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Study of acidogenic fermentation conditions for VFAs production

Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Invited Assistant Professor at Department of Chemistry, University of Aveiro, and Dr. Simon Bengtsson, Researcher at AnoxKaldnes AB.

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palavras-chave

Fermentação Acidogénica, Digestão Anaeróbia, Culturas Microbianas Mistas, Ácidos Gordos Voláteis, HSSL, Soro de Queijo, CSTR, SBR

resumo

A fermentação acidogénica é a segunda fase da digestão anaeróbica. Ácidos gordos voláteis (AOVs) são os principais produtos da fermentação acidogénica e podem servir como substratos na produção de hidrogénio ou polihidroxialcanoatos (PHA).

Neste trabalho, culturas microbianas mistas (MMC) recolhidas numa estação de tratamento de águas residuais foram usadas para acidificar alguns componentes do licor de cozimento ao sulfito ácido (HSSL), um subproduto da indústria papeleira, num reator contínuo perfeitamente agitado (CSTR), e permeado de soro de queijo (CWP) num reator descontínuo sequencial (SBR), em condições não estéreis.

Na fermentação acidogénica do HSSL, foi testado o efeito de HRTs de 1,6 e 2 dias com uma alimentação de 15 g COD/L, com 22 % de glucose e xilose. O pH não foi controlado, mas manteve-se estável em cerca de 5,0 ± 0,24 devido ao efeito tampão do HSSL. A maior conversão e grau de acidificação obtidos foram ambos de 10% com uma HRT de 2 dias e OLR de 7,7 g de COD/(L·d). O consumo de açúcares foi de 68 %.

Na fermentação de CWP foi atingida uma conversão elevada (> 80%), mesmo com pH controlado a um valor inferior ao ótimo (4,5). O pH e o HRT influenciaram a conversão de açúcares em VFA e o perfil de produtos. A conversão melhorou com o pH e a HRT e fermentação do tipo etanólico e propiónico foram identificadas a pHs diferentes. Verificou-se que o conjunto de condições testadas que conduziu a uma redução do consumo de base, com elevada conversão, foi pH 4,5, HRT 24 h e 30 ºC.

keywords Acidogenic Fermentation, Anaerobic Digestion, Mixed Microbial Cultures, Volatile Fatty Acids, HSSL, Cheese Whey, CSTR, SBR

abstract

The acidogenic fermentation is the second phase of anaerobic digestion. Volatile fatty acids (VFAs) are the main products of acidogenic fermentation and can act as substrates for hydrogen or polyhydroxyalkanoates (PHA) production.

In this work, mixed microbial cultures (MMC) collected in a wastewater treatment plants were used for acidogenic fermentation of hardwood spent sulphite liquor (HSSL), a by-product of paper and pulp industry, in a continuously stirred tank reactor (CSTR), and cheese whey permeate (CWP) in a sequential batch reactor (SBR) in non-sterile conditions.

In HSSL fermentation, HRT of 1.6 and 2 days were tested with a feeding concentration of 15 g COD/L. pH was left uncontrolled, but was stable around 5.0 ± 0.24 due to the buffer effect of HSSL. The highest conversion of 10 %, with 68% of sugars, and degree of acidification of 10 % were obtained at HRT 2 days and OLR of 7.7 g COD/(L·d).

By using CWP a high conversion of acidogenic fermentation (> 80 %) was achieved, even at a pH below the optimal value (4.5). pH and HRT influenced sugars to VFA conversion and the profile of products. Conversion improved with pH and HRT and ethanol-type and propionic-type fermentation were identified at different pHs. Finally from the conditions tested for acidogenic fermentation of CWP, pH 4.5, HRT 24 h and 30 ºC led to the reduction of base consumption at high conversion.

CONTENTS

ABBREVIATIONS

ACP	**Acyl carrier protein**
ADF	Aerobic dynamic feeding
AN/AE	Anaerobic-aerobic
ATP	Adenosine triphosphate
CoA	Coenzyme A
COD	Chemical oxygen demand
CSTR	Continuous-stirred tank reactor
CWP	Cheese whey permeate
DA	Degree of acidification
EBPR	Enhanced biological phosphorous removal
EtOH	Ethanol
FBR	Fluidized bed reactor
FP	Fermentation products
FP/SCOD	Percentage of fermentation products in the effluent
GAO	Glycogen-accumulating organisms
Glc	Glucose
HA	Hydroxyalkanoates
HAc	Acetic acid
HB	Hydroxybutyrate
HBu	Butyric acid
HDPE	High density polyethylene
HPr	Propionic acid
HRT	Hydraulic retention time
HSSL	Hardwood spent sulphite liquor
HV	Hydroxyvalerate
lcl-PHA	Long-chain-lenght polyhydroxyalkanoates
LDPE	Low density polyethylene
LS	Lignosulphonates
MA/AE	Microaerophilic-aerobic
mcl-PHA	Medium-chain-length polyhydroxyalkanoates

MMC	Mixed microbial culture
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) (reduced form)
NaOH	Sodium hydroxide
NaOH/FP	Ratio of sodium hydroxide on produced fermentation products
NaOH/VFA	Ratio of sodium hydroxide on produced volatile fatty acids
OLR	Organic loading rate
P(3H2MB)	Poly(3-hydroxy-2-methylbutyrate)
P(3H2MV)	Poly(3-hydroxy-2-methylvalerate)
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-co-3HD)	Poly(3-hydroxybutyrate-co-3-hydroxydecanoate)
P(3HB-co-3HHx)	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(3HB-co-3HP)	Poly(3-hydroxybutyrate-co-3- hydroxypropianate)
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HHx)	Poly(3-hydroxyhexanoate)
P(3HP)	Poly(3-hydroxypropianate)
P(3HV)	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
P(5HV)	Poly(5-hydroxyvalerate)
PAO	Polyphosphate-accumulating organisms
PBBR	Packed bed biofilm reactor
PET	Poly(ethylene terephthalate)
PFP	Produced fermentation products
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
Poly-P	Polyphosphate
PP	Polypropylene
$Prod_{max}$	Maximum productivity
PS	Polystyrene
PSS	Pseudo-stationery state
q_{EtOH}	Ethanol specific production rate
q_{FP}	Fermentation products specific production rate
q_{HAc}	Acetic acids specific production rate

$\mathbf{q_{HPr}}$	Propionic acid specific production rate
–qS	Substrates specific uptake rate
$\mathbf{r_{FP}}$	Fermentation products volumetric production rate
$\mathbf{-r_S}$	Substrates volumetric uptake rate
RT	Retention time
SBR	Sequential batch reactor
scl-PHA	Short-chain-length polyhydroxyalkanoates
SCOD	Soluble chemical oxygen demand
$\mathbf{S_{cons.}}$	Substrates consumption
SRT	Solids retention time
SSL	Sulphite spent liquor
SSSL	Softwood sulphite spent liquor
SSV	Settled sludge volume
SVI	Sludge volume index
TSS	Total suspended solids
TSSL	Thick sulphite spent liquor
UASBR	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WWTP	Wastewater treatment plant
X	Biomass
Xyl	Xylose
$\mathbf{Y_{FP/S}}$	Yield of fermentation products on substrates
$\mathbf{Y_{FP/SCOD}}$	Yield of fermentation products on soluble chemical oxygen demand

FIGURES

TABLES

1. Introduction

In the last decade, special attention has been paid to energy and environmental issues raised by the decreasing availability of petroleum deposits and pollution problems. The society based its economy on fossil fuels, from large-scale agriculture to fuels for transportation of people and supplies, chemicals for plastics, clothing and other basic needs, giving rise to a dependence on petroleum that is continuously increasing since the industrial revolution. Associated problems such as the increase of greenhouse gases and air pollution, and the accumulation of persistent petroleum-based plastics on the soils and seas, affecting both human health and the ecosystems and biodiversity of the planet, have generated great political debate.[1,2]

Recently, the Energy 2020 Strategy on the utilization of renewable energy sources in the European Union was discussed. In this context, new solutions pointing towards a more sustainable use of resources have been presented as the renewable energies (wind, solar, hydroelectric, tidal and geothermic power), biofuels (biodiesel, bioethanol, hydrogen) and new raw materials or new uses of known raw materials, that can partially substitute oil and other fossil fuels.[3]

The most abundant bio-based renewable raw materials are lignocellulosic biomass materials. However, their potential is yet to be fully exploited. Recent trends point to a new concept of biorefinary, which transforms biomass-based feedstocks into a vast range of products such as fuels, chemicals, materials and energy. Also, industrial by-products and agricultural and urban waste, considered as residues, can be further processed to produce value-added products. At this level, a great effort has been made to investigate the use of sub-products from the biorefinary point of view.[4,5] For instance, the hardwood sulphite spent liquor (HSSL) is a by-product from the pulp and paper industry, produced in high quantities with a considerable concentration of monomeric sugars. HSSL is usually burned for energy production, therefore a considerable part of the organic carbon substrate still present in the liquor is underexploited.[4] To utilize sugars from HSSL, several strategies have been used, namely ethanol and bioplastics production.[4,6]

Polyhydroxyalkanoates (PHA) are bioplastics produced by microorganisms as carbon and energy reserve. These polymers are advantageous over conventional plastics

and other bioplastics because PHA are both bio-based (produced from bio-based renewable raw materials by direct fermentation) and truly biodegradable (Figure 1)[7].

