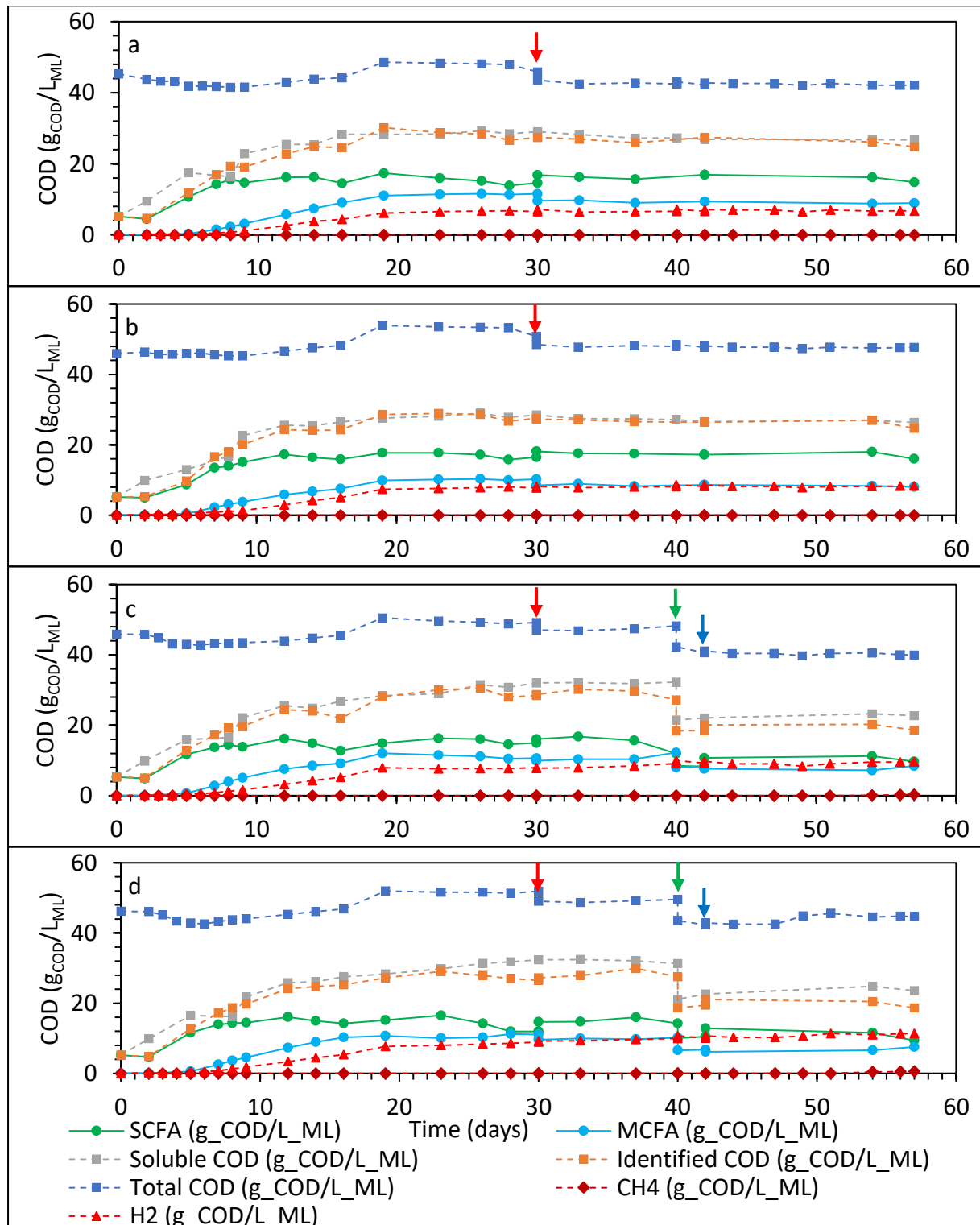
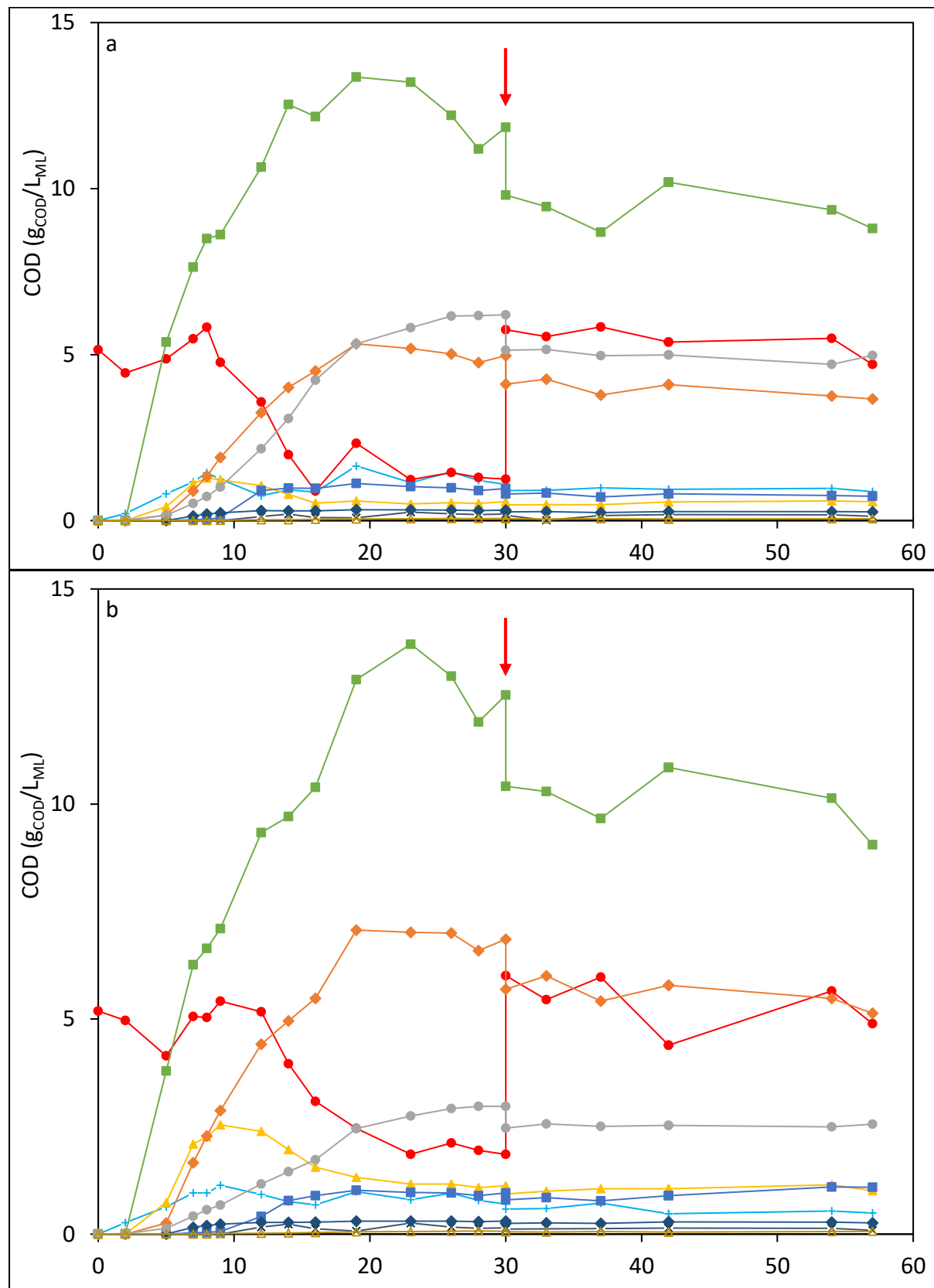


Figure 17 : Overview of the metabolite pools during the fermentation of BP in 1 L PR. a) & b) is the monitoring of the reactors 1 and 2 respectively, at pH 5,5 and c) & d) of the reactors 3 and 4 at pH 6,5. The red arrow indicates a dilution on day 30: 150 mL_{supernatant}/L_{ML} are replaced by 150 mL_{acetic acid}/L_{ML}. A second dilution is made on day 40 indicated by a green arrow in the two reactors at pH 6,5: 350 mL_{supernatant}/L_{ML} are replaced by 90 mL_{acetic acid}/L_{ML} and 260 mL_{distilled water}/L_{ML}. The blue arrow shows the third and last dilution: 85 mL_{supernatant}/L_{ML} are replaced by 85 mL_{acetic acid}/L_{ML}. H₂ curve is the COD of H₂ consumed from the head space of the reactors.

Figure 18 shows the evolution of the metabolite concentration over time in the 1 L PR.



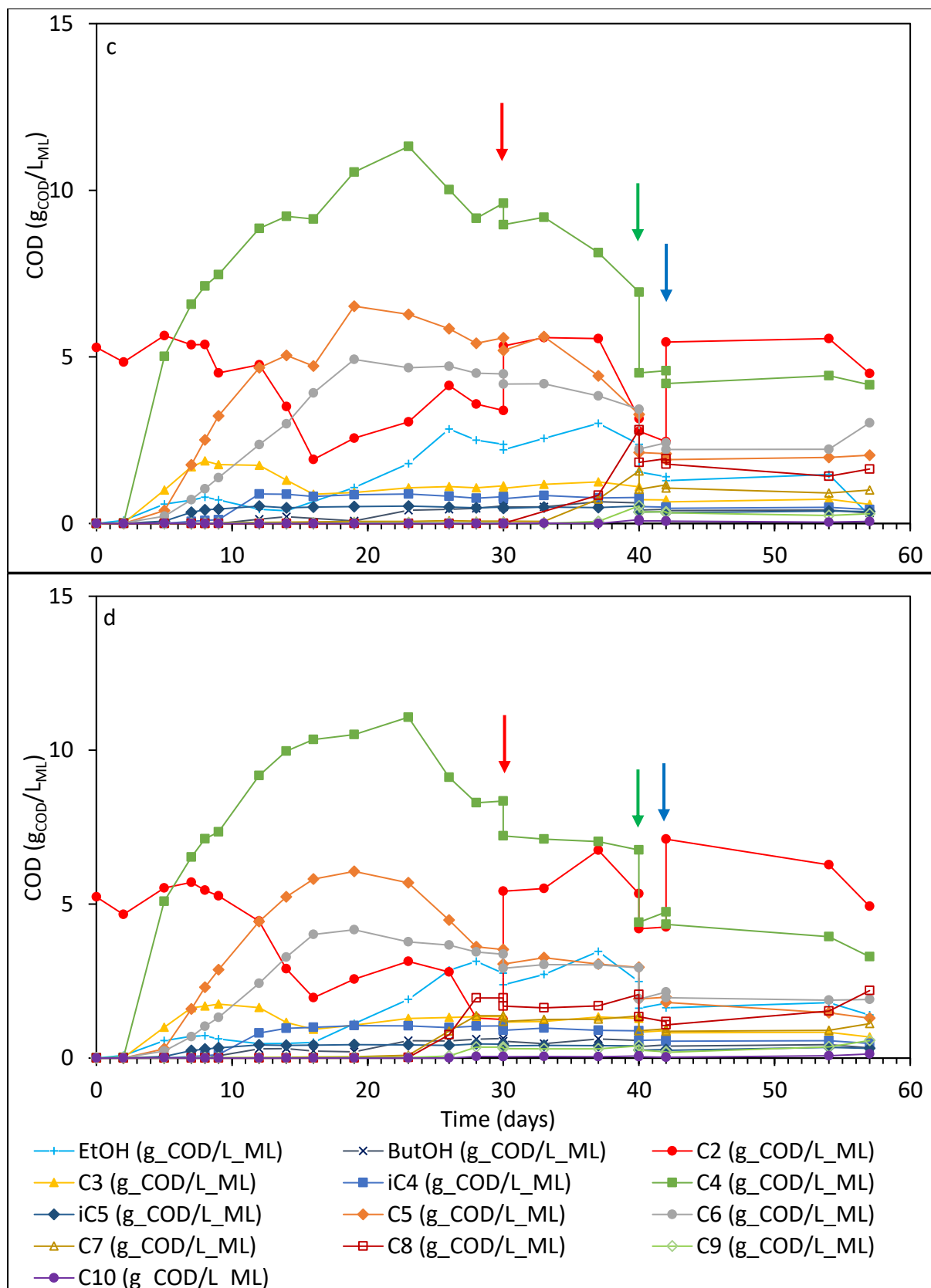


Figure 18 : Evolution of the metabolite concentration over time in the 1 L PR. a) & b) show the metabolite concentration in the medium of the reactors 1 and 2 at pH 5,5 and c) & d) of reactors 3 and 4 at pH 6,5. No production of MCFA of eight or more carbon chain was observed in reactor 1 and 2. A dilution is made on day 30, replacing 50 mL of the liquid phase by 50 mL of acetic acid. A second dilution was made on day 40 in the reactors 3 and 4 at pH 6,5 by replacing 100 mL of the supernatant by 25 mL of acetic acid and 75 mL of water. The meaning of the colored arrows is explained in the legend of Figure 17.

Figure 19 show the evolution of the net cumulated metabolite production over time in the 1 L PR.

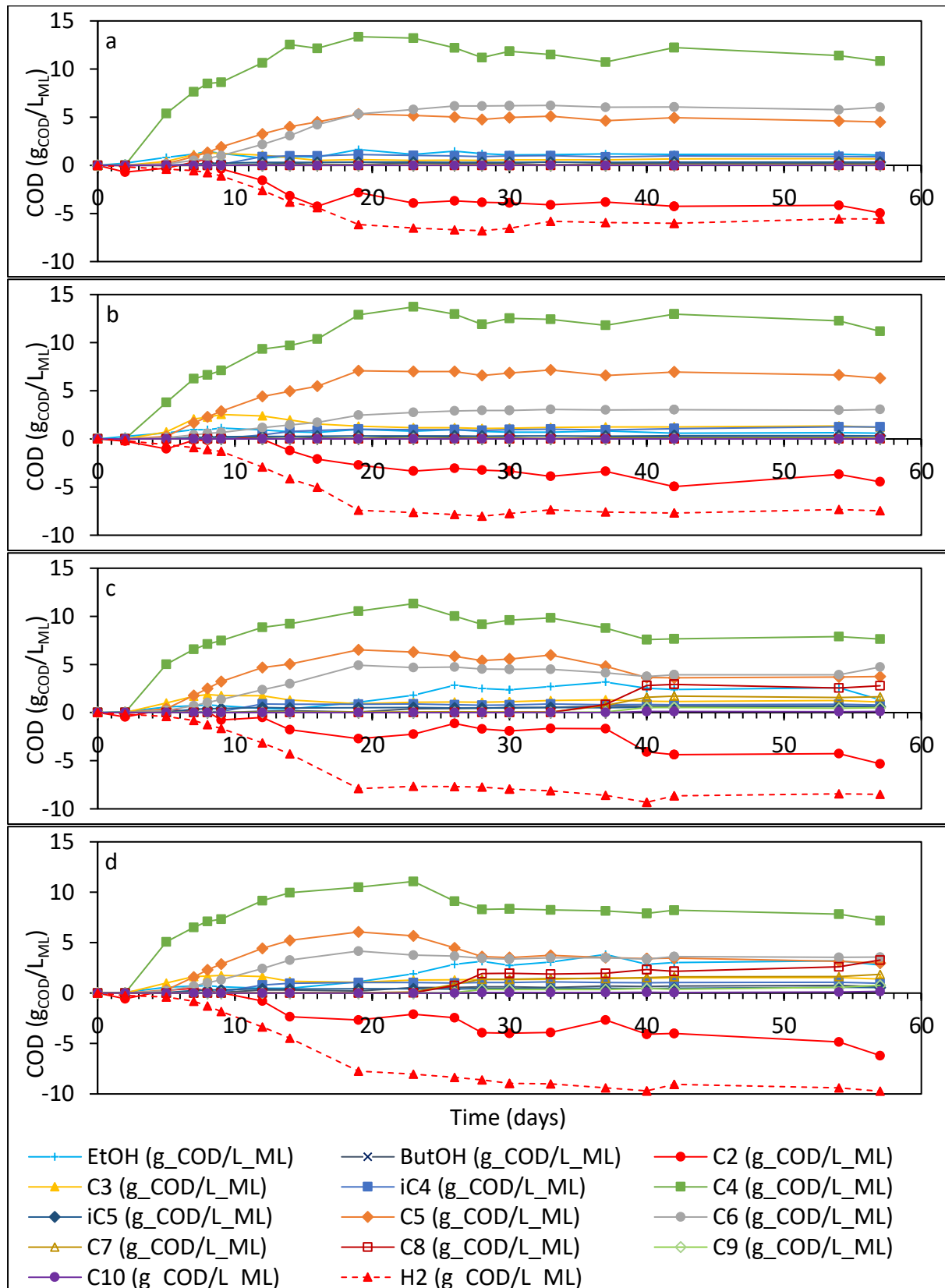


Figure 19 : Net cumulated metabolite production evolution over time in the 1 L PR. a) & b) show the production of the reactors 1 and 2 at pH 5,5 and c) & d) of reactors 3 and 4 at pH 6,5.