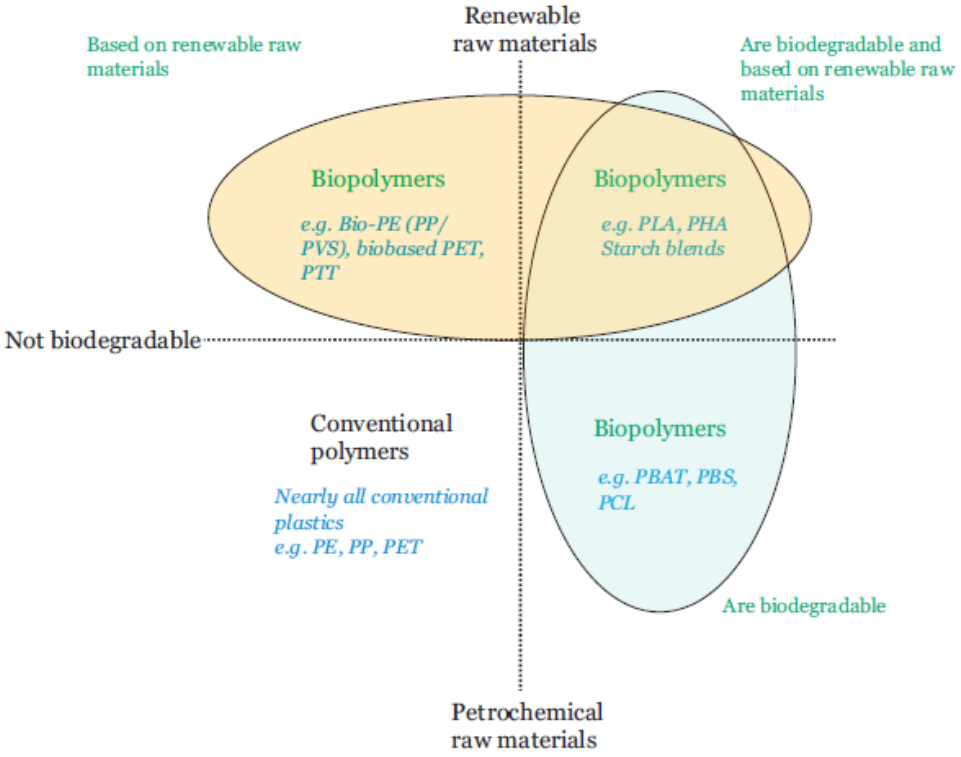

Figure 1: Types of plastics based on their raw materials and biodegradability[7].

PHA are still more expensive that conventional plastics, which dissuades its widespread use. In attempting to decrease the production costs of PHA, fermentation of complex waste feedstocks with mixed microbial cultures (MMC) have been tested. MMC allow for the utilization of complex substrates without the need of sterile conditions.[8] However, MMC accumulate PHA mainly from volatile fatty acids (VFA), so an acidogenic fermentation process is required to convert carbohydrates in wastewater to VFA.[9]

The objective of this work was the reduction of the costs of acidogenic fermentation through the utilization of industrial by-products as substrates, such as HSSL and cheese whey permeate, and MMC as inoculum. It was also aimed to study the operational conditions that were more suitable for production of VFA in both the reactors used – the CSTR for the fermentation of HSSL and the SBR for the fermentation of CWP – and, in the case of fermentation of CWP, to reduce sodium hydroxide consumption while maintaining a high conversion. In the context of this work, the main purpose of producing VFA was their use as substrate for PHA production.

2. State of the Art

2.1. Polyhydroxyalkanoates: bio-based and biodegradable plastics

Polyhydroxyalkanoates (PHA) are a group of bio-based aliphatic polyesters which are completely biodegradable, biocompatible and present thermoplastic properties similar to those of conventional petroleum-based plastics. These characteristics make them suitable for substitution of conventional plastics not only in traditional applications but also in many novel applications where non-toxicity, biodegradability and the use of renewable resources are mandatory.[10] PHA are synthesized intracellularly as carbon/energy or reducing-power storage material in microbial cells, when the growth is limited by depletion of an essential component such as nitrogen, phosphorous, sulphur, oxygen or magnesium in the presence of an excess amount of a carbon source.[11] These polymers are accumulated as insoluble cytoplasmic inclusions. This strategy is advantageous for bacteria since they are able to store high concentrations of these materials within the cell, without substantially alter its osmotic state, thus preventing the leakage of these valuable compounds at low maintenance costs.[12,13]

The general chemical structure of PHA is shown in Figure 2. Table 1 shows the most common PHA homopolymers.

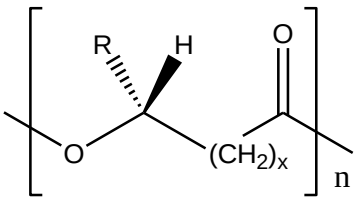

Figure 2: General chemical structure of PHA (adapted from Verlinden et al.[12]).

Table 1: Most common PHA homopolymers (adapted from Verlinden et al.[12]).

x	R	Full name	Short name
1	H	Poly(3-hydroxypropianate)	P(3HP)
	CH_3	Poly(3-hydroxybutyrate)	P(3HB)
	C_2H_5	Poly(3-hydroxyvalerate)	P(3HV)
	C_3H_7	Poly(3-hydroxyhexanoate)	P(3HHx)
2	H	Poly(4-hydroxybutyrate)	P(4HB)
3	H	Poly(5-hydroxyvalerate)	P(5HV)

The most common monomers in PHA are 3-hydroxyalkanoates (3HA), where the pendent group (R) varies from methyl (C_1) to tridecyl (C_{13}), but alkanoates with the hydroxy group at position 4, 5 and 6 and pendent group containing substituents or unsaturations are also known.[12] So there is a broad range of possible variations in the structure of HA that leads to over 150 different monomer units of hydroxyalkanoic acids known as constituents of PHA polymers.[14] Due to the stereo-specific enzymes involved in the biosynthesis, the monomers present the stereochemical (*R*)-configuration in all PHA that have been characterized so far, which makes PHA perfectly isotactic and optically active.[12,13] The value of n depends on the pendent group and the microorganism, but it is typically between 100 and 30000, and the molecular weights are usually between 50,000 and 1,000,000 Da.[15] Furthermore, it is possible to tailor the type of monomers produced by manipulating the process conditions, which originates PHA copolymers with different relative compositions.[16] The general chemical structure of the PHA copolymers is presented in Figure 3 and some examples of PHB copolymers are shown in Table 2.[12,17]

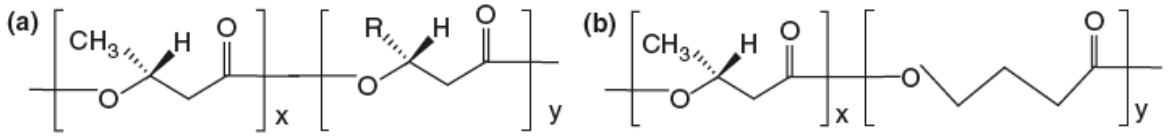

Figure 3: General chemical structure of PHA copolymers: (a) Poly(3-hydroxybutyrate) copolymers; (b) Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB)). x and y are the number of respective monomeric units in the copolymer Verlinden et al. 2007.

Table 2: Examples of PHB copolymers whose structure was given in Figure 3 (a) (adapted from Sudesh et al.[17]).

R	Full name	Short name
H	Poly(3-hydroxybutyrate-co-3- hydroxypropianate)	P(3HB-co-3HP)
C_2H_5	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	P(3HB-co-3HV)
C_3H_7	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)	P(3HB-co-3HHx)
C_7H_{11}	Poly(3-hydroxybutyrate-co-3-hydroxydecanoate)	P(3HB-co-3HD)

PHA can be divided into three main groups based on the number of carbon atoms in their monomers: the short-chain-length PHA (scl-PHA) that contain monomers with 3–5 carbon atoms (e.g. P(3HB) and P(3HB-co-3HV)); the medium-chain-length PHA (mcl-PHA) that contain monomers with 6–14 carbon atoms (e.g. P3HHx and poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)); and the long-chain-length PHA (lcl-PHA) that contain monomers with more than 14 carbon atoms.[18]

The properties of PHA polymers are greatly influenced by the molecular structure and composition of their monomers (Table 3).[15–17,19–21] P(3HB) has high molecular weight and level of crystallinity (60-80%), high melting point and low glass transition temperature. Mechanical properties like Young's modulus and tensile strength are close to that of polypropylene though extension to break is markedly lower. Hence, P(3HB) is stiffer and becomes brittle over a period of several days upon storage under ambient conditions.[19] On the other hand, mcl-PHA and their copolymers have low level of crystallinity (20–40%) and low tensile strength, but high elongation to break (do not break easily).[15] These copolymers have ductility, toughness, low shrinkage and brittleness which are required for many practical applications.[21] Yet, they have lower melting points when compared with scl-PHA and polypropylene, which makes them easier to process.[21]

The major advantage of PHA is the diversity of materials that can be produced by changing the type, content, and distribution of co-monomer units comprising the polymer chains, as well as the average molecular weight and molecular weight distribution, thus allowing for a vast range of potential applications.[16] Even though there is a great diversity of PHA polymers, the most common and, so far, industrially produced are P(3HB), P(3HB-co-3HV), P(3HB-co-3HHx), which are thought to have more potential for substitution of conventional plastics, particularly polypropylene (PP), high density polyethylene (HDPE), low density polyethylene (LDPE) and poly(ethylene terepthalate) (PET).[20]

Table 3: Thermal and mechanical properties of some PHA polymes and most common petroleum-based polymers.[16,19,20,22]