Figure 20 shows the COD balance of the pressurized reactors.

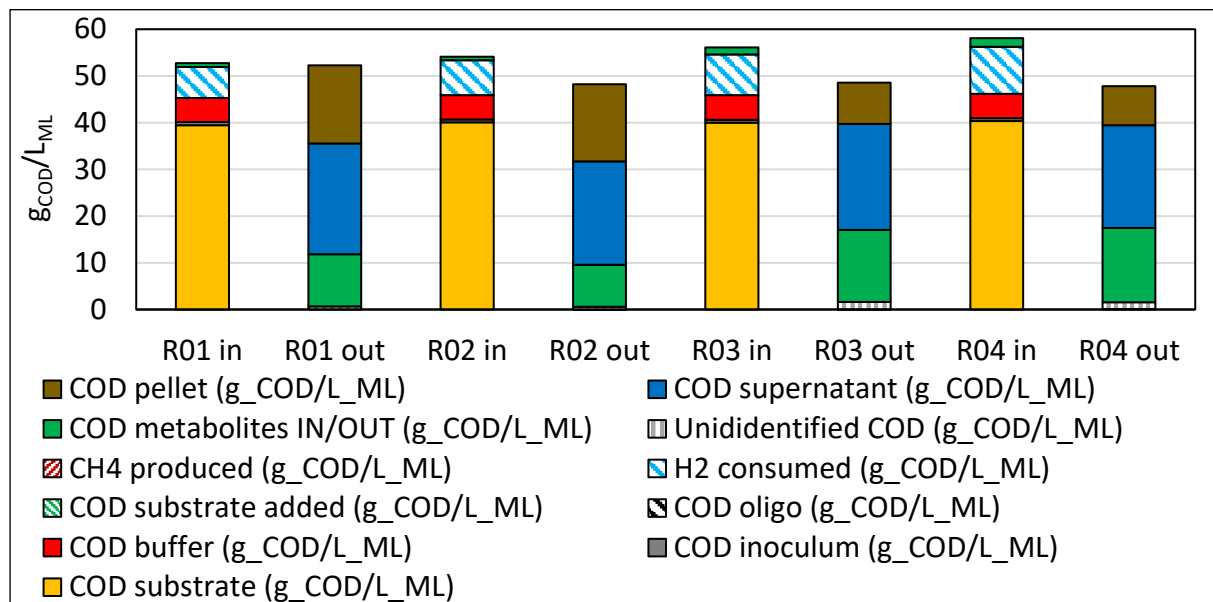


Figure 20 : COD balance of the pressurized reactors, reactors 1 and 2 at pH 5,5 and 3 and 4 at pH 6,5.

Between day 2 and day 19, SCFA and MCFA accumulated in the four reactors, as can be seen in Figure 17. As shown on Figure 15, CO₂ partial pressure increased, up to 0,7 bars before decreasing. At the same time, H₂ partial pressure also dropped and H₂ was added in the reactor to maintain a global pressure above 1,3 bars. pH dropped, down to 4,7, and was readjusted every 2-3 days (Figure 16). C₂ started to be consumed while Ethanol, C₄ and C₆ were produced (Figure 19).

C₈ was produced in both pH 6,5 reactors but starting at different times. In reactor 4, C₈ was produced, up to 2 g_{COD}/L_{ML}, along with ethanol between day 23 and 28. Simultaneously, C₂, C₄ and C₆ were consumed (Figure 19d). CO₂ partial pressure rose until day 8 after which it decreased to reach almost 0 bar around day 19. During this time laps, H₂ partial pressure dropped and had to be readjusted (Figure 15d). Ethanol started to be produced between day 14 and 28 and its concentration remained high (>2,5 g_{COD}/L_{ML}) until the end. pH went down and was readjusted on day 28 (Figure 16d). The same observations can be made for reactor 3 where C₈ was produced along with ethanol between day 33 and 40 (Figure 18c). During this time laps, C₂, C₄ and C₆ were consumed. CO₂ and H₂ partial pressure dropped between day 33 and 40 (Figure 15c) and the pH tended to increase (Figure 16c). In both reactors, C₁₀ production started 5 to 7 days after C₈. No C₈ was detected in reactors at pH 5,5.

A dilution was performed in pH 6,5 reactors on day 40 (Figure 18c & d). After the dilution, the metabolite concentration curves remained constant until the end.

5.2 Experiment 2

In this experiment, 2 L reactors were used, to increase the reservoir of H_2 available in the headspace.

Figure 21 below shows the evolution of the headspace gas composition over time in the 2 L reactors.

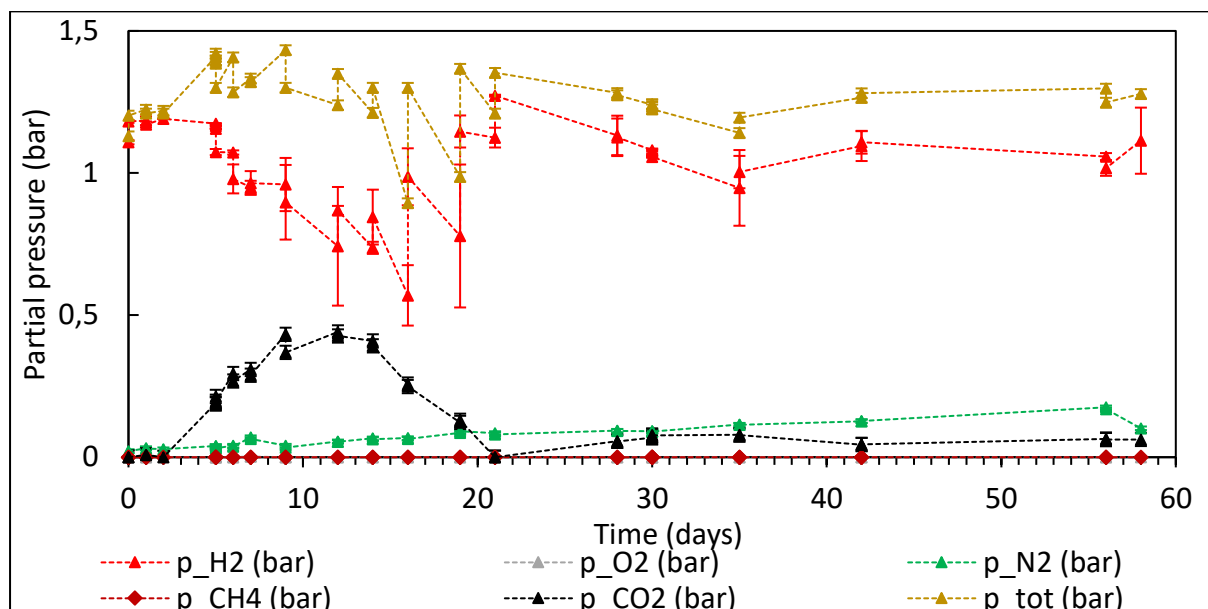


Figure 21 : Evolution of the headspace gas composition over time in the 2 L reactors. Mean $\pm$ standard deviation of duplicated reactors.

Figure 22 shows the evolution of pH and the requirements for neutralization as a function of time in the 2 L reactors.

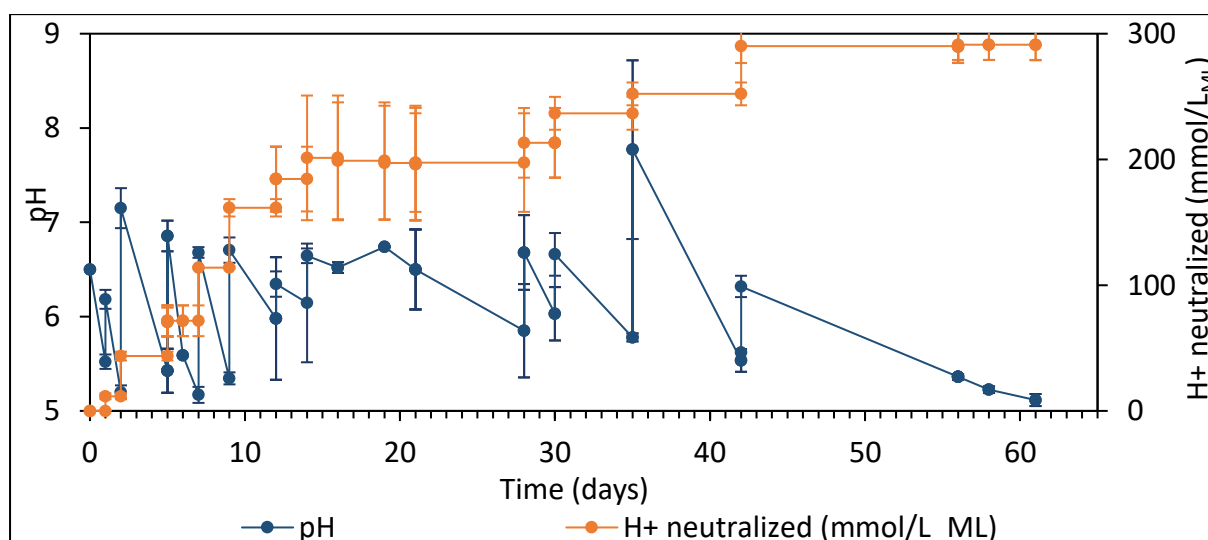


Figure 22 : Evolution of pH and the requirements for neutralization as a function of time in the 2L reactors. Mean $\pm$ standard deviation of duplicated reactors.

Figure 23 shows an overview of the metabolite pools during the fermentation of BP in 2 L reactors.

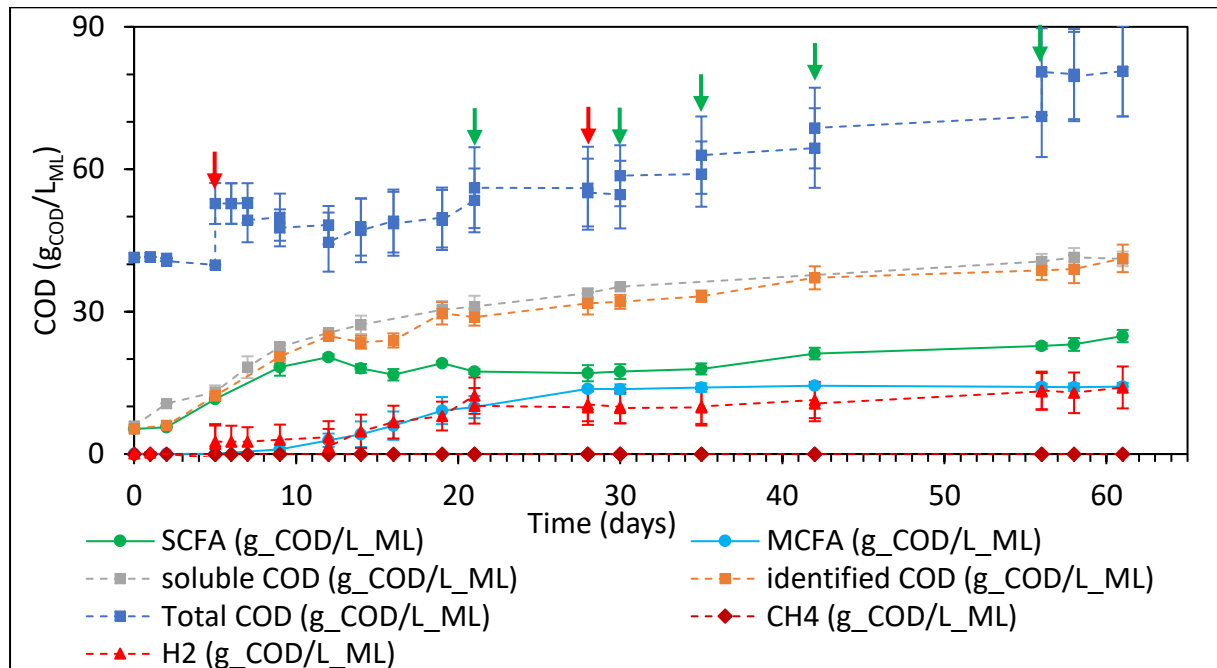


Figure 23 : Overview of the metabolite pools during the fermentation of BP in 2 L reactors. Red arrows indicate an addition of substrate with a dilution of the liquid phase while green arrows indicate an addition of substrate without dilution of the liquid phase. On day 5: $9,1 \pm 1,5$ g_{COD}/L_{ML} of substrate is added and 118 ± 4 mL_{supernatant}/L_{ML} is replaced by distilled water. Day 21: $4,4 \pm 0,2$ g_{COD}/L_{ML} of substrate is added in the reactor. Day 28: $2,9 \pm 0,2$ g_{COD}/L_{ML} of substrate is added and 99 ± 5 mL_{supernatant}/L_{ML} is replaced by distilled water. On day 30, 35, 42 and 56, $5,8 \pm 0,4$, $5,7 \pm 0,2$, $6,4 \pm 0,5$, $8,8 \pm 0,3$ g_{COD}/L_{ML} of substrate are respectively added in the reactors without dilution of the mixed liquor. Mean $\pm$ standard deviation of duplicated reactors.

Figure 24 shows the evolution of the metabolite concentrations over time in the 2 L reactors.

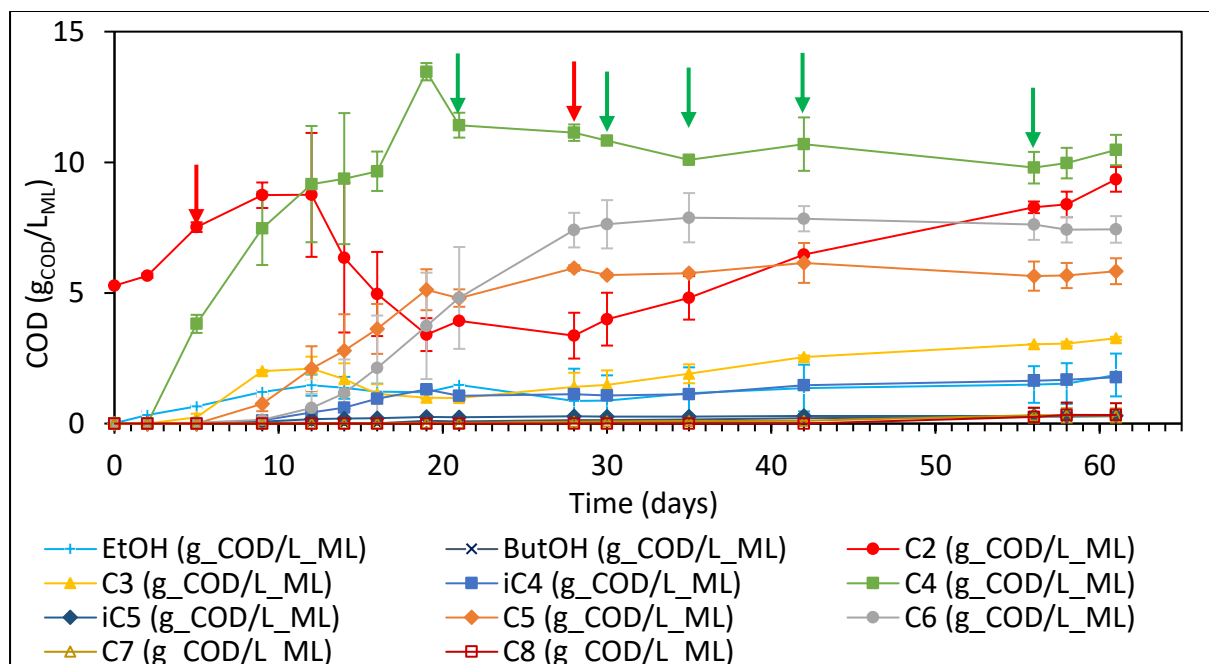


Figure 24 : Evolution of the metabolite concentrations over time in the 2 L reactors. No production of MCFA of C9+ chain is observed. The meaning of the arrows is described in the legend of Figure 23. Mean $\pm$ standard deviation of duplicated reactors.

Figure 25 shows the evolution of the net cumulated metabolite production over time in the 2 L reactors.