Polymers		Melting point (ºC)	Glass transition temperature (ºC)	Young's Modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)		180	4	3.5	40	5
P(4HB)		53	-48	149	104	1000
P(3HB-3HV)						
mol % 3HV	**3**	170	–	2.9	38	–
	9	162	–	1.9	37	–
	14	150	–	1.5	35	–
	20	145	-1	1.2	20	50
	25	137	–	0.7	30	–
P(3HB-4HB)						
mol % 4HB	**3**	166	–	–	28	45
	10	159	–	–	24	242
	16	150	-7	–	26	444
	64	50	–	30	17	591
	90	50	–	100	65	1080
P(3HB-3HHx)						
10% 3HHx		127	-1	–	21	400
PP		176	-10	1.7	34.5	400
LDPE		130	-30	0.156	13	126
PET		262	3400	2.2	56	7300
PS		110	21	3.1	–	576

Today there are a few companies that produce and commercialize PHA (Table 4), mainly for added value specialized applications such as medical/pharmaceutical (biocompatible devices, drug delivery, tissue regeneration/repair, etc), short term packaging and agriculture, since the current price of PHA is an ongoing impediment to its widespread use.[15,20]

The PHA production price is far above the market price of conventional plastics (comparing PHA prices in Table 4 against €0.75 kg^{-1} for oil-derived plastics). Although the latest market price of Mirel™ is quoted at about €1.50 kg^{-1}, it is still three times the price of PP.[7,20]

However, a substantial effort has been devoted to reducing the production cost through the development of more efficient fermentation (improved yields and productivity) and recovery processes (higher PHA content in the produced biomass). It is also known that the raw materials account for 40–50% of the total operational costs and have the largest influence on the cost of production of PHA. So the utilization of cheaper substrates is mandatory for the reduction of the market price of PHA.[11,23] Companies are making efforts to expand and optimize the PHA production. It is estimated that, by 2020, the bioplastics market in the European Union will have increased to 2–5 million tons and expanded to the textile, automotive, and agricultural sectors, including many durable applications.[20]

Table 4: The current and potential large volume manufacturers of PHA (adapted from Chanprateep et al.[20]).

Manufacturer	Country	Trade name	Polymer	Price (kg^{-1})
Mitsubishi Gas Chemical Company Inc.	Japan	Biogreen®	P(3HB)	€2.5-3.0
Metabolix Inc.	USA	Mirel™	P(3HB)	€1.50
PHB Industrial Company	Brazil	Biocycle®	P(3HB)	n/a
Biomer Inc.	Germany	Biomer®	P(3HB) P(3HB-co-3HV)	€3.0-5.0
Tianan Biologic, Ningbo	China	Enmat®	P(3HB-co-3HHx) Ecoflex blend	€3.26
P&G	USA	Nodax™	P(3HB-co-3HHx)	€2.50
Lianyi Biotech	China	Nodax™	P(3HB-co-3HHx)	€3.70
Kaneka Corporation	Japan	Kaneka PHBH	P(3HB-co-3HHx)	n/a
Tianjin Green Bio Science Co./DSM	China	GreenBio	P(3HB-co-4HB)	n/a
Meredian	USA	Meredian	PHA from P&G	n/a

2.2. Production of PHA

2.2.1. Microbial communities: pure vs. mixed microbial cultures

The occurrence of PHA in bacteria was first observed in 1920s when the French bacteriologist Maurice Lemoigne isolated and characterized P(3HB) from inclusion bodies of *Bacillus megaterium.*[24] Since that time more than 300 different species of Gram-positive and Gram-negative bacteria as well as a wide range of Archaea were identified as PHA producing bacteria.[13,14,20] However, only a few, such as *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Alcaligenes latus*, *Protomonas extorquens*, *Pseudomonas oleovorans*, *Azotobacter vinelandii*, and recombinant *Escherichia coli*, are able to produce sufficient quantity of PHA for large-scale production.[15,20]

In the last decades, PHA production has been based on fermentation by pure cultures and use of simple carbon substrates as glucose, sucrose, methanol, acetic acid, lactic acid and n-alkanoates.[16,20] Nevertheless, costs associated with the use of axenic cultures, due to need for sterile conditions and refined substrates, have limited a more widespread use of PHA.[25,26] Consequently, in the last years, fermentation using mixed microbial cultures (MMC) has been studied as a strategy to reduce the costs of PHA production (reviewed by Dias et al. 2006).[14]

MMC are inocula with high microbial diversity that occur naturally.[27] In 1974, Wallen and Rohwedder reported for the first time the synthesis of PHA in MMC occurring in wastewater treatment plants designed for enhanced biological phosphorus removal (EBPR).[13,28] In MMC-based processes it is possible to use strategies that favour organisms with elevated PHA storage capacity, and thus selectively enrich a culture in PHA-accumulating organisms.[13] Also MMC are able to adapt to mixtures of substrates of variable composition (complex feedstocks), such as urban or industrial waste, since there is a great metabolic diversity for substrate uptake and degradation in the consortia.[25,27] Furthermore, MMC allows for an economically advantageous and environmental-friendly process since no rigorous sterilization is required, it is possible to use cheaper substrates and the PHA production can be integrated in complex waste treatment processes.[25,29,30]

However, waste-based feedstocks, which are usually carbohydrate-rich streams, are often previously fermented to transform carbohydrates into volatile fatty acids (VFA) and other carboxylic acids, which can be readily stored as PHA by mixed cultures. So most studies on PHA production by MMC have been carried out using organic acids, such as acetate, propionate, butyrate, valerate and lactate, as feedstocks.[29,31–33]

2.2.2. Metabolism for PHA synthesis by MMC

The metabolism for PHA synthesis is well established for pure cultures, although experimental metabolic studies for MMC are still lacking.[14] Therefore, it is assumed that the metabolic pathways for PHA synthesis in MMC are similar to those reported for pure cultures using the same carbon substrate.[8] There are eight known pathways for PHA production in different microorganisms that result in PHA with different structure and composition depending on the substrate or mixture of substrates.[13] Figure 4 represents the three best studied pathways for PHA production.

The carbon substrates used for PHA production are mainly organic acids and/or sugar-based compounds. For MMC is common to use a mixture of VFA (acetic, propionic, butyric and valeric acids) as feedstock.[8]

Sugars enter the glycolysis and are converted into acetyl-CoA, which can be used for PHA production or channelled to the tricarboxylic acid cycle for growth and reducing power (NAD(P)H). Also acetate and propionate are converted to the corresponding acyl-coenzyme A (acyl-CoA) after being transported across the cell membrane. At this point, two acetyl-CoA molecules are condensed to acetoacetyl-CoA that suffers a (R)-specific reduction to give (R)-3-hydroxybutyryl-CoA, which is then converted by PHA synthase into P(3HB) (Pathway I). On the other hand, propionyl-CoA can be used to obtain different PHA precursors: (1) acetyl-CoA by decarboxylation; (2) hydroxyvalerate (originates P(3HV)), or in less extent, hydroxymethylbutyrate (originates P(3H2MB)) by condensation with acetyl-CoA; and (3) 3-hydroxy-2-methylvaleryl-CoA (precursor of P(3H2MV)) by condensation of two molecules of propionyl-CoA. Also butyrate and valerate can be directly converted to the corresponding hydroxyacyl-CoA that are the precursors of P(3HB) and P(3HV), respectively.[13,14]

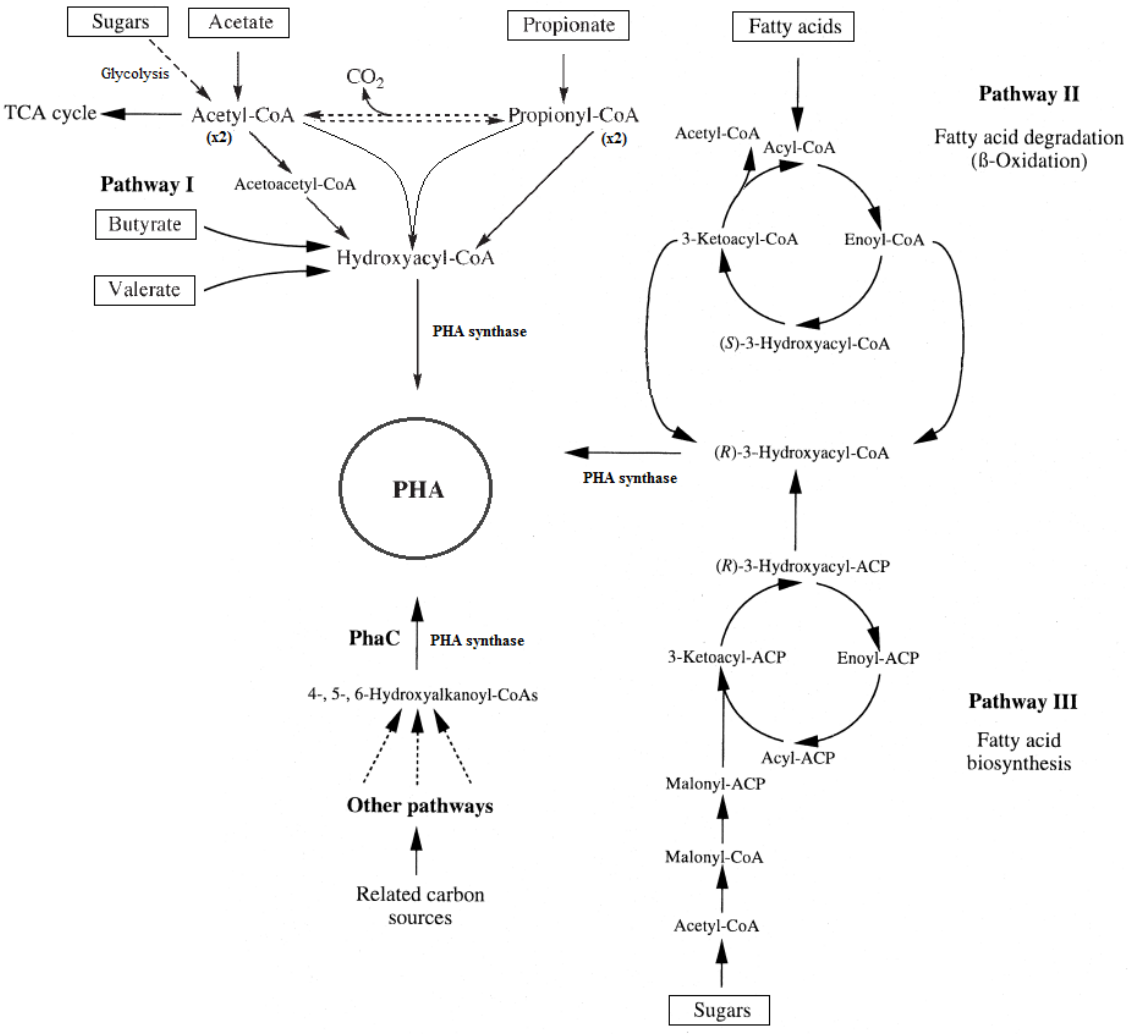

Figure 4: Simplified scheme of the metabolic mechanisms for PHA production by MMC from sugars, VFA and fatty acids (adapted from Sudesh et al. 2000[17] and Dias et al. 2006[14]). Dashed arrows indicate that more than one metabolic step can be involved.