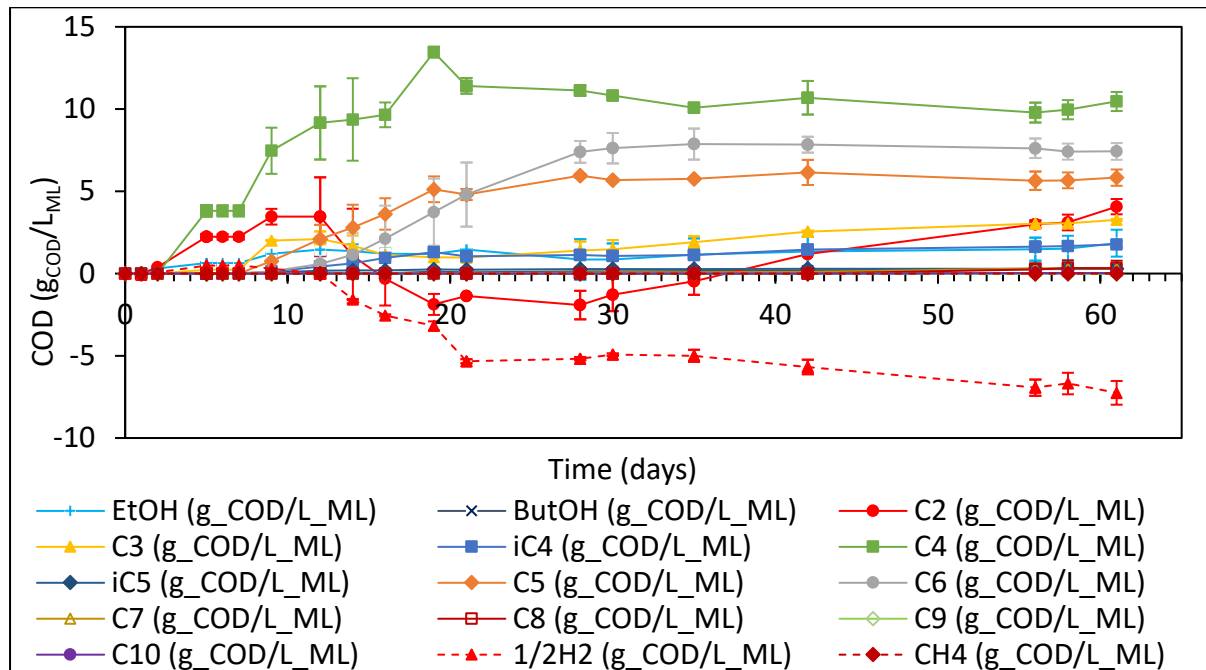


Figure 25 : Evolution of the net cumulated metabolite production over time in the 2 L reactors. Mean $\pm$ standard deviation of duplicated reactors.

Figure 26 shows the COD balance of the 2 L reactors.

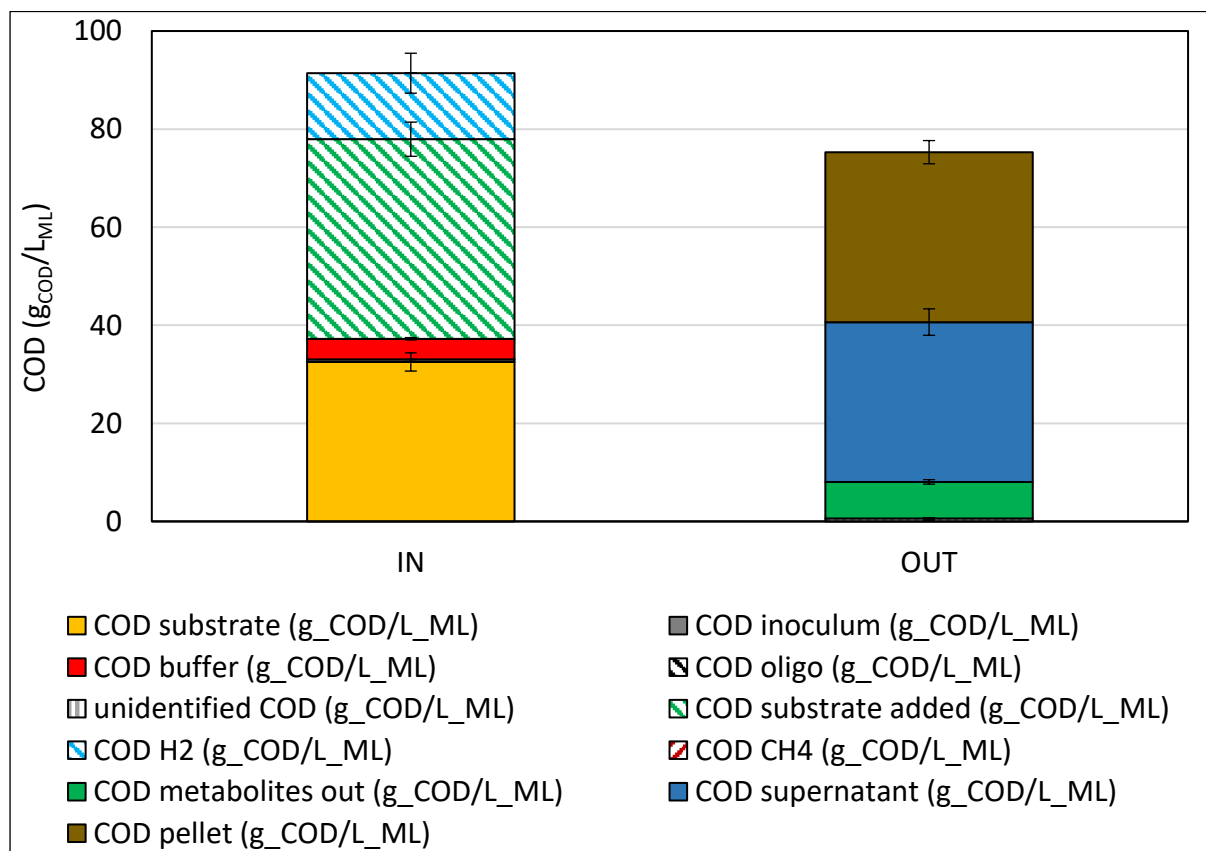


Figure 26 : COD balance of the 2 L reactors. Mean $\pm$ standard deviation of duplicated reactors.

Substrate was added on day 5 (Figure 23), CO₂ partial pressure increased from day 2 to 12 and reached 0,4 bars. C2 and EtOH accumulated until day 12, C4 concentration rose from day 2 until day 19. pH went down and was readjusted until day 14 (Figure 22).

From day 12, CO₂ partial pressure decreased and reached 0 on day 21 (Figure 21). H₂ started to be consumed until day 21 (Figure 25). pH remained stable around 6,5 until day 21. C2 was consumed until day 21, while C4 started to be consumed on day 21. Ethanol concentration remained constant until day 21. C6 concentration rose from day 9 to day 28 (Figure 24).

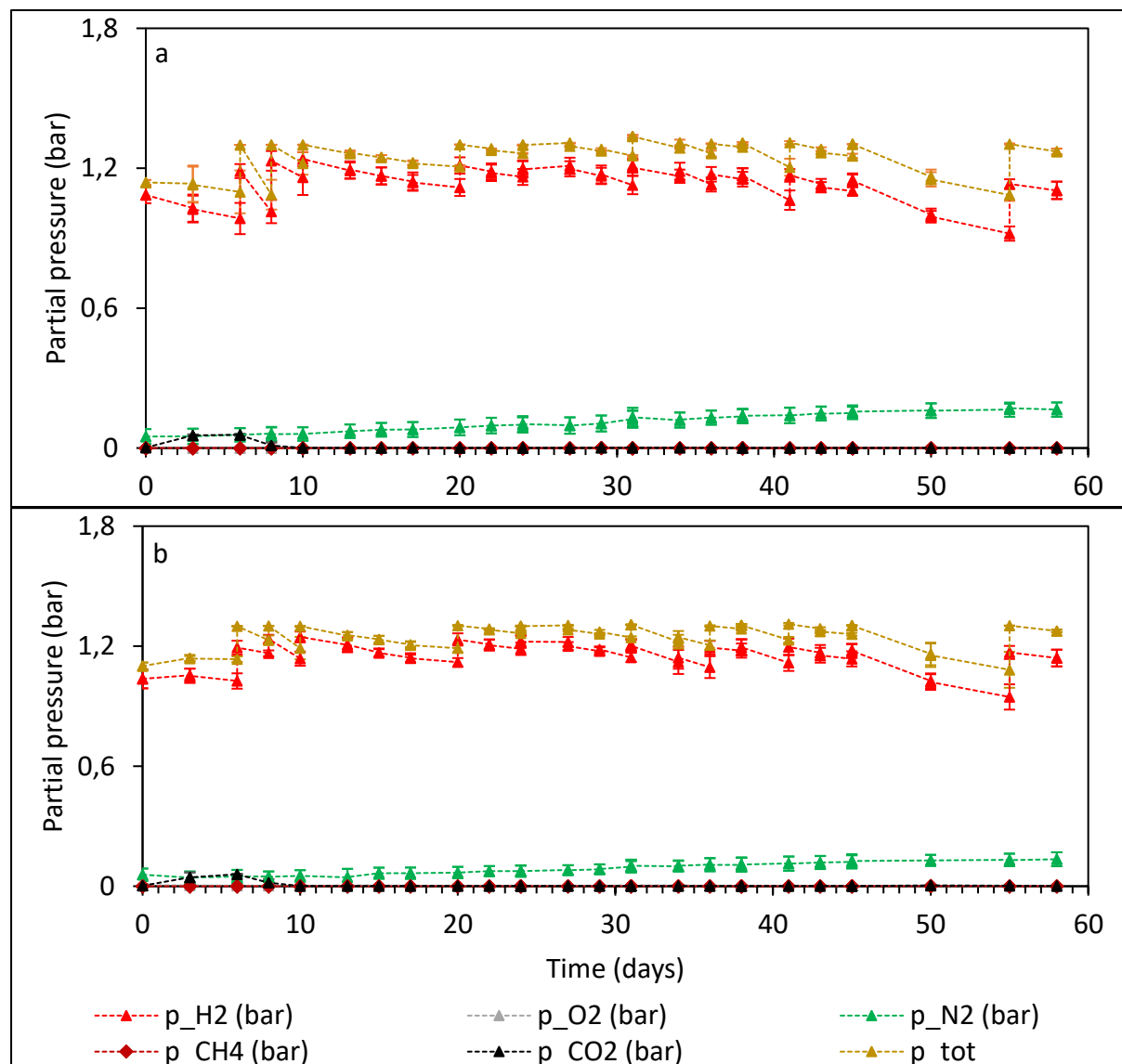
Additional substrate was fed again on day 21, CO₂ partial pressure rose again until day 42 to reach 0,04 bars and was a bit consumed thereafter but remained present in the gas phase. pH decreased again from day 21 until the end. C2 concentration started to rise again on day 28 until the end. Other metabolite concentrations remained stable from day 28 until the end, only ethanol concentration slowly rose to reach 1,8 g_{COD}/L_{ML} on day 61. C8 concentration started to rise on day 42 and reached a final concentration of 0,3 g_{COD}/L_{ML}.

5.3 Experiment 3

In this experiment, 2 substrates were compared, BP and BSG. The effect of H₂ and acetic acid was also tested. The operating conditions are as follow: acetic acid 5g/L_{ML}, H₂ or N₂, BSG 13,3 g_{COD}/L_{ML} or BP 40 g_{COD}/L_{ML}.

5.3.1 Brewer's spent grain (BSG) reactors

Figure 27 shows the evolution of the headspace gas composition over time in the BSG reactors.



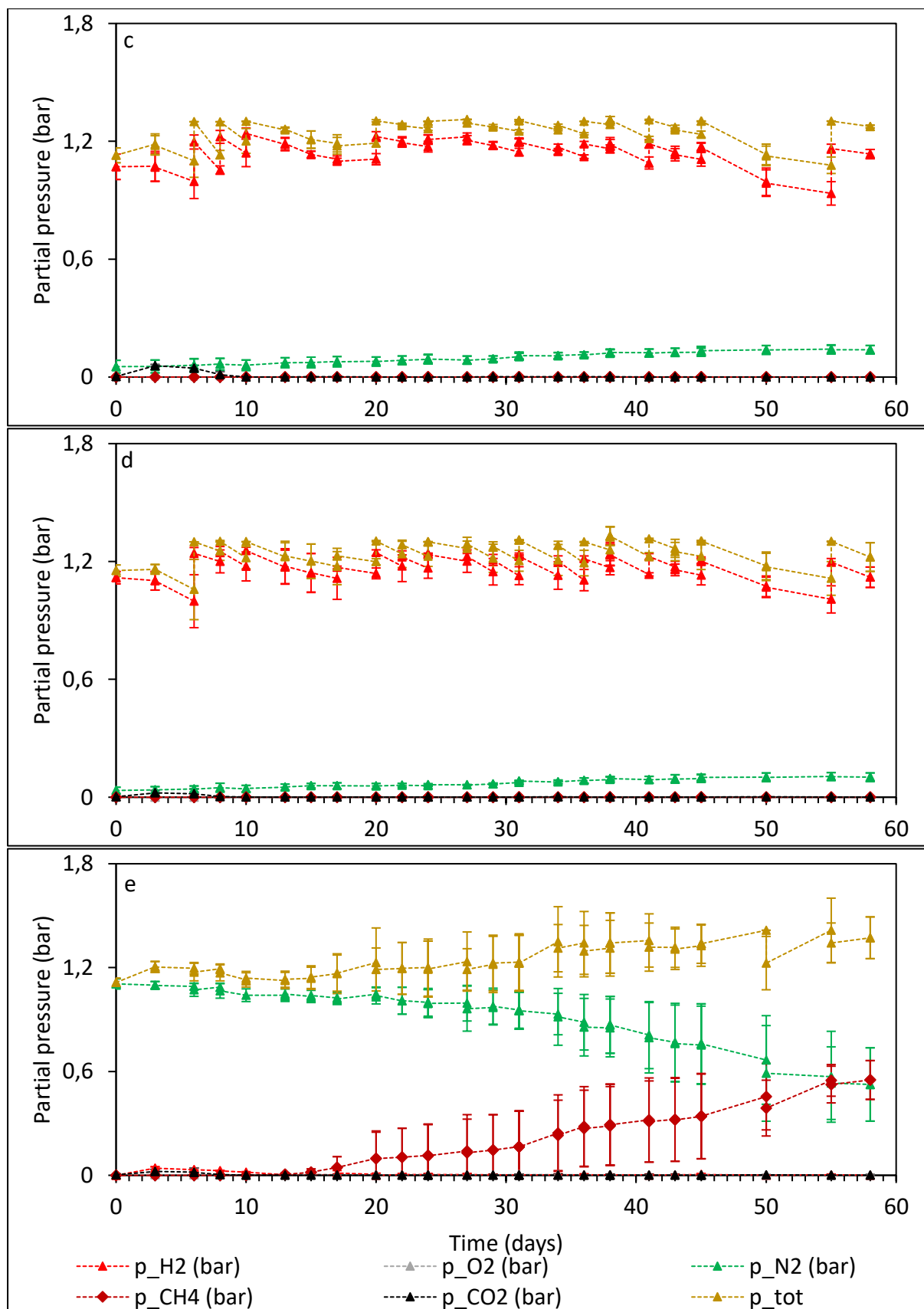


Figure 27 : Evolution of the headspace gas composition over time in the BSG reactors. a) reactor with fresh brewer spent grain, hydrogen and acetic acid. b) reactor with fresh brewer spent grain and trub, with hydrogen and acetic acid. c) reactor with frozen brewer spent grain, hydrogen and acetic acid. d) reactor with frozen brewer spent grain, hydrogen and without acetic acid. Figure 27e, reactor with frozen brewer spent grain, nitrogen and acetic acid. Every condition is done in triplicate.

Figure 28 shows the evolution of pH and the requirements for neutralization as a function of time in the BSG reactors.