When microorganisms uptake longer-chain fatty acids these substrates enter the β-oxidation pathway and are converted to (R)-3-hydroxyacyl-CoA that is used for the synthesis of mcl-PHA (Pathway II). Furthermore, these longer-chain fatty acids can give rise to acetyl-CoA, for an even number of carbons, or to acetyl-CoA and propionyl-CoA, for an odd number of carbons, that follow the metabolic mechanisms described above.[13,14] Valerate can also follow this metabolic pathway.[14]

In Pathway III the acetyl-CoA from glycolysis is drifted for the production of malonyl-CoA, and then malonyl-acyl carrier protein (malonyl-ACP), which is involved in the *de novo* fatty acids biosynthesis. Malonyl-CoA is converted to 3-hydroxyacyl-ACP that can then form 3-hydroxyacyl-CoA, the precursor of PHA.[8]

Other pathways are used for the synthesis of alternative copolymers: Pathways V and VII generate 4-hydroxybutyrate monomers; Pathway VI originates 4,5-hydroxyacyl-CoA precursors; and Pathway VIII produces 6-hydroxyhexanoyl-CoA precursors.[13,34]

2.2.3. Strategies for PHA production using MMC

In the last decade, MMC PHA production has been widely studied as reviewed by Dias et al. (2006)[14] and Serafim et al. (2008)[31]. The configuration of most of those studies consists in a two-step process which includes a culture selection stage for selection of PHA-accumulating organisms and a following stage of PHA accumulation by the selected culture. The physical separation of the culture selection stage from the PHA accumulation stage allows for process optimization, as different optimal conditions are required in each step.[8]

However, when waste-based substrates with high carbohydrate content are used, an additional upstream acidogenic fermentation step is needed in order to covert sugars into VFA and other organic acids, resulting in a three-step process, adopted by Dionisi et al. (2005)[35] and later by Albuquerque et al. (2007, 2010)[9,36]. This additional step is needed because, unlike pure cultures, mixed cultures do not store carbohydrates as PHAs but rather as glycogen.[9] The experimental design utilized by Albuquerque et al. is shown in Figure 5, where the fermented stream is used as substrate in both the subsequent steps.

As the acidogenic fermentation is the main theme of this research work, it will be discussed in greater detail in chapter 2.3, while in this chapter it is presented a description of the culture selection and PHA accumulation steps.

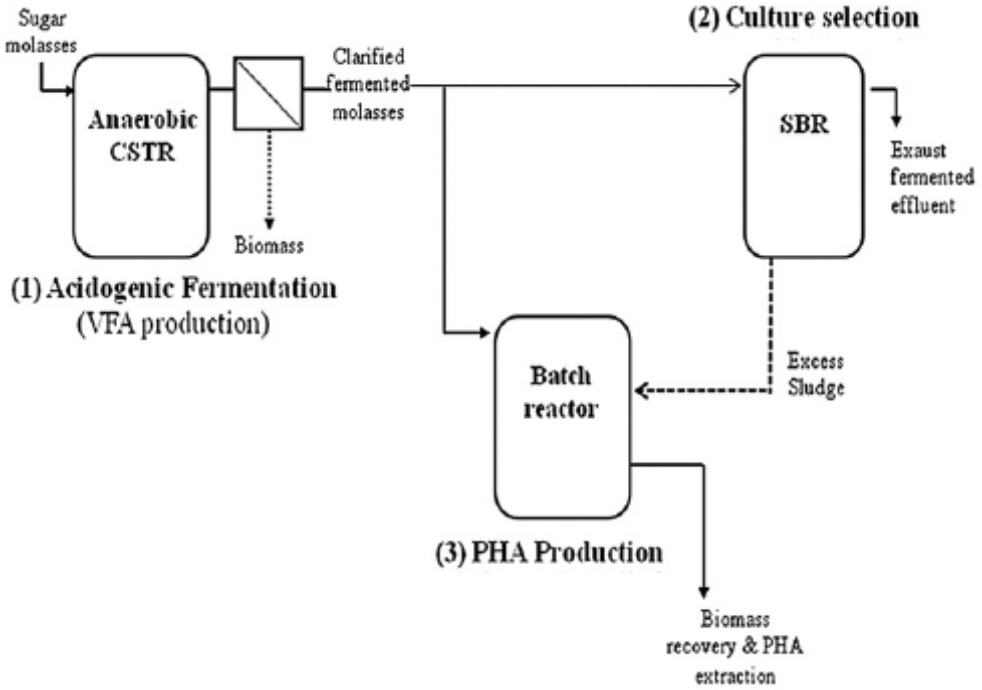

Figure 5: Schematic representation of the 3-step experimental design used by Albuquerque et al. 2007, 2010 for PHA production from sugar cane molasses. [9,36]

I. Culture selection

This stage aims at selecting a homogeneous population of microorganisms with high and stable PHA accumulation capacity. Therefore, in an ideal selected culture all or almost all the microorganisms have high PHA storage capacity and also a high cell growth rate in order to ensure the inoculation of a larger reactor for PHA production with the excess sludge obtained in this stage.[8,14] Yet, the homogeneity of the culture has a major importance in the downstream process, since microorganisms presenting low or no storage capacity contribute for the reduction of the average PHA cell content that results in an increase of the PHA extraction costs.[8]

The strategies commonly used for the culture selection are based on the application of transient conditions, such as, the oxygen cycling, which consists in an alternation of anaerobic and aerobic conditions (AN/AE) or microaerophilic and fully aerobic conditions (MA/AE), or transient substrate supply, with alternation of periods of external substrate abundance (feast) and periods of external substrate limitation (famine).[23]

In the AN/AE process, where electron donor and acceptor availability are separated, two types of microorganisms present in mixed cultures are mainly selected: the

polyphosphate-accumulating organisms (PAO), and the glycogen-accumulating organisms (GAO).[11,37] For both PAO and GAO cultures, under anaerobic conditions and in presence of an external carbon substrate, occurs the uptake of the substrate(s) and polymerization of PHA by the metabolic mechanisms discussed in the chapter 2.2.2. The major difference between those two groups of microorganisms is how they obtain energy for this process. While PAO hydrolyse polyphosphate (poly-P), a reserve polymer, for ATP production and, in less extent, hydrolyse glycogen mainly for reducing power, GAO obtain ATP exclusively from glycogen hydrolysis.[11] In aerobic conditions, the stored PHA is hydrolysed and the resulting acetyl-CoA is used in the tricarboxylic acid cycle for growth, storage of glycogen and energy production by PAO and GAO. In PAOs, part of the energy is used to restore the polyphosphate pool.[23] Also GAO are believed to be more robust and able to reach a higher content, since more PHA is synthesized during the anaerobic phase in order to maintain the redox balance within the cells.[8,38] By applying this strategy, several authors were able to select cultures with PHA accumulation capacity within 10 to a maximum of 37% of cell dry weight, using different types of by-products and wastewaters as carbon source, such as, synthetic wastewater,[38] fermented municipal wastewater,[39] fermented sugar cane molasses[40,41] and fermented paper mill effluent.[42] However, the highest PHA accumulation capacity in GAOs, 60 %, was obtained by Bengtsson (2009) using a defined culture medium with acetate as carbon source.[43]

Saito et al. (1995)[44] reported that laboratory-acclimatized anaerobic–aerobic activated sludge accumulated more PHB under aerobic conditions (36% of the sludge dry weight) than under anaerobic conditions (17% of the sludge dry weight).[37] Considering these results, Satoh et al. (1998)[45] introduced the microaerophilic-aerobic (MA/AE) process where a limited amount of oxygen is supplied to the first phase of the process.[11] The cause of the enhancement is attributed to the difference in the availability of energy for PHA accumulation. While anaerobic substrate uptake is stopped when the stock of energy such as poly-P and glycogen are exhausted, under microaerophilic conditions, microorganisms can produce more energy by oxidative degradation of substrates.[37] Although this strategy allows for the selection of sludge able to accumulate PHA up to 62% of its dry weight, its general use is dependent on a very strict oxygen control in order to keep microaerophilic conditions.[23,45]

In recent years, many studies refer the production of PHA by mixed cultures when exposed to transient substrate supply, a process commonly known as feast and famine (FF).[9,29,31,35] In this process the substrate is fed during a short period of time (feast), followed by a longer period of substrate limitation (famine), resulting in an often called unbalanced growth; during the excess of external carbon substrate, the uptake is driven to simultaneous cell growth and polymer accumulation, and after substrate depletion, the stored polymer is used as carbon and energy source.[11,23] It is important to note that these conditions are not growth limiting, although the accumulation phenomena is usually dominant (70%) over growth.[23] Under these conditions, the microorganisms able to accumulate internal reserves have a competitive advantage over those without this ability and are selected by using this strategy.[37]

Although this process can be performed under aerobic or anoxic conditions, with similar yield, the specific PHA production rate is apparently higher for the aerobic process, frequently referred as aerobic dynamic feeding (ADF).[11] Thus, the ADF process seems to be the best strategy for culture selection since it allows for the selection of a sludge with high PHA content, over three times higher than for AN/AE processes, and high productivity.[14,23] Different authors have tested several waste-based feedstocks under ADF conditions, such as, fermented palm oil mill effluents,[46] olive oil mill effluents,[35,47] fermented paper mill wastewater,[29] tomato cannery waste,[48] fermented brewery wastewater,[49] and fermented sugar cane molasses.[9,36,50]

The most common reactor configuration is the sequential batch reactor (SBR).[9,31,35,36] SBRs are ideal reactors for a selection of robust populations with high PHA accumulation capacity, because biomass grows under transient conditions. Furthermore, it is easy to control and is highly flexible, allowing for a quick modification of the defined process conditions, namely length of feed and cycle length (which define the feast-famine ratio),[11] as well as other conditions, such as, substrate concentration, carbon to nitrogen ratio, hydraulic retention time (HRT), solids retention time (SRT), organic loading rate (OLR), dissolved oxygen concentration, pH, and temperature, which, for the same type of substrate, influence the polymer yield and productivity.[8,14,23]

Moreover, Bengtsson et al. (2008) fed a fermented wastewater to a continuous flow system inoculated with activated sludge. This system includes two continuous stirred-tank reactors (CSTR) in series followed by a settler, that mimic the feast and famine phases

separately, and reached a maximum PHA content of 48% of the sludge dry weight.[29] Albuquerque et al. (2010) used a system with a similar configuration for culture selection using fermented molasses as substrate and obtained a maximum PHA content of 61%.[50] Also polymer yield on substrate and specific productivity were similar to those obtained with SBR configuration. These results confirm the possibility of using the facilities already existing in conventional wastewater treatment plants (WWTP) for PHA production from industrial or municipal wastes.[8]

II. PHA accumulation

Batch PHA accumulation assays are carried out to maximize and ascertain the PHA cell content, polymer yield on substrate and specific and volumetric productivities in order to obtain the best quantity and quality of the polymer. Thus, the PHA accumulation performance reflects not only the optimization of the accumulation step itself, but also the storage capacity of the selected culture (meaning the efficiency of the strategy applied in the previous step).[8]

During the PHA accumulation step, the carbon substrate is often added by pulses (resulting in a fed-batch configuration), so an on-line parameter to determine a new addiction is needed. The dissolved oxygen concentration is used to follow the storage process, once the oxygen uptake rate is related with the PHA storage rate. When the oxygen uptake rate decreases, it indicates the depletion of the external substrate, used namely for PHA production, so it indicates the need of a new substrate pulse.[31]

As mentioned above, the strategy applied previously, greatly influences the PHA accumulation step. In the AN/AE process, the accumulation step may be under anaerobic or aerobic conditions. However, Dai et al. (2007)[38] obtained a maximum PHA content of 41% in aerobic conditions when using acetate as carbon source, while in anaerobic conditions it was only 28%. Also the storage yields obtained under AN/AE (from 0.29 to 0.94 g COD PHA/g SCOD) were slightly higher than the values obtained under ADF conditions (from 0.53 to 0.84 g COD PHA/g SCOD), which was not expected since glycogen is used as an internal substrate for PHA synthesis. On the other hand, specific productivities obtained from cultures selected under AN/AE conditions with real substrates

(0.014 – 0.28 g COD PHA/g COD X.h) are higher than those selected under ADF conditions (0.0082 – 0.42 g COD PHA/g COD X.h).[8]

Volumetric productivities are usually low when compared with those of pure cultures, but can be improved by increasing the PHA content or cell concentration, which can be achieved by optimization of the culture selection. Another option is the utilization of design systems with high cellular densities, for example membrane reactors, that allow for an effective retention of biomass inside the reactor.[8,23]

Nevertheless, the PHA cell content is one of the most important parameters in the PHA production process, since it has great influence in the polymer extraction costs, and in the economic viability of the process. Serafim et al. (2004) were able to obtain a PHA content of 65% of cell dry weight by using acetate as carbon source.[31] Recently, Albuquerque et al. (2010) achieved a maximum PHA cell content of 74.6% using MMC and sugar cane molasses as carbon source,[36] thus proving to be possible to achieved with MMC a PHA cell content within the range obtain with pure cultures (70 – 80%).[23]

The stability of the polymer composition is also an important aspect of PHA production from real complex wastes by MMC.[8] As waste streams are often sensitive to seasonal and process variations, the three-step process is advantageous since it is possible to manipulate the operational conditions in the acidogenic fermentation step to obtain a constant VFA profile, which influences both polymer quality (HB/HV ratio) and quantity (PHA cell content).[23]

2.3. Acidogenic fermentation for VFA production

Acidogenic fermentation is a stage of anaerobic digestion, where the substrates contained in the wastewaters are transformed into VFA and other organic acids (e.g. lactate) and alcohols (e.g. ethanol).[51] VFA are short-chain fatty acids consisting of six or fewer carbon atoms which can be distilled at atmospheric pressure, being the most common acetic, propionic and butyric acids.[52]

The anaerobic digestion is a complex process, which can be divided up into four individual degradation stages: hydrolysis, acidogenesis, acetogenesis, and methanognesis. (Figure 6).[53] This is a mature technology that has been employed within full-scale facilities worldwide for the treatment of industrial and agricultural wastewater treatment and organic

solid waste (e.g. waste activated sludge (WAS); municipal solid waste), with the ultimate production of methane and carbon dioxide (biogas).[54–56] This process is advantageous over aerobic active sludge systems because of its high organic removal rates, low energy-input requirement, energy production, and low sludge production.[55]

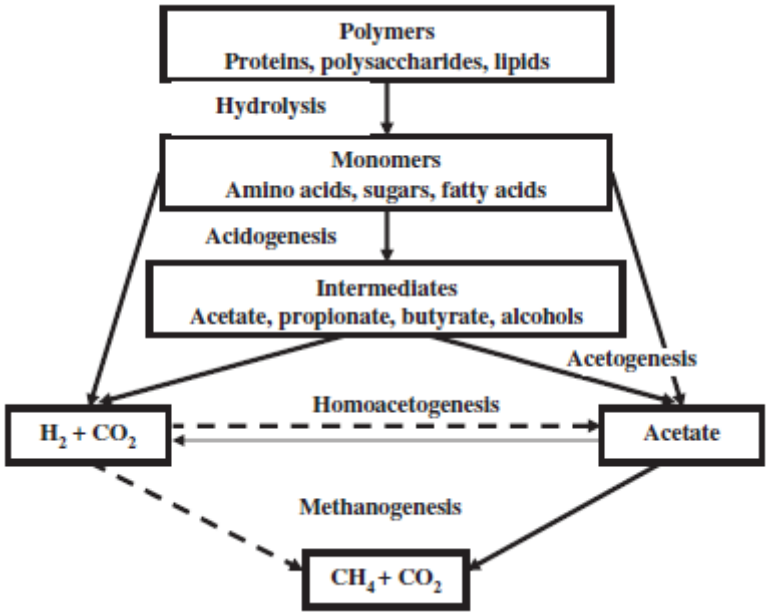

Figure 6: Schematic representation of anaerobic digestion steps and by-products.[53]

As represented in Figure 6, the production of VFA involves hydrolysis and acidogenesis. In hydrolysis, complex organic polymers in waste streams are broken down into simpler organic monomers (amino acids, sugars, fatty acids) by the enzymes excreted from the hydrolytic microorganisms. Subsequently, acidogens (or acidogenic bacteria) ferment these monomers into VFA, alcohols, and dioxide carbon and hydrogen (by-products). Both processes involve a complex consortium of obligate and facultative anaerobes, such as, *Bacteriocides*, *Clostridia*, *Bifidobacteria*, *Streptococci*, and *Enterobacteriaceae*.[57] Even though hydrolysis and acidogenesis are described as two sequential reactions, acidification is thought to occur immediately when the soluble substrate from the hydrolysis is available, making hydrolysis the rate limiting fermentation step, so these reactions are carried out simultaneously in a single anaerobic reactor.[58]

The waste-derived VFA have important downstream applications, such as, carbon substrates to produce PHA (as previously discussed in this work)[32,59] and to assist the biological removal of nitrogen and phosphorous from wastewater;[60] building blocks to use

as starting materials for the chemical industry;[61] and intermediates for bioenergy production, namely, direct electricity by microbial fuel cell,[62,63] biogas by one-phase or two-phase anaerobic digestion,[64,65] hydrogen obtained by photo fermentation,[66] electrohydrolysis,[67] or microbial electrolysis cell,[68] and microbial lipids, synthesized by oleaginous microorganisms, for biodiesel production.[69]

The acidogenic fermentation of organic waste streams has particular interest since it provides both treatment of waste matters and simultaneous generation of value-added products (i.e. VFA) and by-products (i.e. carbon dioxide, hydrogen, other organic acids and alcohols) from low cost waste materials.[70] Figure 7 represents the most important and expected metabolic pathways used by MMC for the production of organic acids and alcohols from sugars (pentoses and hexoses) usually found in wastewater from lignocellulosic materials.[71]