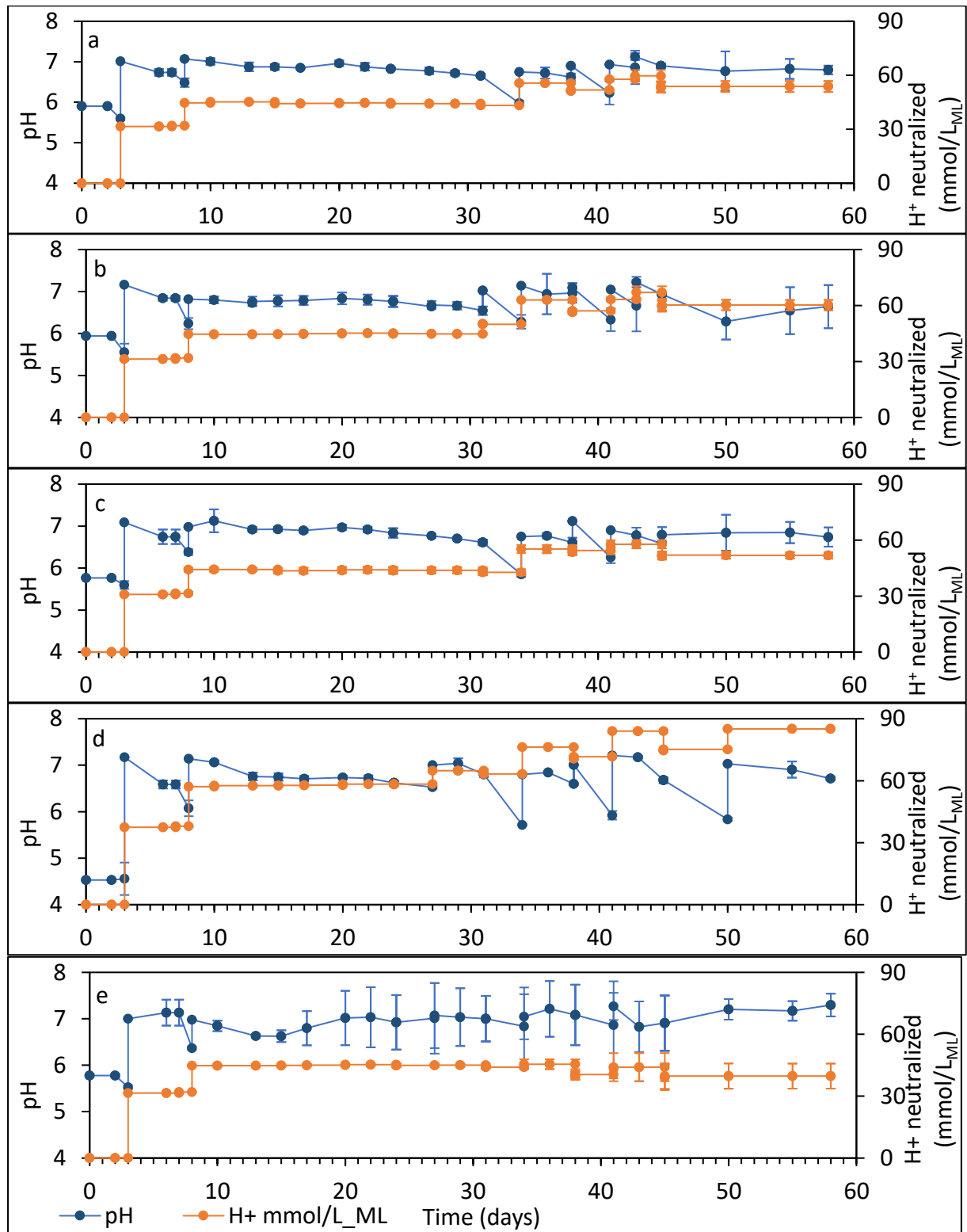
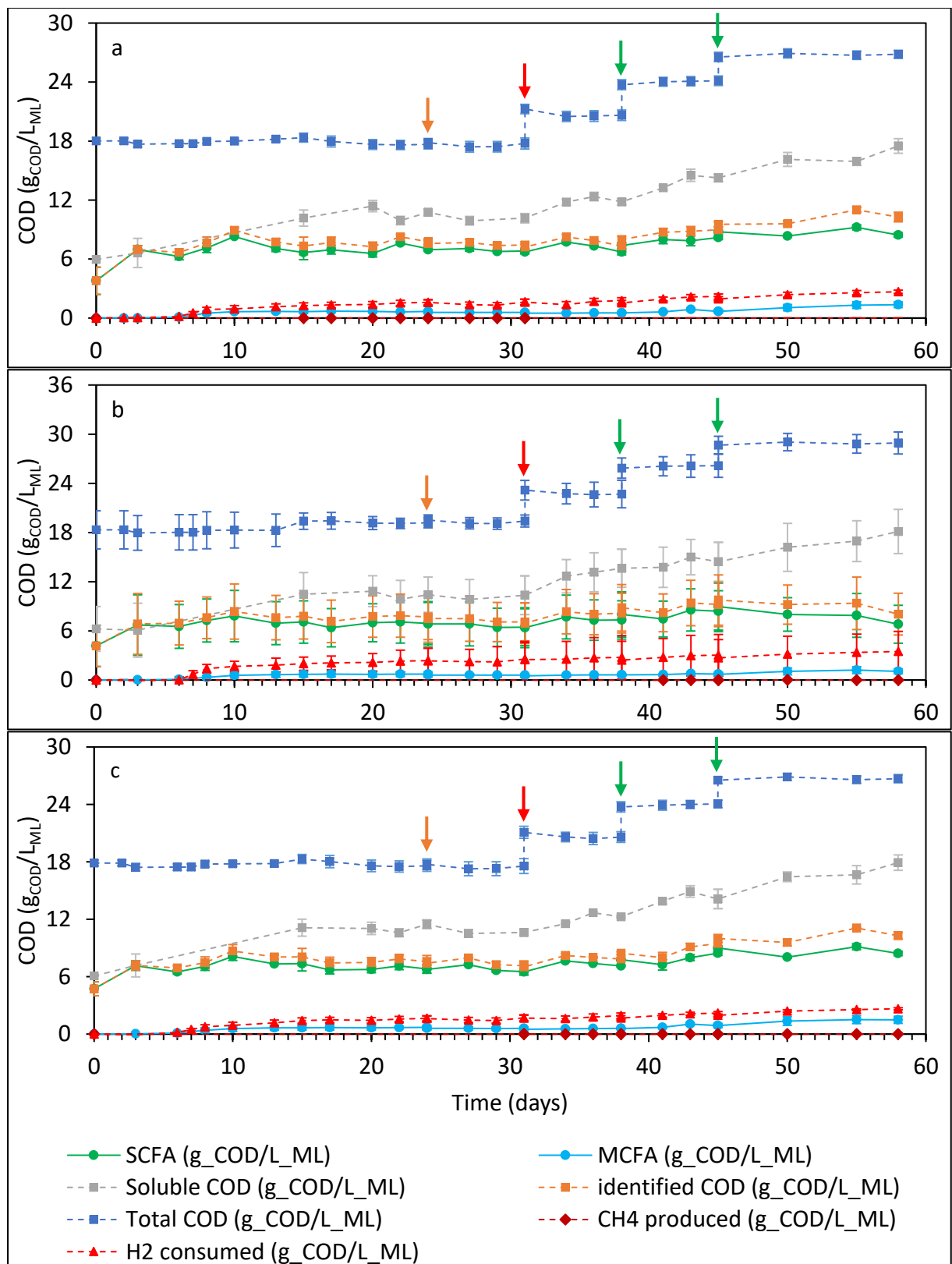


Figure 28 : Evolution of pH and the requirements for neutralization as a function of time in the BSG reactors. a) reactor with fresh brewer spent grain, hydrogen and acetic acid. b) reactor with fresh brewer spent grain and trub, with hydrogen and acetic acid. c) reactor with frozen brewer spent grain, hydrogen and acetic acid. d) reactor with frozen brewer spent grain, hydrogen and without acetic acid. e) reactor with frozen brewer spent grain, nitrogen and acetic acid. Mean and standard deviation of triplicate reactors.

Figure 29 shows an overview of the metabolite pools during the fermentation of BSG.



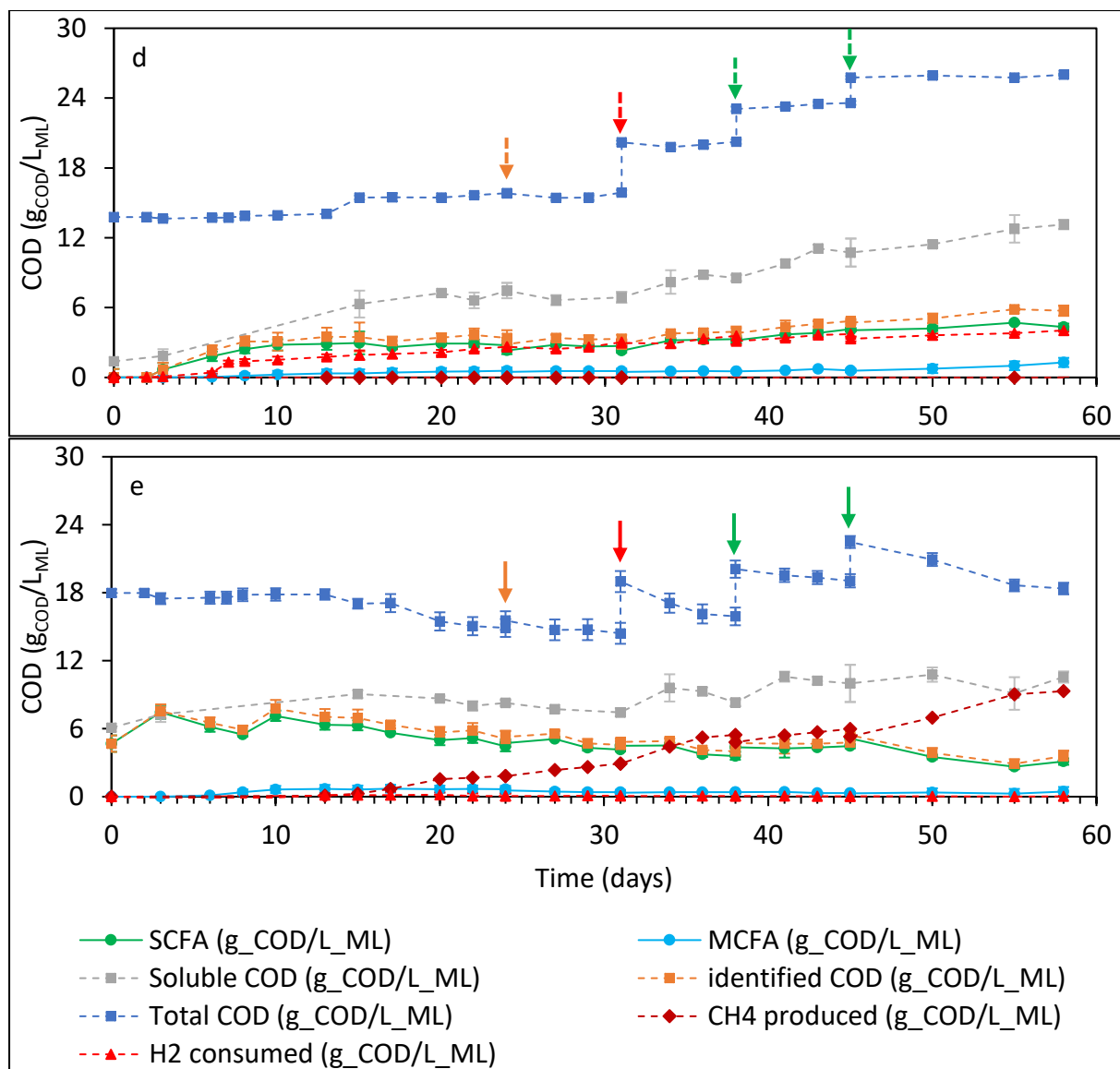
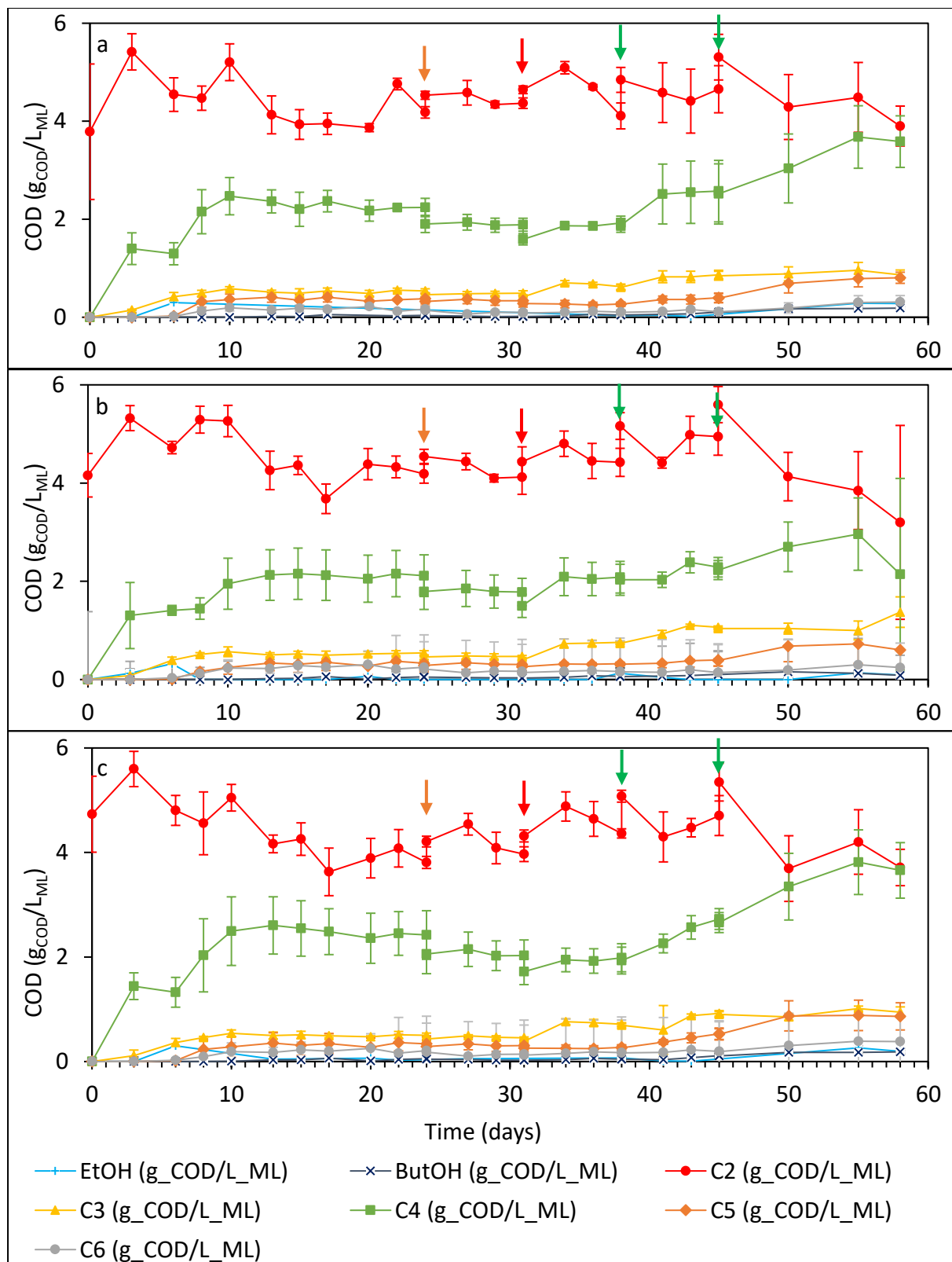


Figure 29 : Overview of the metabolite pools during the fermentation of BSG. a) reactor with fresh brewer spent grain, hydrogen and acetic acid. b) reactor with fresh brewer spent grain and trub, with hydrogen and acetic acid. c) reactor with frozen brewer spent grain, hydrogen and acetic acid. d) reactor with frozen brewer spent grain, hydrogen and without acetic acid. e) reactor with frozen brewer spent grain, nitrogen and acetic acid. The orange arrow on day 24 indicates an addition of $1,2 \pm 0,1$ g_{COD}/L_{ML} and a dilution where 153 ± 9 mL_{supernatant}/L_{ML} are replaced by the same volume of buffer. The red arrow on day 31 indicates an addition of $6,6 \pm 0,2$ g_{COD}/L_{ML} and a dilution where 156 ± 7 mL_{supernatant}/L_{ML} are replaced by the same volume of buffer. The arrows on day 38 and 45 both indicate an addition of $5,8 \pm 0,2$ and $5,2 \pm 0,2$ g_{COD}/L_{ML} respectively without addition of 50 mL of buffer, increasing the volume of mixed liquor in the reactor. The dotted arrows on Figure 29e indicates an addition of substrate as for the orange, red and green arrows, but the dilution is made using distilled water instead of the buffer. Mean and standard deviation of triplicate reactors.

Figure 30 shows the evolution of the metabolite concentration over time in the BSG reactors.



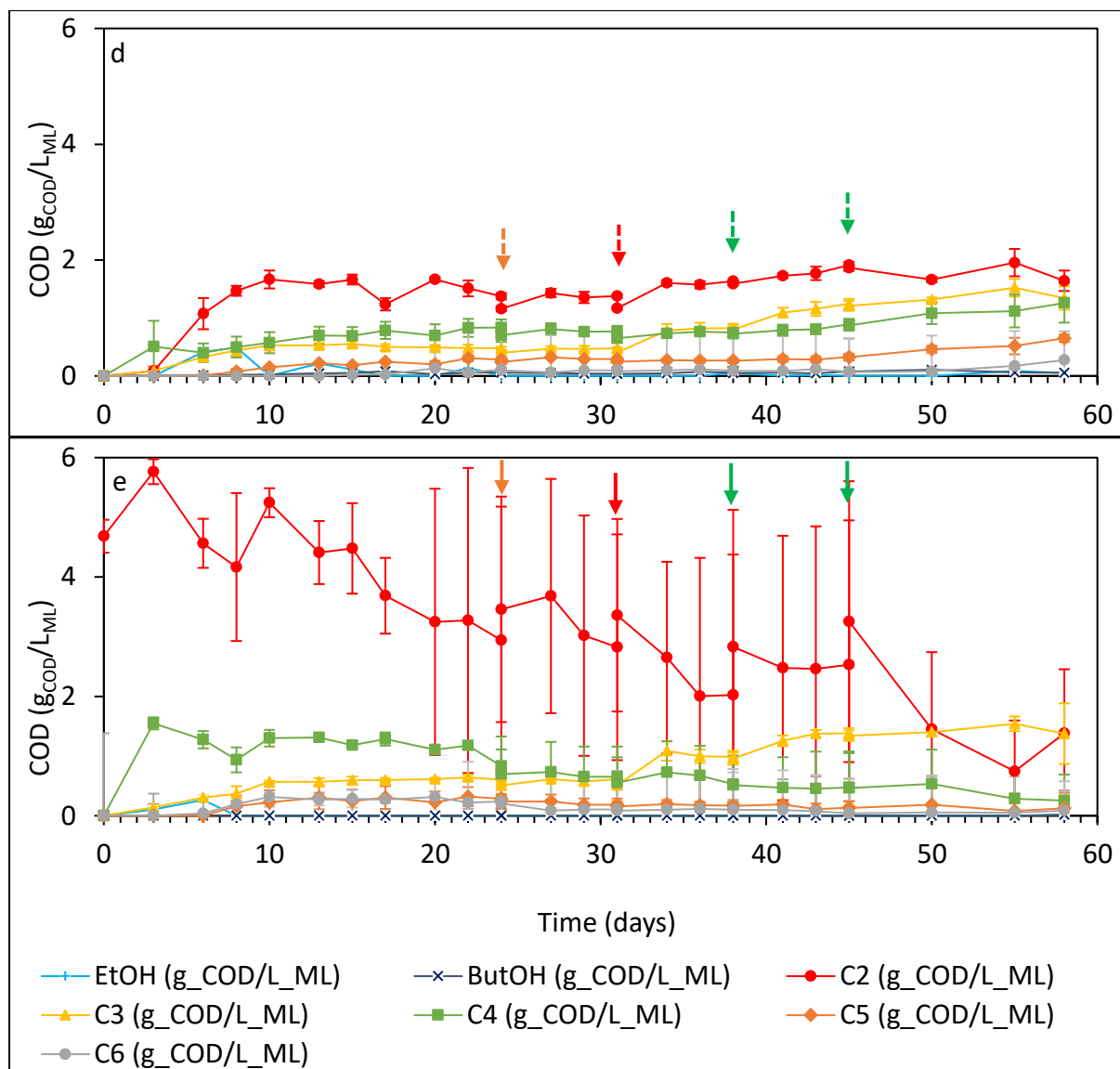
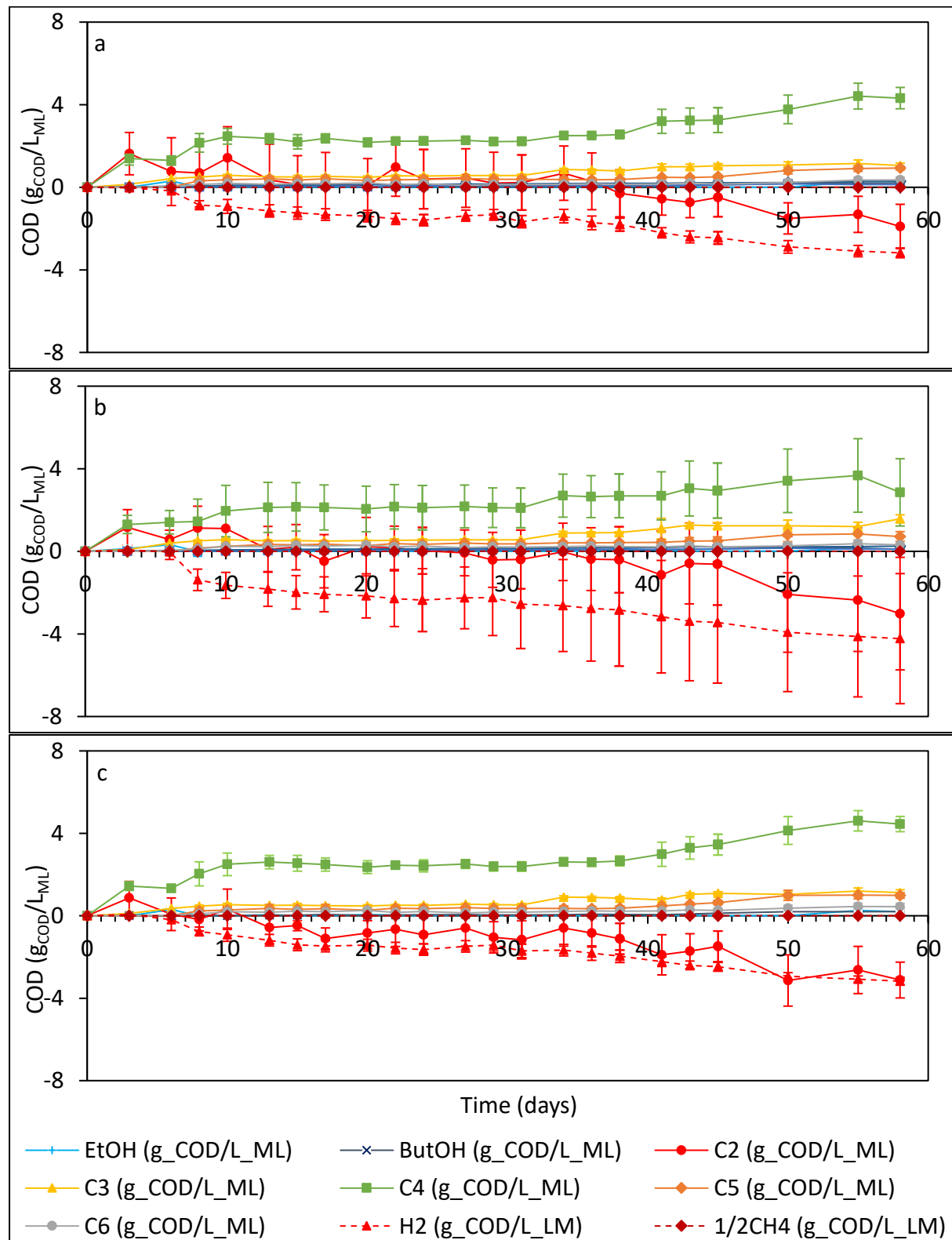


Figure 30 : Evolution of the metabolite concentration over time in the BSG reactors. a) reactor with fresh brewer spent grain, hydrogen and acetic acid. b) reactor with fresh brewer spent grain and trub, with hydrogen and acetic acid. c) reactor with frozen brewer spent grain, hydrogen and acetic acid. d) reactor with frozen brewer spent grain, hydrogen and without acetic acid. e) reactor with frozen brewer spent grain, nitrogen and acetic acid. The meaning of the arrows is described in the legend of Figure 29. Mean and standard deviation of triplicate reactors.

Figure 31 shows the evolution of the net cumulated metabolite production over time in the BSG reactors.



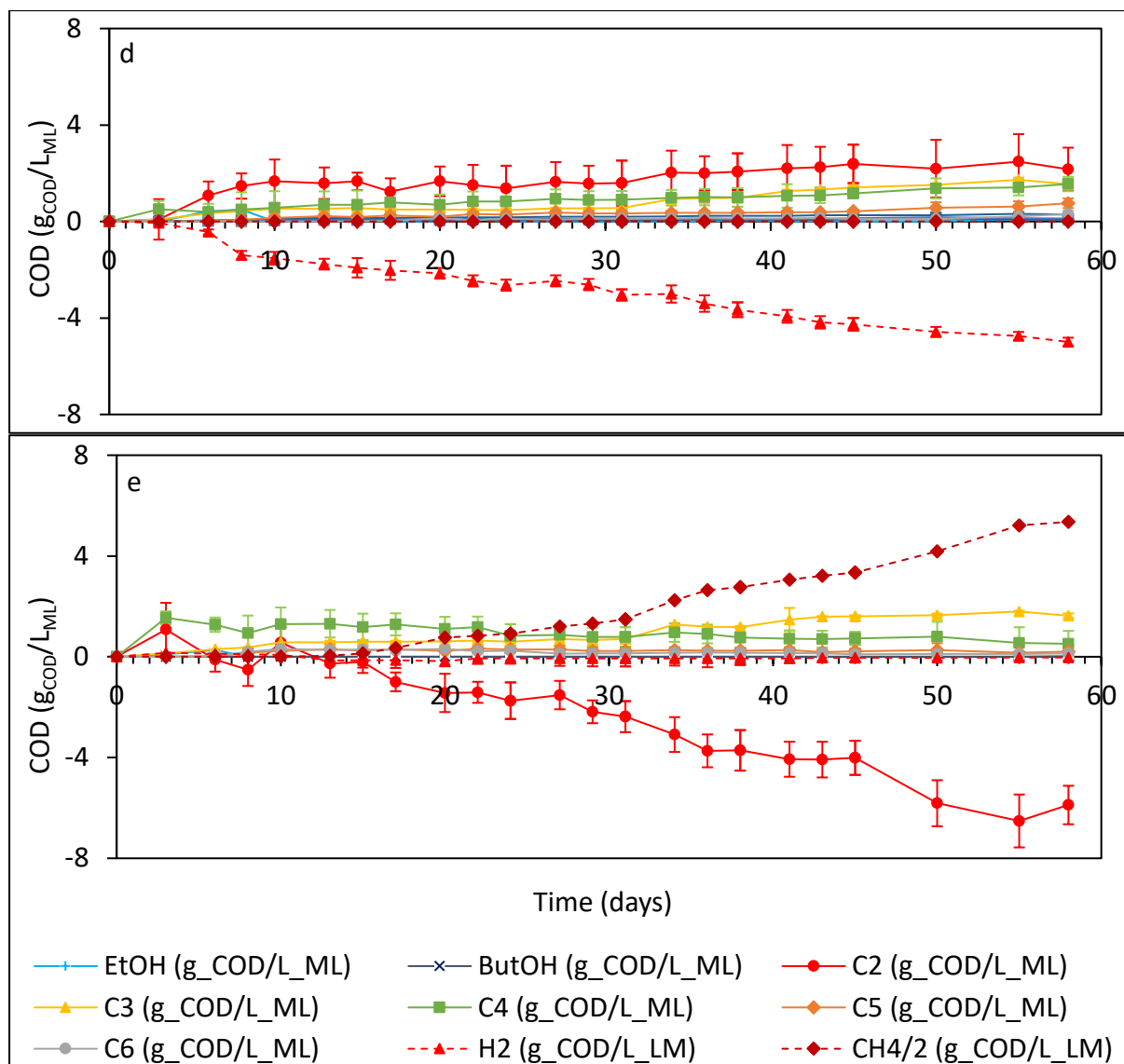


Figure 31 : Evolution of the net cumulated metabolite production over time in the BSG reactors. a) reactor with fresh brewer spent grain, hydrogen and acetic acid. b) reactor with fresh brewer spent grain and trub, with hydrogen and acetic acid. c) reactor with frozen brewer spent grain, hydrogen and acetic acid. d) reactor with frozen brewer spent grain, hydrogen and without acetic acid. e) reactor with frozen brewer spent grain, nitrogen and acetic acid. Every condition is done in triplicate. For the sake of good presentation of the other metabolites, the data of CH₄ are divided by a factor of 2. Mean and standard deviation of triplicate reactors.

Figure 32 shows the COD balance of the BSG reactors.

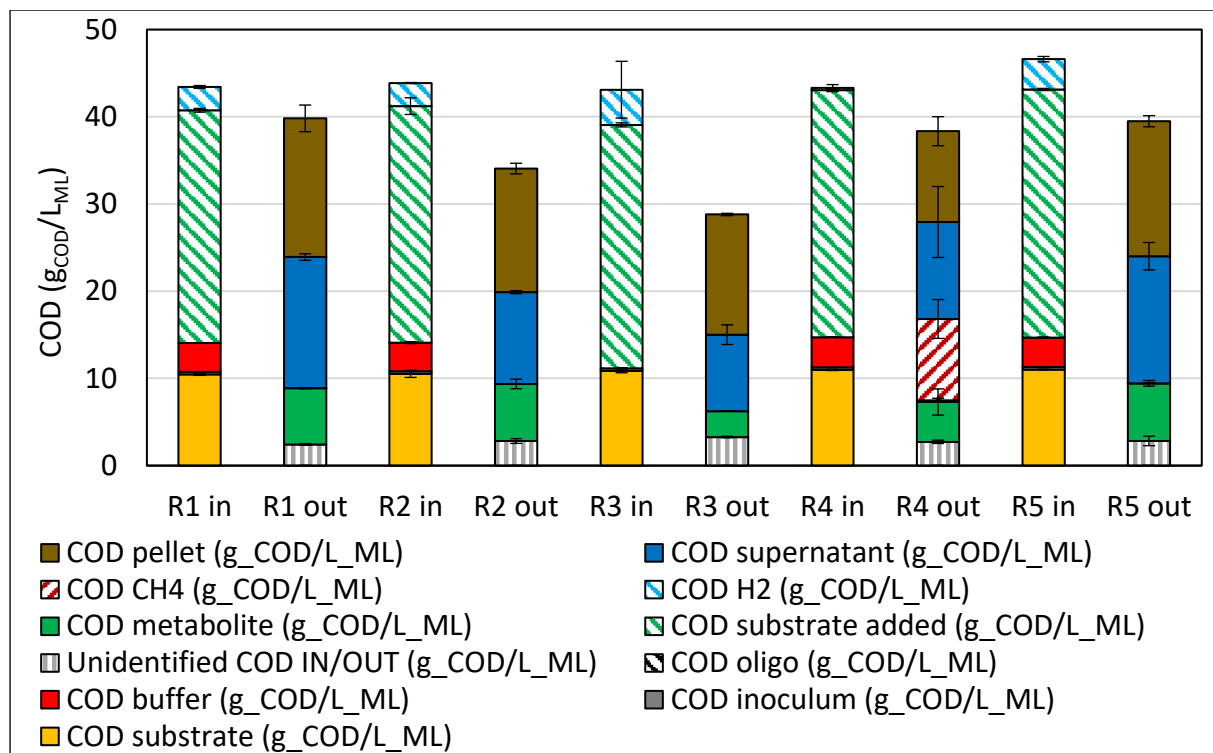


Figure 32 : COD balance of the BSG reactors. R1, reactors with fresh BSG, acetic acid and hydrogen. R2, reactors with frozen BSG, acetic acid and with hydrogen. R3 reactors with frozen brewer spent grain, hydrogen and without acetic acid. R4, reactor with frozen brewer spent grain, acetic acid and nitrogen. R5, reactor with fresh BSG + trub, acetic acid and hydrogen. Mean and standard deviation of triplicate reactors.

5.3.1.1 Reactors with fresh BSG, frozen BSG or fresh BSG + trub, acetate, H_2

Between day 0 and day 10, CO_2 partial pressure rose, up to 0,06 bars before decreasing and reaching 0 bars. H_2 partial pressure started to drop on day 6 and pressure was readjusted with H_2 to remain above 1,3 bars. pH goes down but was readjusted to remain around 7. C4 concentration rose to reach 2,5 g_{COD}/L_{ML} and C2 concentration remained constant.

From day 31, substrate was added every week. pH went down and was readjusted frequently to remain around 7. As can be seen in Figure 27, no CO_2 was present in the gas phase, but H_2 started to be consumed on day 34 (Figure 31). C2 was consumed on day 45 while C4 and ethanol were produced.

5.3.1.2 Reactor with frozen BSG, no acetate, H_2

Between day 0 and 10, CO_2 partial pressure roses, up to 0,02 bar before decreasing and reaching 0. H_2 partial pressure dropped and was readjusted by adding H_2 to keep a global pressure above 1,3 bars. pH went down and was readjusted to remain around 7. C4 concentration rose from the start until day 3 and remained constant thereafter while C2

concentration started rising on day 3 until day 10. Ethanol concentration also rose, up 1,7 g_{COD}/L_{ML} before decreasing, down to 0 g_{COD}/L_{ML} on day 10.

From day 31, substrate was added every week. pH went down and was readjusted to 7. No CO₂ was detected in the gas phase. C₂ and H₂ consumption increased while C₄ remained constant (Figure 31d). C₆ started to be produced on day 50.

There is a difference between the in and out COD of reactor 3 as shown on Figure 32

5.3.1.3 Reactors with frozen BSG, acetate, no H₂

Between day 0 and day 8, pH went down and was readjusted. C₂ and C₄ were produced until day 3 before being consumed (Figure 31). CH₄ started to be produced on day 15 and CO₂ was not produced.

From day 31, substrate was added every week. pH remained stable, C₂ and C₄ were still consumed and CH₄ production increased.

As shown in Figure 32, COD balance of the BSG reactors without acetic acid (reactor 4) matches.

5.3.2 Beet pulp (BP) reactors

Figure 33 shows the evolution of the headspace gas composition over time in the BP reactors.