It is important to note that, in an acidogenic fermentation process operating with MMC and complex substrates, many types of microorganisms and biochemical pathways are involved, so a large number of by-products can be formed with different rates and yields. Also, it is widely recognized that operational conditions and environmental factors, such as pH, temperature, oxidation–reduction potential (ORP), HRT, SRT, OLR, reactor configuration, wastewater characteristics and availability of trace minerals strongly affect the final VFA composition.[72] In the case of PHA production, by controlling the VFA profiles, polymers with different monomer structure and composition can be produced, resulting in the tailored synthesis of PHA with controlled properties.[32] Therefore, further research is needed in order to establish relations between process conditions during acidogenic fermentation and VFA production for different potential feedstocks.[73]

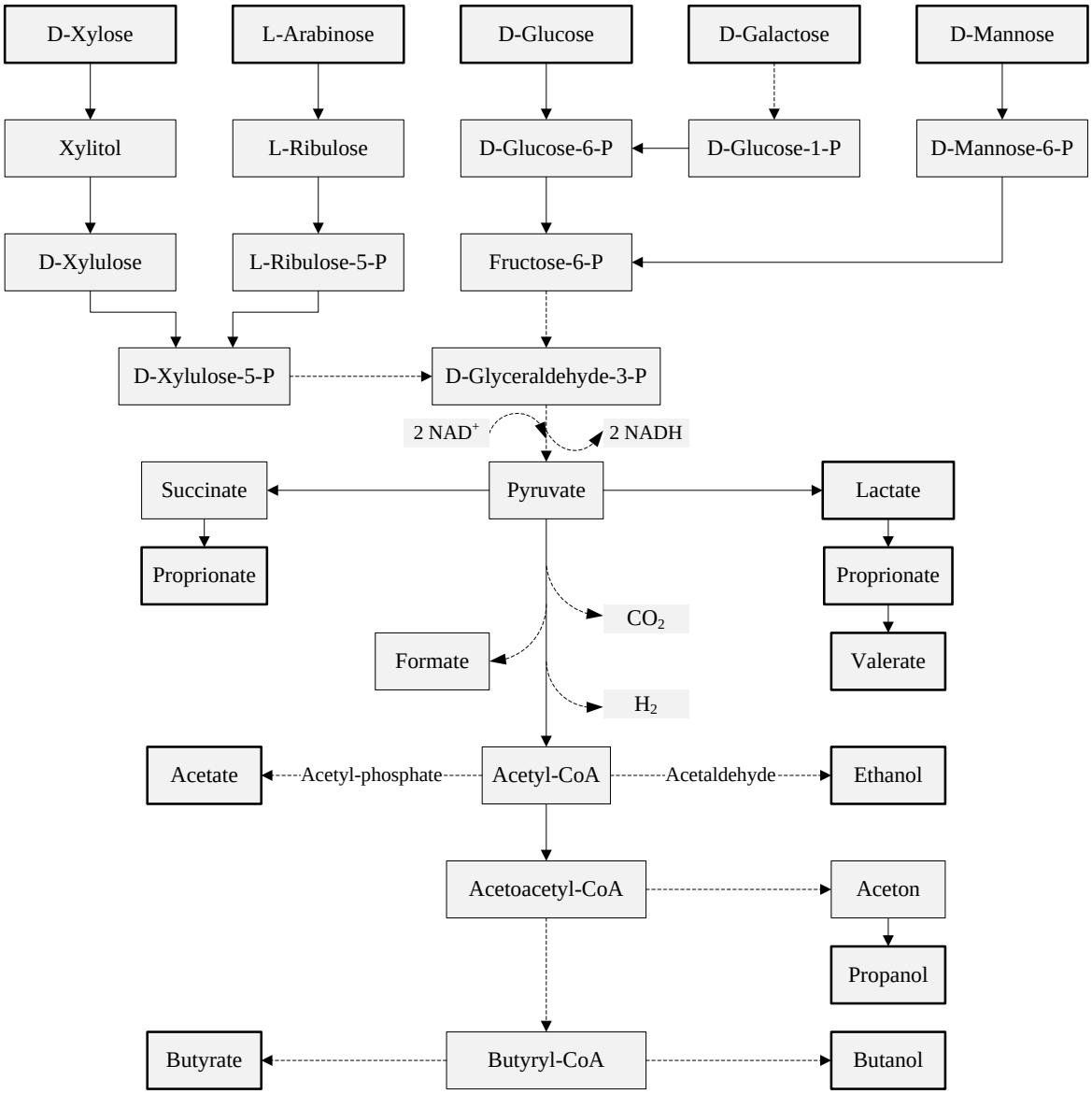

Figure 7: Schematic representation of the most important and expected pathways of sugars (pentoses and hexoses) metabolism during acetogenenis and formed products (organic acids and alcohols) by MMC (alternative pathways for the same product are possible, depending on the type of microorganism). Dashed arrows indicate that more than one metabolic step can be involved. Adapted from Lehninger et al. 2005, Hahn-Hägerdal et al. 2007, Temudo et al. 2009, and Saady, Noori 2013.[53,71,74,75]

2.3.1. Anaerobic technologies for VFA production

The geometry of the reactor greatly influences the performance of the biological processes, in this case, the VFA production, thus being an important choice to be aware of when planning the process design. Different types of reactors have been used in the production of VFA from waste-based feedstocks (Figure 8). For instance, packed bed biofilm reactor (PBBR) and fluidized bed reactor (FBR) are operated based on attached growth technology (biomass grows attached to support), while upflow anaerobic sludge blanket reactor (UASBR) and continuous stirred-tank reactor (CSTR) are operated based on suspended growth technology (biomass grows freely in suspension).[52]

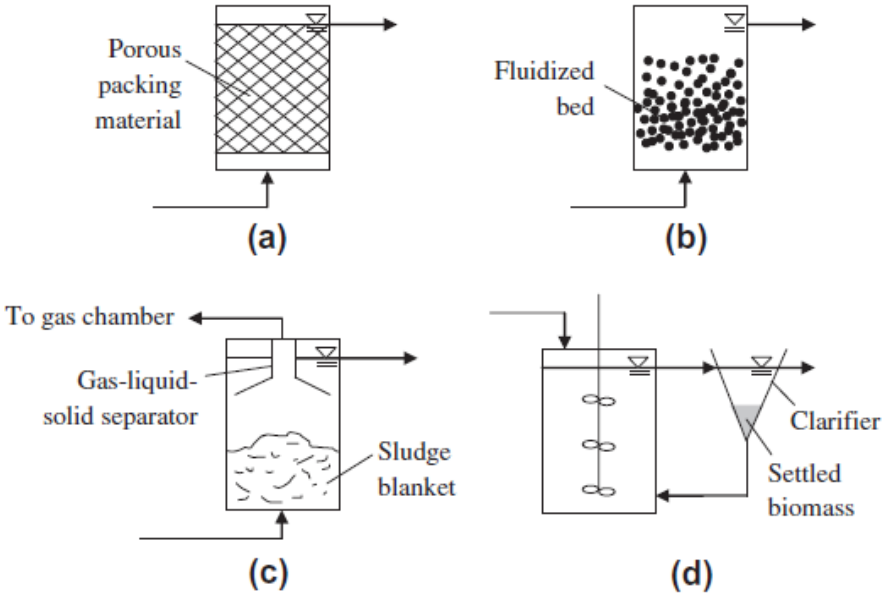

Figure 8: Common types of reactors for anaerobic VFA production: (a) PBBR; (b) FBR; (c) UASBR; and (d) CSTR with biomass recirculation.[52]

In PBBR (Figure 8 (a)), the biomass grows attached to porous packing material inserted in the reactor (immobilization system), such as, alumina-based ceramic cubes,[47] 'Manville' silica beads,[76] and granular activated carbon.[77] This reactor geometry allows for the application of the high flow rate feedings without great risks of shock loading and/or washout problems, which can negatively affect the productivity of suspended growth reactors operating with low-growth rate biomass and high organic loads.[47] Also, the high

flow rate feedings applied contribute to minimize the VFA-consuming methanogenic activity, which is normally mediated by bacteria with very low specific growth rates.[78]

FBR has been developed to avoid clogging in PBBR by wastes containing high concentrations of suspended solids (Figure 8 (b)). In this reactor, the biomass grows attached to small solid medium such as sand that remains in suspension by the upward flowing motion of the fluid.[52] Also, it has very large surface areas for biomass attachment, enabling high OLR and short HRT during operation, and minimal problems of channeling, plugging or gas hold-up.[79]

Despite being based on a suspended growth technology, the successful operation of the UASBR relies on the formation of granular biomass, that is retained by sedimentation, forming a sludge blanket at the bottom of the reactor (Figure 8 (c)).[52] Even though UASBR is a well-established technology for anaerobic treatment of a wide range of industrial effluents, including those with inhibitory compounds, with simultaneous biogas production, it is not the most studied approach for VFA production.[80]

Finally, CSTR involves complete mixing of waste stream and biomass, which can be approximately achieved by well-designed impellers, baffles and reactor shape. In this case, the speed of agitation is a crucial operational parameter so the suspended microbes are not damaged by the shear stress.[52] Typically, a gravity settling clarifier is used to separate and recycle the biomass from the effluent of the CSTR (Figure 8 (d)),[52] or it can be used as a chemostat: the influent is supplied continuously and no biomass retention is applied.[73]

2.3.2. Operational conditions influencing VFA production

The operational conditions pH, temperature, retention time, OLR, as well as additives used have great effects on the concentration, yield and composition of VFA produced from waste-based materials.[52] In the literature, most of the researchers examine these factors one at a time and there are only a few works evaluating their interactive effects.[81,82]