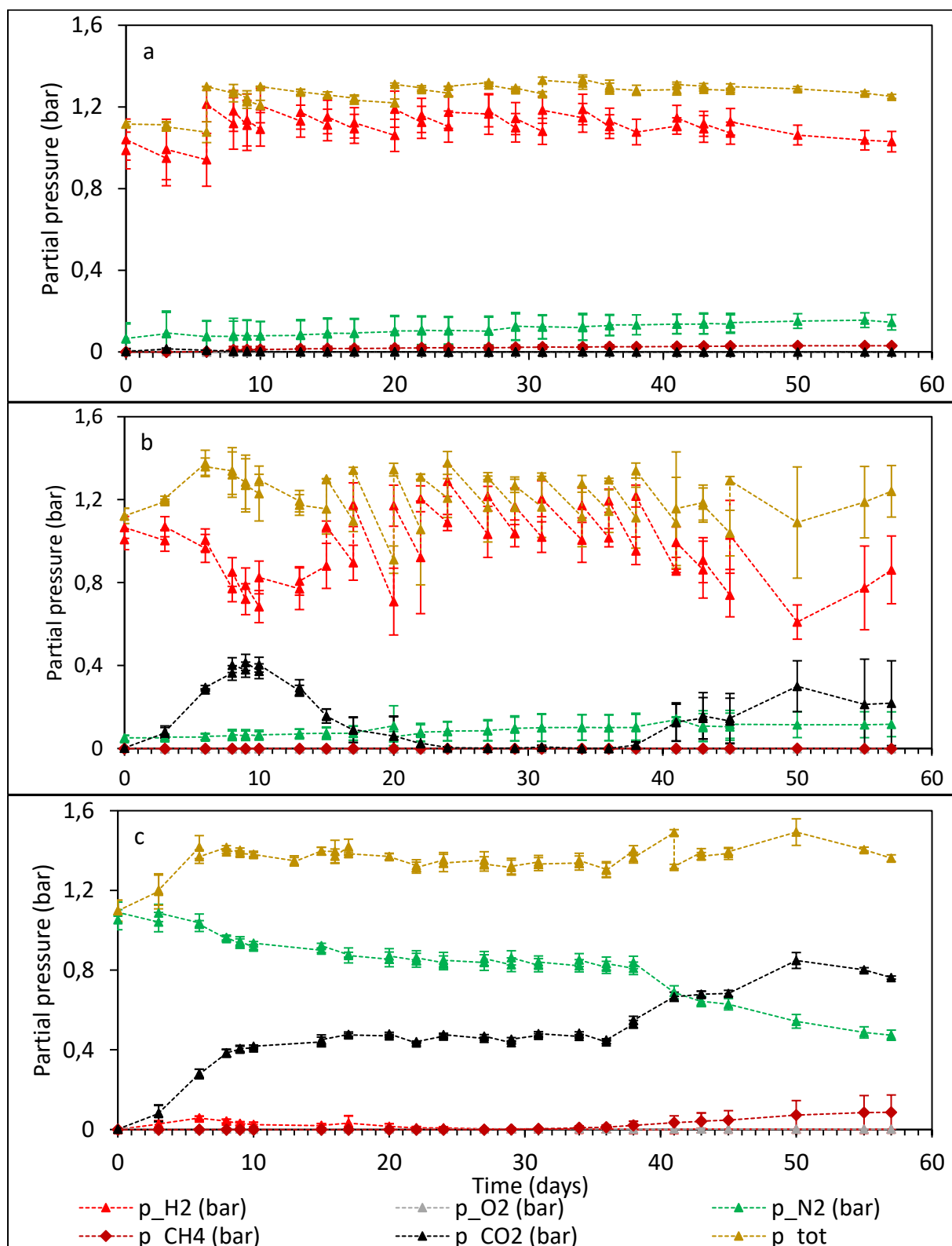


Figure 33 : Evolution of the headspace gas composition over time. a) reactor without substrate, with hydrogen and acetic acid. b) reactor with BP, hydrogen and acetic acid. c) reactor with BP, nitrogen and acetic acid. Mean and standard deviation of triplicate reactors.

Figure 34 shows the evolution of pH and the requirements for neutralization as a function of time in the BP reactors.

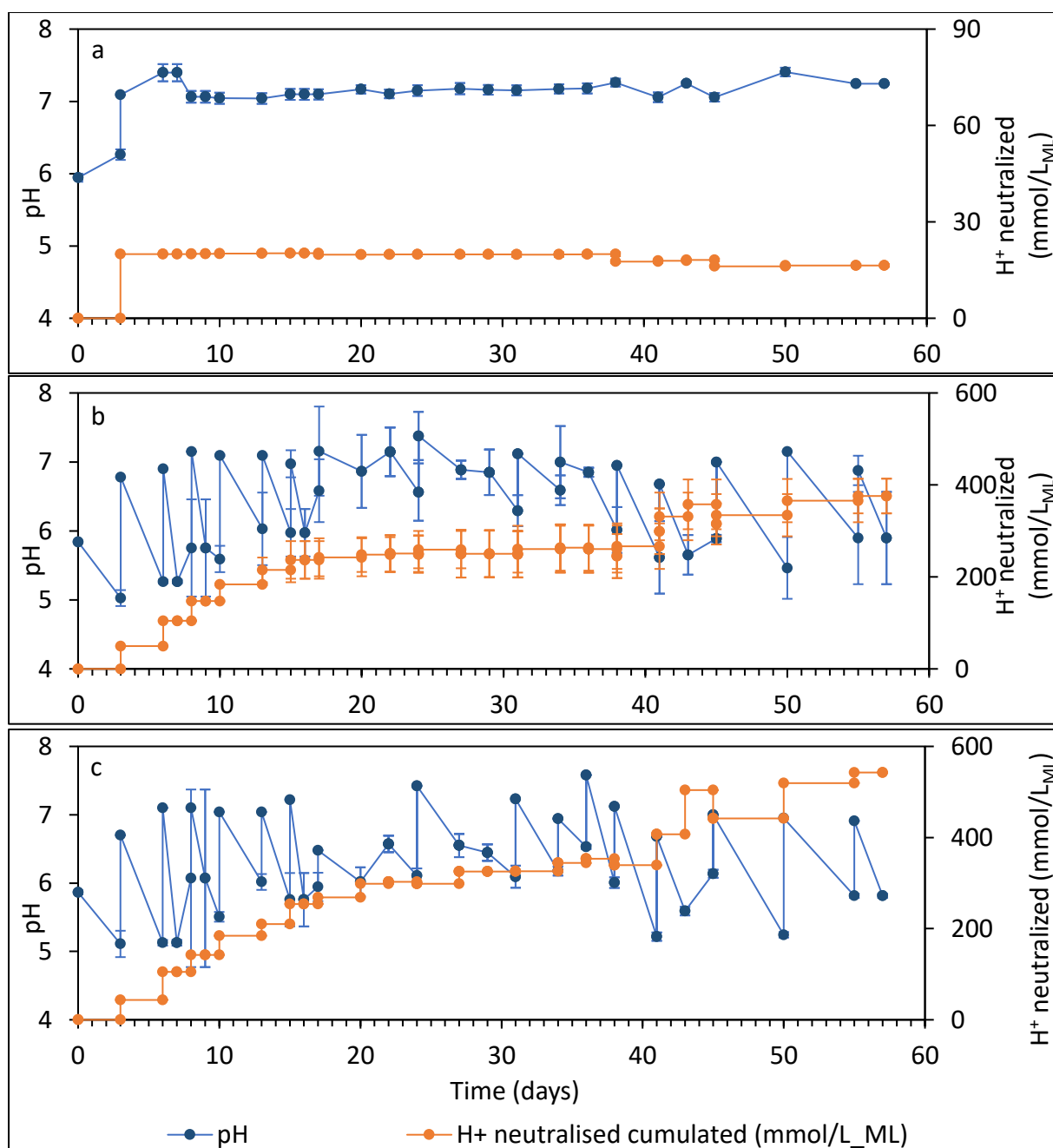


Figure 34 : Evolution of pH and the requirements for neutralization as a function of time in the BP reactors. a) reactor without substrate, with hydrogen and acetic acid. b) reactor with BP, hydrogen and acetic acid. c) reactor with BP, nitrogen and acetic acid. Mean and standard deviation of triplicate reactors.

Figure 35 shows an overview of the metabolite pools during the fermentation of BP.

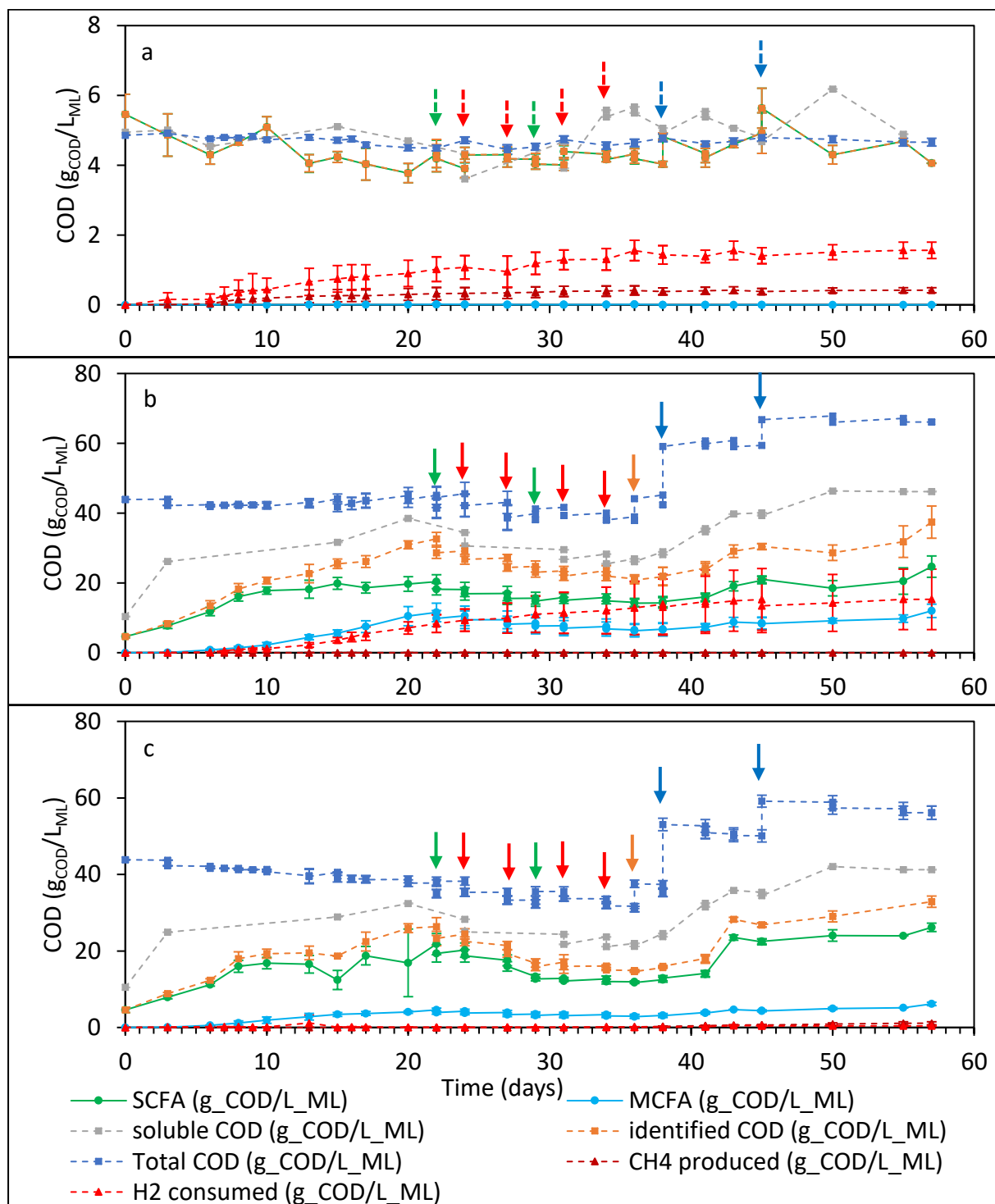


Figure 35 : Overview of the metabolite pools during the fermentation of BP. a) reactor without substrate except the inoculum sludge, with hydrogen and acetic acid. b) reactor with BP, hydrogen and acetic acid. c) reactor with BP, nitrogen and acetic acid. Notice that the scale in Figure 35a is different from Figure 35b and c. The green arrows on day 22 and 29 represent an addition of $3,2 \pm 0,1$ g_{COD}/L_{ML} and $3,2 \pm 0,3$ g_{COD}/L_{ML} of substrate respectively and a replacement of 152 ± 4 mL_{supernatant}/L_{ML} and 89 ± 6 mL_{supernatant}/L_{ML} respectively by the same volume the buffer. Red arrows on day 24, 27 31 and 34 indicate a dilution of the liquid phase: 105 ± 3 , 134 ± 9 , 93 ± 3 and 89 ± 6 mL_{supernatant}/L_{ML} respectively were replaced by the same volume of buffer. The orange arrow on day 36 indicates that $6,4 \pm 0,4$ g_{COD}/L_{ML} of substrate is added in the reactors. The blue arrows on day 38 and 45 indicates an addition of 17 ± 1 and $15,0 \pm 0,9$ g_{COD}/L_{ML} respectively and an addition of 125 ± 9 and 110 ± 6 mL_{buffer}/L_{ML} respectively. The dotted arrows on Figure 35a indicate that no substrate is added, but the dilutions are made as for the other reactors. Mean and standard deviation of triplicate reactors.

Figure 36 shows the evolution of metabolite concentration over time in the BP reactors.

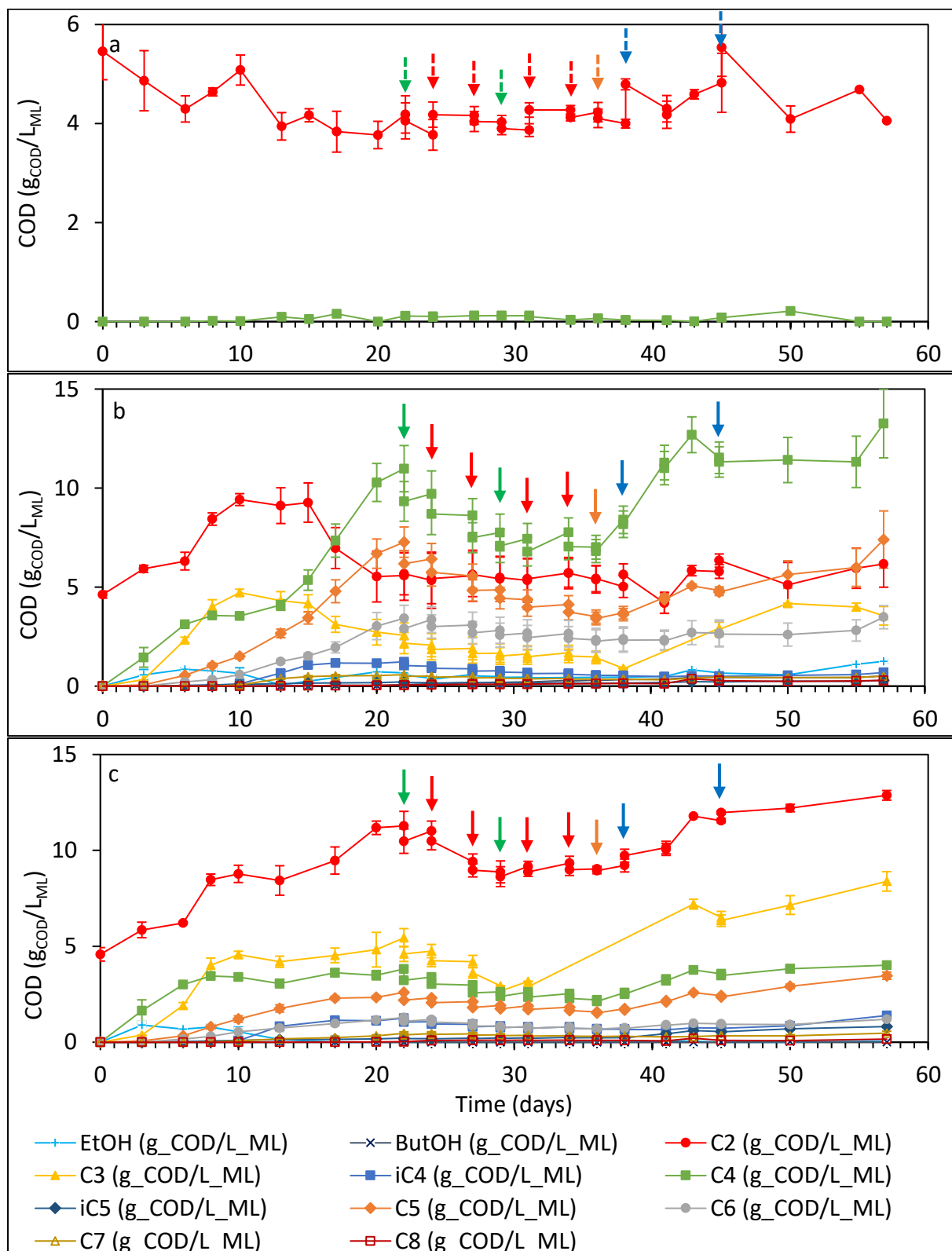


Figure 36 : Evolution of metabolite concentration over time in the BP reactors. a) reactor without substrate, with hydrogen and acetic acid. b) reactor with BP, hydrogen and acetic acid. c) reactor with BP, nitrogen and acetic acid. Notice that the scale in Figure 36a is different from Figure 36b and c. The meaning of the colored arrows is described in the legend of Figure 35. Mean and standard deviation of triplicate reactors.

Figure 37 shows the evolution of the net cumulated metabolite production over time of BP reactors.

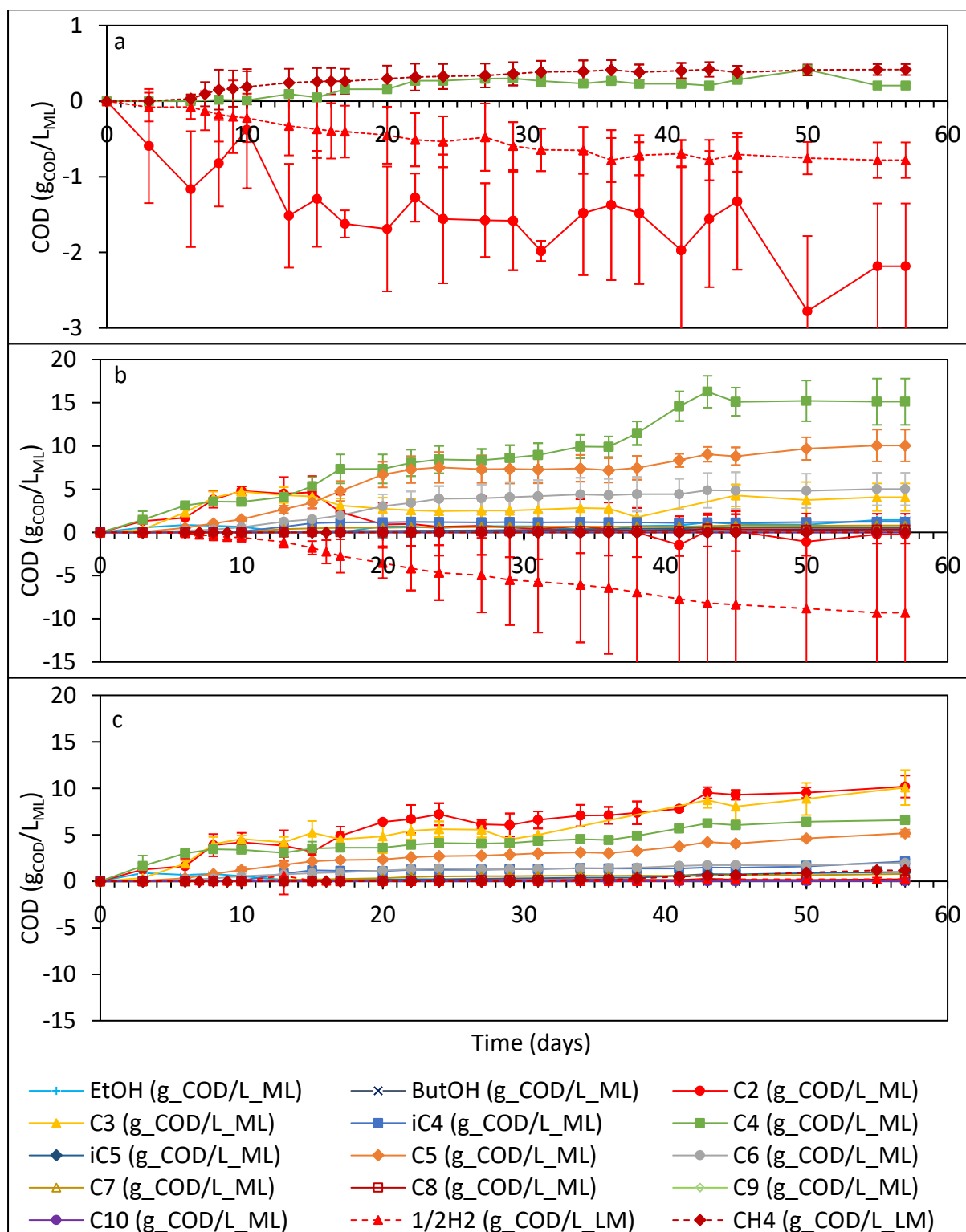


Figure 37 : Evolution of the net cumulated metabolite production over time of BP reactors. a) reactor without substrate, with hydrogen and acetic acid. b) reactor with BP, hydrogen and acetic acid. c) reactor with BP, nitrogen and acetic acid. Notice that the scale in Figure 37a is different from Figure 37b and c. Mean and standard deviation of triplicate reactors.

Figure 38 shows the COD balance of the BP reactors.

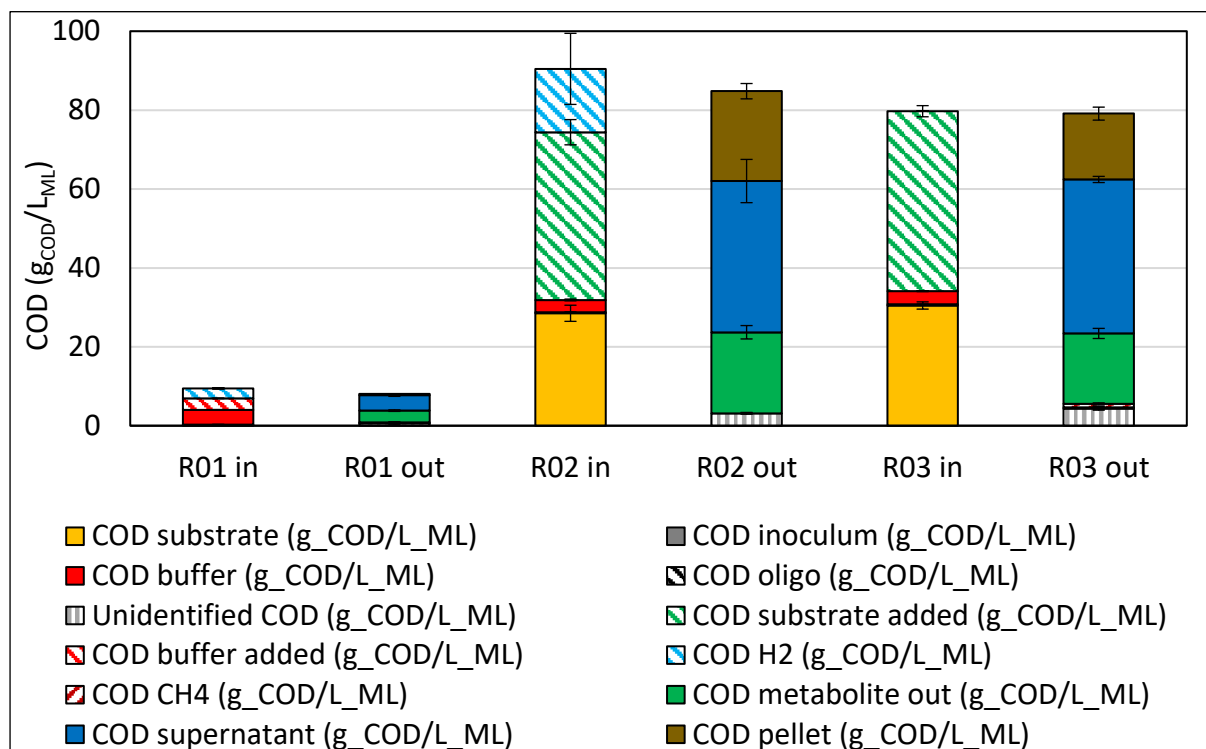


Figure 38 : COD balance of BP reactors. R1, reactor without substrate, with hydrogen and acetic acid. R2, reactor with BP, hydrogen and acetic acid. R3, reactor with BP, nitrogen and acetic acid.

5.3.2.1 Reactors with no substrate, acetate, H₂

The only available substrate was the COD brought by the inoculum sludge. pH had to be adjusted on day 3 and remained stable thereafter. As shown on Figure 37, C2 was consumed from the start and C4 production started to rise on day 6. On the same day, H₂ consumption and CH₄ production started.

As can be seen on Figure 38, the balance between in and out COD matches well.

5.3.2.2 Reactors with substrate, acetate, H₂

Between day 0 to 15, pH decreased and had to be readjusted to 7 frequently. CO₂ partial pressure rose up to 0,4 bars, before decreasing. H₂ partial pressure decreased and H₂ was added to keep a total pressure above 1,3 bars. C2 and C4 were produced but C2 accumulation stopped on day 15 around 5,4 g_{COD}/L_{ML} and C6 production started on day 6 as shown in Figure 37.

On day 15, pH still needed to be adjusted, H₂ consumption increased a bit and CO₂ partial pressure continued to drop until it reached 0 bars and C2 started to be consumed. On day 17, C4 production stabilized and C6 production increased until day 24.

Starting from day 24, substrate was added every week. CO₂ partial pressure increased from 0 to 0,3 bars between day 38 to 50 before decreasing. H₂ was consumed. Between day 41 and 43, C₂, C₄ and ethanol concentration rose, C₆ concentration remained stable and C₈ concentration rose from 0 to 0,4 g_{COD}/L_{ML}.

As shown on Figure 38, in and out COD were balanced.

5.3.2.3 *Reactors with substrate, acetate, no H₂*

Between day 0 and 22, the pH decreased and was readjusted (Figure 34). CO₂ partial pressure rises and stabilized around 0,4 bars on day 10. H₂ partial pressure rises, up to 0,06 bars on day 6, and decreased thereafter (Figure 33). C₂ and C₄ concentrations increased until day 10 where they stabilized around 8,8 and 3,4 g_{COD}/L_{ML}, C₂ concentration increased again from 8,4 to 11,3 g_{COD}/L_{ML} between day 13 and 22. Ethanol concentration rose to 0,9 g_{COD}/L_{ML} until day 3 and slowly decreased to 0 on day 17. C₆ concentration rises between day 6 and 22 where it reached 1,3 g_{COD}/L_{ML} (Figure 36).

From day 24, substrate was added every week (Figure 35), pH decreased again and was readjusted (Figure 34). CH₄ partial pressure rose on day 36 and reached a final concentration of 0,1 bar. CO₂ partial pressure increased from 0,4 to 0,8 bar between day 36 and 50 (Figure 33). SCFA concentration rose from 13,9 to 23,5 g_{COD}/L_{ML} between day 41 and 43 before remaining stable until the end of the experiment with a final concentration of 26,2 g_{COD}/L_{ML}. MCFA remained at low concentration from day 24 until the end, with a final concentration of 6,2 g_{COD}/L_{ML}.

As shown on Figure 38, the in and out COD were balanced.

6 Discussion

6.1 Preliminary discussion

6.1.1 Fermentations profile

In all reactors with fermentable substrate (BP, BSG), a first phase is observed where CO_2 , C_2 , C_4 and Ethanol are produced. pH also decreases, probably linked to the production of C_2 and C_4 . This suggest that a significant acidogenic fermentation occurs. This phenomenon is not observed in the reactor without substrate (except the organic matter present in the inoculum), indicating that easily digestible substrate is necessary. A second phase is observed after CO_2 has accumulated in the reactor. During this phase, CO_2 and H_2 are consumed, while butyrate is produced and acetate remains almost constant. This suggest two simultaneous phenomena: an homoacetogenesis that consumes CO_2 and H_2 to produce acetate, which is consumed in an elongation process to produce butyrate.

Acidogenesis is probably the dominant reaction as long as CO_2 is produced and CO_2 can be considered as a process indicator. Once CO_2 starts to be consumed, homoacetogenesis would become the dominant pathway until no more CO_2 is observed in the gas phase. This suggest that the CO_2 production is a limiting factor for the homoacetogenesis. The elongation process then becomes the dominant pathway.

Possibly, the two first phenomena continue to happen during the whole experiment, but are no longer the dominant ones.

6.1.2 pH jump in the bio-electro-reactor

A jump in pH was observed between day 14 and 16, when the applied potential was raised from 2300 to 2700 mV (Figure 8). Protons of the catholyte compartment are transformed at the surface of the biocathode in H_2 while protons are produced in the anodic compartment. The pH peak observed on day 16 is due to the protons that couldn't go from the anodic to the catholyte compartment as fast as they were consumed in the catholyte, resulting in a progressive alkalization of the cathodic compartment. There was thus a kinetic phenomenon and as can be seen on Figure 8 once the potential difference was lowered, the consumption of H^+ in the catholyte decreased and the pH came back to normal.

6.1.3 Cyclic voltammetry

Figure 39 shows the cyclic voltammetry results of the bio-electro-reactor (BER) on different days of the experiment. Microorganisms are expected to catalyze the electron transfer from the cathode, reducing the cathode overpotential and diverting the electrons from hydrogen synthesis. However, as can be seen on Figure 39, the cathode potential where current started increasing is lower on day 0 than later in the experiment. Hydrogen is also produced in the BER as shown in Figure 7, suggesting the microorganisms don't use electrons directly.

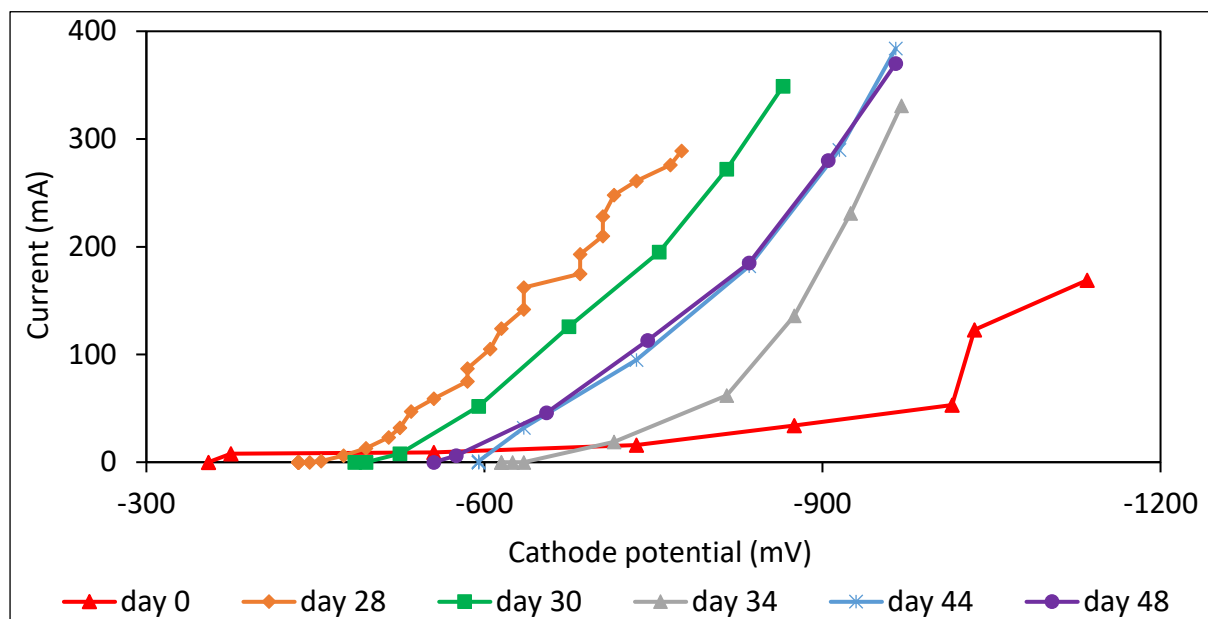


Figure 39: Evolution of the current as a function of the cathode potential over time.

However, in order to understand the influence of the biofilm development, voltammetry measurements should have been done from the beginning of the experiment and with an abiotic reactor as negative control. Between day 28 and 48, the biofilm is expected to be well developed and it's more likely that the cathode potential variation is caused by a change of composition of the liquid phase (Figure 12), than to the further development of the biofilm.

6.1.4 COD balance of the bio-electro-reactor

As shown on Figure 14, 1/3 of the COD introduced in the BER is not recovered at the end of the experiment.

A first hypothesis could be that a part of the solid remaining substrate stayed stuck on the inner side of the reactor and thus couldn't be collected when the experiment was stopped. However, by looking at the COD of the solid, which is $0,22 \pm 0,01 \text{ g}_{\text{COD}}/\text{g}_{\text{fresh matter}}$, $10 \text{ g}_{\text{COD}}$ represent $45,5 \text{ g}_{\text{fresh matter}}$ that should stay stuck on the inner side of the reactor. Only $16,2 \text{ g}$

of solid fresh matter were retrieved when the BER was stopped. It's thus impossible that almost three times this amount was forgotten in the reactor.

A second hypothesis is that the reactor could have lost part of its airtightness over time and some H₂ could have leaked. However, multiple airtightness tests had been done during the experiment by immersing the reactor and looking for bubbles. The reactor had been proven airtight every time.

The third hypothesis is that the gas outflow could be underestimated. The H₂ outflow was measured every 2-3 days but not continuously. Interpolation of measurements leaves the risk of missing peaks of production and underestimating the total production. As shown in Figure 10, the electron consumption and H₂ outlet doesn't match at all. This supports the idea of an underestimation of the H₂ outlet and explains the lack of COD balance in the BER.

6.1.5 Dilution of the mixed liquor in the one-liter batch bioreactors (R1L)

MCFA can be toxic for the microorganisms by incorporating and disrupting the phospholipid membrane (Sherkhanov *et al.*, 2014). Since important amounts of MCFA (>12 g_{COD}/L_{ML}) were observed in the pH 6,5 reactors, a dilution was made on day 40 (Figure 17) to avoid a potential intoxication of the microbiota. As can be seen in Figure 18, in the case of reactor 4, no MCFA production was observed during 10 days before dilution, while reactor 3 was in an important MCFA production phase. In both cases, no production was observed after the dilution, indicating that the MCFA toxicity wasn't the limiting factor.