- **pH**

The pH value in the reactor is important to the production of VFA because it determines the biodiversity and bacterial community in the anaerobic reactor, and so the degree of acidification and profile of VFA produced.[56] pH has been studied extensively to investigate its role in acidification, but it is still not consensual which pH values are optimal for VFA production. VFA production from wastewater is mostly conducted under acidic condition with optimum pH ranges from 5.25 to 6.0.[72,73] However, several authors have reported that anaerobic fermentation under alkaline pH conditions can significantly improve VFA production from sewage sludge, since it enhances the hydrolysis of carbohydrates and proteins of sludge, resulting in more soluble substrates available.[56,83,84] Moreover, the VFA yields and the acetate percentage were higher under alkaline pH conditions than those under neutral or acidic pH conditions.[56] Besides, optimal pH values seem to depend on the type of waste used.[52,73]

Also, pH can affect the VFA profile obtained from acidogenic fermentation, particularly, concentrations of acetic, propionic and butyric acids. Bengtsson and co-workers (2008)[73] have evaluated the effect of pH on acidogenic fermentation of two waste-based feedstocks: cheese whey and paper mill effluent in a chemostat at a HRT of 48h.[73] In this study, the VFA production was dramatically reduced below pH 5 for cheese whey and below pH 5.5 for the paper mill effluent, while optimum pH with respect to amount of VFA produced was around pH 5.25–5.5 for cheese whey and pH 5.5–6 for the paper mill effluent.[73] Also, the composition of VFA changed with pH increase; the amount of acetate and butyrate decreased while the amount of propionate increased for cheese whey (pH range 5.25–6), and butyrate and propionate increased for the paper mill effluent (pH range 4.9–6). A similar VFA composition shift was also observed by other authors.[9,56] Albuquerque et al. (2007) explained that under lower operating pH there are more reducing equivalents available to be incorporated into the fatty acid chains, thus resulting in the production of longer chain fatty acids,[9] while Liu et al. (2012) associated the VFA composition change with the shift in the dominant microbial population.[56]

Nevertheless, it is suggested that the optimal pH for the production of a specific VFA is highly dependent on the type of waste used.[52] Thus, studies on this particular

subject are fundamental to control the balance between the types of VFA, and consequent control of the composition and physical properties of PHA in a downstream process.[73]

- **Temperature**

The effect of temperature in the production of VFA from waste has been ascertained under different temperature ranges, viz. psychrophilic (4–20 ºC) and mesophilic (20–50 ºC),[58,85–87] thermophilic (50–60 ºC),[87–89] and extreme/hyper-thermophilic (60–80 ºC) conditions.[65,90] Increasing the temperature within the psychrophilic and mesophilic temperature ranges is beneficial as it increases the concentration of VFA produced,[58,86] the rate of VFA production[85] and the VFA yield.[58] For instance, Zhang et al. (2009) reported that raising the temperature from 10 ºC to 35 ºC resulted in an increase of total VFA concentration due to improved WAS hydrolysis at higher temperature.[86] Likewise, Xiong et al. (2012) showed that the total VFA concentration obtained from WAS increased substantially when raising the temperature from 40 ºC to 50 ºC, slightly decreasing at 60 ºC.[89] On the other hand, Zhou el al. (2012) worked with semi-continuous-flow reactors for hydrolysis and acidification of ultrasonic-pretreated WAS under alkaline conditions and found that when temperature increased from 37 ºC to 55 ºC, the total VFA concentration was 40% lower that at 37 ºC, which was probably related with the fact that the acid-forming enzymes activities at thermophilic temperature (55 ºC) were lower than that at mesophilic temperature (37 ºC).[87] In conclusion, although the increasing temperature can improve the efficiency of VFA production, it needs to be balanced with the associated energy costs, for heating and maintenance of high temperature, inhibition of enzyme activity or killing fermentation bacteria.[89]

Unlike pH, the effect of temperature on VFA composition seems minor. Yuan et al. (2011), Yu & Fang (2003) and Zhou et al. (2012) found no significant variation in VFA composition during the fermentation of WAS in mixed reactors from 14 ºC to 24.6 ºC,[58] the acidogenesis of gelatin-rich wastewater from 20 ºC to 55 ºC,[88] and in the fermentation of ultrasonic-pretreated WAS from 10 ºC to 55 ºC,[87] respectively.

- **Retention time**

Firstly, it is common to distinguish between two critical operational parameters in the anaerobic reactor for the production of VFA: the retention time of the waste, hydraulic retention time (HRT) and the retention time of the MMC, solids retention time (SRT). While HRT is closely related to the volume of the reactor, the SRT is associated with the selection of predominant microbial species in the reactor.

Despite several authors have reported that applying higher HRTs could be advantageous to the production of VFA,[73,91] prolonged HRT could lead to stagnant VFA production.[92–94] For instance, Fang & Yu (2000) showed that the production of VFA from dairy wastewater nearly doubled as the HRT increased from 4 h to 12 h, but a further increase to 16–24 h only improved VFA production by 6%. This might be due to the increased time microorganisms have to react with the waste, i.e., prolonged hydrolysis of wastes.[93]

The effect of HRT is most likely associated with the type of substrate, since changes in HRT did not seem to affect the fermentation of simple soluble substrates, but acidogenic fermentation of complex substrates (such as wastewater sludges and pharmaceutical wastes) were greatly influenced by HRT.[95] Anyhow, operation with higher HRT requires a larger reactor and consequently greater initial capital investment and energy and maintenance costs, so the gains in terms of total VFA concentration and yield needs to be balanced with the overall process costs.[95]

As for the SRT, most of the studies found that lower SRT is beneficial to the production of VFA from WAS.[86,89,96,97] Xiong et al. (2012) studied the effect of SRT on VFA yield and composition, when varying the SRT from 1 to 13 days in the acidogenic fermentation of WAS. The authors observed that the VFA production was maximum at the SRT of 5 days and that when it was further increased, the VFA yield declined rapidly.[89] The reported findings suggest that lower SRT can prevent the dominance of methanogens in the anaerobic reactor as the growth rate of methanogens is lower than that of acidogens.[89,96] Nevertheless, SRT should be long enough to promote hydrolysis of WAS, thus increasing the soluble protein and carbohydrates, but not so long that the VFA concentration decreases due to the methanogens activity.[86] Relatively to the the influence of SRT in the VFA profile, Xiong et al. (2012) also reported that after SRT of 1 day, iso-

valeric acid production was much greater than any other VFA at any fermentation time. Moreover, acetic acid was the second major VFA at SRT of 1 to 5 days; n-butyric acid at SRT from 6 to 8 days and propionic acid at SRT from 10 to 13 days. Thr authors also stated that the VFA profile can be widely affected by the type of substrate.[89]

However, there are a very limited number of studies on the influence of retention time (RT) on the VFA production from wastewater. Salmiati et al. (2007) evaluated the effect of SRT on the VFA production from palm oil mill effluent: total VFA concentration increased when the SRT was increased from 2 to 6 days, and then decreased when the SRT was further increased to 7 days.[46] Bengtsson et al. (2008) studied the effect of RT on the degree of acidification and VFA profile of the acidogenic fermentation of paper mill effluent and cheese whey on a CSTR.[73] For cheese whey fermentation, increasing the RT from 20 to 95 hours favored the production of propionic acid but not the formation of butyric acid. For paper mill effluent, when RT increased from 11 to 24 hours a similar tendency was verified.[73]

Thereby, the optimal retention time should be evaluated for different waste-based feedstocks, considering also the effect of other operational conditions, as the effect of the retention time on the degree of acidification and VFA profile still cares for concordant results and rigorous conclusions.[52,72]

- **Organic loading rate**

OLR reflects the amount of carbon substrate fed into the reactor per reactor volume unit and per time unit. In the literature, the influence of OLR on VFA production seems inconsistent but can be rationalized by the presence of an optimum value.[52] Oktem et al. (2006) verified that the optimal OLR during the acidogenic fermentation of chemical synthesis-based pharmaceutical wastewater was 7 g COD/(L·d), within a range of 1 to 14 g COD/(L·d), and the VFA concentration suffers a drastic drop when OLR slight increased to 14 g COD/(L·d).[72] Lim et al. (2008) investigated the total VFA concentration produced from food waste; it increased with OLR from 5 to 13 g/(L·d), to a maximum of 28.9 – 30.0 g/L, but the operation of the reactor at 13 g/(L·d) was unstable because the loading rate was too high and fermentation broth was very viscous.[92] Ferrer et al. (2010) and Yu (2000) revealed a linear positive correlation between the total VFA concentration in the effluent

and the OLR in the range from 0.47 to 5.2 g COD/(L·d)[96] and from 1 to 32 g COD/(L·d),[98] respectively. Kundu et al. (2013) tested the effect of HRT and OLR at 37 and 55 ºC. It was asserted that the total VFA concentration increased with increasing OLR (2.22 – 10 g/(L·d)) at a fixed HRT (5 days), and beyond a certain OLR limit (8 g/(L·d) at 37 ºC and 6 g/(L·d) at 55 ºC), severe VFA production was observed.[99] Furthermore, the maximum total VFA concentration at 55 ºC (8188 mg/L) was higher than the corresponding value at 37 ºC (5119 mg/L) under stable performance.[99]

As other parameters mentioned above, the OLR applied in the acidogenic fermentation has significant influence on the composition profile of the VFA. Yu (2001) studied the influence of OLR, in the range from 2 to 26 g COD/(L·d), on acidogenesis of starchy wastewater in a UASB reactor. Acetic acid was the major acid accounting for 60 – 80% of the total VFA concentration and propionic acid was the second major acid, but it was substituted by butyric acid at the higher OLR of 26 g COD/(L·d).[98]

The effects of OLR should be seen in the light that there are a few other factors, notably pH and HRT, which strongly affect the distribution of the VFA, and thus more multi-factorial studies should be performed.