6.2 Influence of pH

During the first experiment in batch mode, pH was deliberately maintained acid (pH 5,5) to inhibit methanogenesis. A pH close to neutral (6,5) was also tested to see if pH had a significant influence on C8 production. Two other experiments were done in fed-batch and semi-continuous mode at pH 6,5 and 7 respectively.

The most important observation is that at pH 5,5, no caprylate or longer chain fatty acids are observed in the R1L, while $2,4 \pm 0,6$ g_{COD}/L_{ML} of caprylate is produced in the R1L batch reactors at pH 6,5. 2,6 g_{COD}/L_{ML} of caprylate were also observed in the BER after the pH was raised from 5,5 to 6,5. Caprylate was only detected in pH 7 semi continuous reactors with H₂ and beet pulp. Those observations suggest that pH has an influence and that too acidic pH (below 6,5) does not support C8 production.

A future perspective could be to test if elongation up to C8 occurs at basic pH.

6.3 Influence of the supply of reducing equivalent on MCFA production

Two types of reducing equivalent were supplied to the bioreactors: electrons as current and hydrogen as gas. Based on the gas composition of the bio-electro-reactor shown in Figure 7, it appears that significant amounts of H₂ are produced and that the liquid phase is most likely saturated with H₂. The main advantage of the BER over the pressurized reactor is that H₂ is produced in situ thanks to the cathode immersed in the liquid phase. The woven stainless-steel cathode enables a large exchange surface area between the gas produced as fine bubbles on the electrode and the liquid phase. In the H₂ pressurized reactors, the exchange surface only corresponds to the only area of the surface in contact with the gas phase, which is linked to the internal diameter of the reactor. As can be seen in Table 11 below, results between the BER and the 1L reactor at pH 6,5 are similar, suggesting that the gas diffusion in the liquid phase is not the limiting factor in the MCFA production.

Table 11 : Mean and maximum concentration of MCFA in the EF and the 1L reactors.

	Operating condition	Final concentration (g _{COD} /L _M L)		Maximum concentration (g _{COD} /L _M L)	
		Mean	Standard deviation	Mean	Standard deviation
Butyric acid	pH 5,5	8,9	0,2	13,4 (day 23)	0,4
	pH 6,5	6,85 (on day 40)	0,08	11,2 (day 23)	0,2
	BER	5,6	-	11,6 (day 28)	-
Caproic acid	pH 5,5	4	2	5 (day 30)	2
	pH 6,5	3,2 (on day 40)	0,4	4,5 (day 19)	0,5
	BER	3,4	-	3,9 (day 40)	-
Caprylic acid	pH 5,5	-	-	-	-
	pH 6,5	2,4 (on day 40)	0,5	2,4 (day 40)	0,5
	BER	2,5	-	2,5 (day 57)	-
Capric acid	pH 5,5	-	-	-	-
	pH 6,5	0,1	0,05	0,1 (day 57)	0,05
	BER	0,07	-	0,08 (day 54)	-

Pressurized two liters bioreactors with larger internal diameter, and thus larger gas exchange surface has been tested but never gave better results in term of caprylate production, confirming that H₂ diffusion is not the limiting factor for elongation of MCFA.

In both pH 6,5 one-liter reactors, high concentrations of ethanol (> 1,9 g_{COD}/L_ML) were recorded, before caproate elongation in caprylate was observed. This suggests that ethanol

is used as electron donor in the elongation process and that H₂ would only be used to produce this ethanol (Steinbusch *et al.*, 2008). However, ethanol concentrations remained always very low in the BER, under 0,6 g_{COD}/L_ML and even reached zero when caprylate started to be produced. This observation can have two meanings i) ethanol is directly consumed during the elongation process and thus can't accumulate in the mixed liquor (this also means that there is no required ethanol threshold for the elongation of caproate in caprylate). ii) the microorganisms can use another reducing equivalent source than ethanol directly in the elongation process. Different microorganisms can use various substrate as electron donor for the elongation (section 2.6). However, no metagenomic analysis was performed during this experiment to determine which microorganism were implied in the elongation. It could be interesting to compare the microbiota of pressurized reactors and BER in the future.

6.4 Influence of substrate

Three substrates were tested in the reactors: beet pulp (BP), brewer's spent grain (BSG) and brewer's spent grain with trub (BSG+T). Beet pulp has the overall best conversion ratio with half of the total COD converted in carboxylates (Table 12). BP has also the best conversion ratio for the production of MCFA, which represent 1/3 of the carboxylates produced in term of COD.

Table 12 : Mean and standard deviation (std) of SCFA, MCFA and total carboxylate conversion ratio for the different substrates in the third experiment (BP or BSG as substrate, pH 7, H₂ in the gas phase). The concentration values of the last day of the experiment are used to calculate the conversion ratio, which is expressed in g_{COD of carboxylate}/g_{total COD in the mixed liquor}.

	SCFA		MCFA		Total carboxylate	
Substrate	Mean	Std	Mean	Std	Mean	Std
BP	0,37	0,08	0,18	0,04	0,5	0,1
Fresh BSG	0,32	0,01	0,05	0,01	0,36	0,02
Fresh BSG+T	0,24	0,08	0,04	0,01	0,27	0,08

There is no significant difference between the SCFA and MCFA conversion rate of BSG and BSG+T, but BSG+T substrate has a significantly lower SCFA and MCFA conversion ration than BP. All the total carboxylate yields are significantly different between the three substrates, with the worst being BSG+T.

The concentration of propionate is significantly higher with BSG+T than with the BSG substrate (Table 13). Trub is a protein rich substrate and it has been observed that protein

rich substrates produce more propionate than other substrates. The conversion ratio of valerate is not significantly different from zero with BSG+T.

Table 13 : Uneven chain fatty acid conversion ratio for BSG and BSG+T substrate. The concentration values of the last day of the experiment are used to calculate the conversion ratio, which is expressed in $g_{COD \text{ of carboxylate}}/g_{total \text{ COD in the mixed liquor}}$.

	Propionate		Valerate	
Substrate	Mean	Standard deviation	Mean	Standard deviation
BSG	0,032	0,003	0,030	0,004
BSG+T	0,05	0,01	0,02	0,02

As shown in Table 14 below, the conversion ratio of butyrate and caproate are not significantly different from zero for BSG+T, while they are superior to zero for the BSG substrate. Those observations suggest that trub can have an inhibitory effect on the elongation process.

The negative control, a reactor without complex organic substrate, has also been tested (Figure 36). In this condition, no elongation of acetate to butyrate is observed, suggesting that the microorganism needs another substrate as complement or another reducing equivalent source. Elongation of acetate with hydrogen has already been observed but with production of ethanol as intermediate electron donor by acetate reduction (Steinbusch *et al.*, 2008). In this experiment, no ethanol is detected, which could be the reason for the absence of elongation.

Table 14 : Even chain fatty acid conversion ratio for BSG and BSG+T substrate. The concentration values of the last day of the experiment are used to calculate the conversion ratio, which is expressed in $g_{COD \text{ of carboxylate}}/g_{total \text{ COD in the mixed liquor}}$.

	Butyrate		Caproate	
Substrate	Mean	Standard deviation	Mean	Standard deviation
BSG	0,13	0,02	0,012	0,005
BSG+T	0,07	0,07	0,01	0,01

6.5 Influence of the operating mode (batch, fed batch, semi continuous)

In the first experiment, the pressurized one-liter bioreactors (R1L) and the bio-electro-reactor (BER) were all operated in batch mode. In the second experiment, the two-liter bioreactors at pH 6,5 (R2L) can be considered as a fed batch mode where substrate is added but the volume of liquid remains the same. In the third experiment, the reactors can be considered as a semi-continuous mode. Only the reactors with beet pulp, H_2 and acetate will be compared to each other since BP has been tested in the three experiments. Table 15 below shows the

conversion ratios to even chain fatty acids. In the fed batch and semi-continuous mode, no solid was removed from the reactor, resulting in a solid retention time (SRT) close to infinity and different from the hydraulic retention time (HRT).

Table 15 : Mean and standard deviation (std) of the conversion ratio of the even chain fatty acids for the different operating conditions. The concentration values of the last day of the experiment are used to calculate the conversion ratio, which is expressed in $\text{g}_{\text{COD of fatty acid}}/\text{g}_{\text{total COD in the mixed liquor}}$

	Butyrate		Caproate		Caprylate	
Experiment	Mean	Std	Mean	Std	Mean	Std
1 BER (batch)	0,09	-	0,055	-	0,04	-
1 R1L (batch)	0,09	0,02	0,06	0,02	0,05	0,01
2 (fed-batch)	0,13	0,02	0,09	0,01	-	-
3 (semi continuous)	0,20	0,05	0,05	0,01	0,005	0,003

In batch reactors, substrate is a finite resource, and the microorganisms have to reduce the substrate as much as possible in order to survive over long time periods. This is likely the reason why the conversion ratio of caprylate is the highest in batch mode reactors. There is no significant difference between the caprylate conversion ratio of the BER and the R1L.

In the second experiment, a part of the liquid phase was replaced 2 times by distilled water over a 51 days period as shown on Figure 24, resulting in an HRT of 106 days. Solid substrate was added 7 times over 51 days resulting in an average OLR of $0,85 \text{ g}_{\text{COD.LML}}^{-1}.\text{d}^{-1}$. Those conditions enabled the highest caproate conversion ratio but no caprylate was detected.

In the third experiment, substrate was added 3 times and liquid replaced 6 times over a 4 week period (Figure 36), resulting in reactors with an average OLR of $0,82 \text{ g}_{\text{COD.LML}}^{-1}.\text{d}^{-1}$ and an HRT of 32 days. Under those condition, the highest butyrate conversion ratio is observed, while the caprylate ratio is ten times lower than in batch reactor. Caproate conversion rate is similar to the batch reactor.

Those three observations indicate that very long HRT enable better elongation and thus the production of more reduced substrate such as caproate. Moreover, low OLR can selectively enhance the production towards longer chain fatty acids such as caprylate.

6.6 Influence of acetate

Table 16 below shows the conversion ratio of carboxylates in the fresh BSG reactor with and without acetate (Figure 30).

Table 16: mean and standard deviation (std) of S&MCFA conversion ratio in the BSG reactors with and without acetate. The concentration values of the last day of the experiment are used to calculate the conversion ratio, which is expressed in g_{COD} of fatty acid/ $g_{total\ COD}$ in the mixed liquor.

	Acetate		Propionate		Butyrate		Valerate	
Condition	Mean	std	Mean	std	Mean	std	Mean	std
No acetate	0,061	0,008	0,050	0,008	0,05	0,01	0,025	0,002
Acetate	-0,12	0,03	0,034	0,004	0,14	0,02	0,03	0,01

The first observation is that without acetate, the microorganisms converts the substrate in acetate and there is a net positive conversion of the substrate to acetate compared to the reactors with acetate (Figure 31). More propionate but less butyrate is converted in the reactors without acetate than in the ones with acetate. No significant difference for valerate conversion ratio can be made in the two conditions. All these observations suggest that elongation of SCFA is favored by acetate.

6.7 Methanogenesis inhibition

The first parameter that enabled a partial methanogenesis inhibition is H_2 . As show on Figure 27d and e, in the reactors without hydrogen, a methanogenesis started after 15 days. Thermodynamically the production of CH_4 is favored in presence of H_2 with a $\Delta G^{0'}$ of -131 kJ/mol $_{CH_4}$ (Hackstein 2018) and it's thus hard to explain why hydrogen has an inhibitory effect on methanation.

Another inhibitory effect is a high concentration of carboxylate in the reactor as shown on Figure 29e and Figure 35c. Methanogenesis started in the BSG reactor with N_2 on day 15 while the total carboxylate concentration in the reactor reached $6,9 \pm 0,8 g_{COD}/L_{ML}$. In the BP reactor with N_2 , from day 15 until the end, the total carboxylate concentration never went below $16 \pm 3 g_{COD}/L_{ML}$ and methanogenesis only started slowly on day 34. This could be linked to a toxic effect of the carboxylates on the methanogens.

At the end of the first experiment, a bit of methane is detected in the two reactors at pH 6,5 and not at pH 5,5 (Figure 15). It has been enlightened in the scientific literature that acidic pH can have an inhibitory effect on the methanogenesis process. However, as shown in the third experiment, even at neutral pH, no methane production is observed in the BSG reactors and BP reactors with H_2 over a 57 days period.

7 Conclusion

The aim of this master thesis was to determine which parameters enable the synthesis of caprylate from complex substrates and acetate in a non-axenic microbial culture. The fermentation results showed good reproducibility, despite significant variability of the lag phase before the start of the elongation process.

Bio-electro-fermentation has been experimented but not proven more efficient than pressurized bioreactors. The results obtained also suggest that electrons were not used directly by the microorganisms but generated hydrogen, which could be used by the microorganisms. Hydrogen as reducing equivalent source has been proven in this work to help inhibiting the methanogenesis process as well as allowing elongation, up to capric acid. However, the real electron donor in the elongation process seems to be the ethanol produced in situ. Gaseous hydrogen diffusion in the liquid phase has also been proven efficiently enough to not be a limiting step of the elongation process. H_2 as reducing equivalent is thus a key parameter for caprylate production.

From the three substrates used, beet pulp has been proven the best one, with the highest conversion ratios towards caproate ($0,09 \pm 0,01 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$ in fed batch mode reactor) and caprylate ($0,05 \pm 0,01 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$ in batch mode reactor). Overall better substrate conversion to carboxylates has been observed with beet pulp ($0,5 \pm 0,1 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$) than with BSG ($0,36 \pm 0,02 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$). However, BSG should be tested in batch reactors to compare its conversion ratio towards caprylate with BP. Reactors with acetate addition enabled better short chain fatty acid elongation compared to reactors without acetate but no effect on longer chain carboxylates has been enlightened. Substrate is not a key parameter toward the production of caprylate. However, protein rich substrate, which will favor uneven chain fatty acid production, should be avoided.

From the three experiments, the best results were obtained in batch mode reactors, with caprylate concentration of $2,4 \pm 0,5 \text{ g}_{\text{COD}}/\text{L}_{\text{ML}}$ ($0,05 \pm 0,01 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$) and capric acid concentrations of $0,1 \pm 0,05 \text{ g}_{\text{COD}}/\text{L}_{\text{ML}}$ ($0,002 \pm 0,001 \text{ g}_{\text{COD}}/\text{g}_{\text{total COD in the ML}}$). The subsequent experiments indicated that high HRT and low OLR enhanced the elongation toward longer chain fatty acids. The operating mode is thus a key parameter for the elongation process toward caprylate.

Caprylate was only produced in reactors at pH 6,5 and 7. pH 5,5 was also investigated but didn't showed any caprylate production, making pH a key parameter for the elongation process towards caprylate. A future perspective could be to investigate the elongation process in alkaline conditions.

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Microbial elongation of short chain carboxylates: investigation with environmental inoculum and complex biomass substrate

Submitted by Jean-Guillaume Heymans

A mixed culture of microorganisms is able to metabolize complex fermentable substrate like beet pulp (BP) into short chain fatty acids (SCFA). Some microorganisms, like *Clostridium kluyveri*, are able to elongate the carbon chain of SCFA to produce medium chain fatty acids (MCFA). They require a reducing equivalent source like ethanol or H₂. The aim of this work is to identify the key parameters that enable the elongation of SCFA towards caprylate by a non-axenic culture under anaerobic conditions.

The influence of several parameters was tested in pressurized bioreactors (PR) or in bio-electro-reactor (BER). Three substrates were used: BP, brewer spent grain (BSG) and BSG + trub. Three different pH were tested: 5,5; 6,5 and 7. Two gas phase compositions were used: H₂ and N₂ at 1,3 bar. Two different electron supply methods were tested: as electric current in the BER or as gas (H₂) diffusing from the headspace in the liquid phase of the PR. The PR were running in three different operating modes: batch, fed-batch and semi continuous mode.

The best conversion ratio ($0,05 \pm 0,01 \text{ g}_{\text{COD caprylate}}/\text{g}_{\text{total COD}}$ in the mixed liquor (ML)) was obtained in the batch mode PR reactors at pH 6,5 with BP as substrate and H₂ as supply gas. The fed batch reactors had the best conversion ratio ($0,09 \pm 0,01 \text{ g}_{\text{COD caproate}}/\text{g}_{\text{total COD}}$ in the ML) under the same conditions and the semi continuous PR reactors at pH 7 with BP and H₂ had the best conversion ratio ($0,20 \pm 0,05 \text{ g}_{\text{COD butyrate}}/\text{g}_{\text{total COD}}$ in the ML) towards butyrate. Globally, BP showed better conversion ratios towards carboxylates than BSG or BSG + trub. No caprylate was observed in PR reactors at pH 5,5. BER did not give better results than pH 6,5 PR using H₂ as reducing equivalent source. When replacing H₂ by N₂ in the gas phase, little elongation was observed and methane production occurred. Based on those experiments, the key parameters enlightened towards the production of caprylate were a batch mode PR at pH > 6,5, H₂ as reducing equivalent source and feeding BP as substrate rather than BSG.