- **Additives**

In recent years, additives have been utilized to improve the production of VFA.[52] The addition of surfactants such as sodium dodecyl sulfate (SDS)[100] or sodium dodecylbenzene sulfonate (SDBS)[101] to WAS enhances the solubilization of the extracellular polymeric substances, mainly protein and carbohydrates, thus boosting the hydrolysis of sludge and production of VFA. Also chemical inhibitors of methanogenesis (recently reviewed by Liu et al. (2011)[102]) might enhance the production of VFA by suppressing the activity of VFA-consuming methanogens. The chemical inhibitors of methanogenesis can be specific or nonspecific. The first are specific inhibitors for enzymes of methagonens (e. g. 2-bromoethanesulfonate, 2-chloroethane-sulfonate, mevastatin and lovastatin), and the later affect the activity of microorganisms in general (e. g. ethylene, acetylene and several halogenated aliphatic hydrocarbons like chloroform, fluoroacetate and methyl fluorid).

The effect of addition of Fe^0 (iron in oxidation state 0 works as a reductive agent) to artificial wastewater in a two-stage anaerobic system was studied by Liu et al. (2012).[103] It is believed that the acidogenic fermentation acids profile is closely related to the ORP of the medium, as propionic-type fermentation is associated with ORP higher than -278 mV (facultative anaerobic environment), while acetic-type or butyric-type fermentations need lower ORP levels (obligate anaerobic environment).[103] Liu et al. concluded that the hydrolysis and acidogenesis processes were accelerated, the degree of acidification and COD removal were enhanced by Fe^0 dosing, and the subsequent acetogenesis and methanogenesis were favored.[103]

Although addictives have been studied to improve the production of VFA from sludge, the associated costs are a huge drawback when the actual tendency is to look for strategies to reduce the production costs of VFA. In this case, the enhanced VFA production has to be balanced against the costs of addictives. Several other factors influence the VFA production, which can have cumulative and synergistic effect on the quantity and quality of the organic acids produced, thus more work needs to be done on this subject.

2.3.3. Complex substrates for acidogenic fermentation

As already discussed, the acidogenic fermentation has the potential to produce value-added compounds with diverse downstream applications from low cost waste-based materials. This aspect has vital importance to the integration of an acidogenic fermentation stage into other processes, once the substrate costs often have a significant contribution for the overall process costs. Particularly, the substrate costs in PHA production are estimated to be 40 to 50% of the total process costs.[14]

Therefore, the biochemical acidogenic potential of an organic waste stream, i.e. the amount of VFA that can be generated from fermentation of the organic constituents, as well as the knowledge of their profiles, are crucial parameters that should be ascertained to select the most suitable waste streams for acidogenic fermentation, and thus to establish a more cost-effective process.[104] This way, a variety of solid and liquid wastes have been studied for their potential to be used for VFA production.[52]

The most investigated solid wastes are WAS, food waste and organic fraction of municipal solid waste. Although these solid residues are permanently available in large amounts and are rich in organic matter with total COD ranging from 14.800 mg/L to 347.000 mg/L, the soluble COD in these wastes is lower than their total COD. This retards the production of VFA as the hydrolysis of the particulate organic matter in WAS, food waste and organic fraction of municipal solid waste is the rate-limiting step, thus needing for various pretreatment methods.[52]

For liquid waste, wastewaters generated from the agricultural, dairy, pulp and paper industries are commonly used for VFA production. Studies have been reported with the use of palm oil mill effluent,[105,106] olive oil mill effluent,[35,47] sugar cane molasses,[9,30,36,41,59] cheese whey permeate,[73] dairy wastewater,[95,107,108] paper mill wastewater,[73] and chemical synthesis-based pharmaceutical wastewater.[72]

In general, wastes commonly used for VFA production are rich in organic matter with COD greater than 4000 mg/L, the ammonium content of waste should be lower than 5000 mg/L to avoid inhibition of VFA production though it is an essential nitrogen source for the growth of microorganisms, and the availability and the amount of the waste generated have to be taken into consideration to ensure stable and continuous waste supply for the production of VFA.[11,52,108]

- **Hardwood sulphite spent liquor**

HSSL is a by-product from the acidic sulphite pulping process of hardwoods in pulp and paper industry.[109] The main objective of wood pulping processes is the removal of lignin from wood, maintaining the cellulose and hemicelluloses integrity, thus providing the cellulosic fibres with desired composition (percentage of cellulose and hemicelluloses) for further use as papermaking raw material or chemical feedstock.[109,110] There are two processes employed by pulp and paper industries to produce cellulosic fibres: the alkaline Kraft pulping and the acid sulphite chemical pulping. Although the Kraft pulping is the dominant process responsible for more than 90% of the cellulosic pulp production worldwide, the by-products that result from this process have a chemical composition unsuitable for further use as substrates for fermentative processes.[109,111]

The sulphite pulping is carried out in batch digesters under extreme conditions, that include high temperature (135 – 145 ºC) and high medium acidity (pH 1 – 2) for 8 to 12 h, causing the sulphonation and removal of lignin from wood as salts of lignosulphonates (LS) and the partial hydrolysis of hemicelluloses, while maintaining cellulose intact.[109] The block scheme of acid sulphite pulp production is shown in Figure 9. After the pulping process, unbleached pulp is washed and spent sulphite liquor (SSL) containing dissolved LS and degraded carbohydrates (monomeric sugars and oligosaccharides from wood) are concentrated by evaporation, resulting in thick spent sulphite liquor (TSSL), that is used for energy and reagents recovery or commercialised.[110,112]

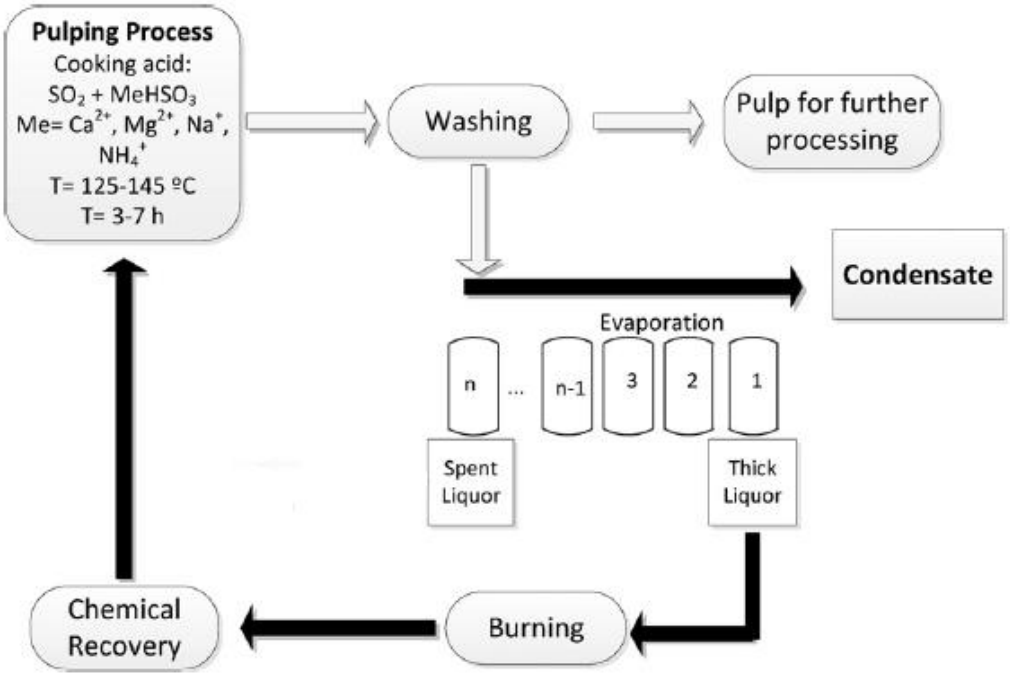

Figure 9: General block scheme of acidic sulphite wood pulping process with release of SSL and TSSL, traditionally burned for energy and chemicals recovery.[109]

The chemical composition of SSL depends strongly on the wood species used for the pulping process, which determines the application and processing of SSL for further purposes.[109] Thus, SSL obtained from pulping of softwoods (SSSL) contain a high proportion of hexoses (> 70%), while those obtained from pulping of hardwoods (HSSL) contain mainly pentoses (> 70%), since hemicelluloses of softwoods and hardwoods are mainly hexosanes and pentosans, respectively.[112]

HSSL contains significant amounts of dissolved organic matter corresponding to a high chemical oxygen demand (COD > 100,000 mg O_2/L).[109] Typically, the major organic

components of HSSL from *Eucalyptus globulus* (liquor utilized in this work) are LS (59.0 – 78.2 g/L) and sugars, mainly xylose (21.0 – 24.6 g/L), from hydrolysed glucuronoxylan, which is the predominant hemicellulose in hardwoods (Table 5).[110] Other monomeric sugars, namely arabinose (1.0 – 7.8 g/L), glucose (2.3 – 3.9 g/L), mannose (1.0 – 8.5 g/L) and galactose (2.4 – 5.0 g/L), and oligosaccharides (up to 25% of the dissolved carbohydrates) are also present in HSSL from *Eucalyptus globulus.* Among the volatile compounds, acetic acid is the most abundant although furfural is also present but at low concentrations (<0.1 g/L) (Table 5).[109,110]

Table 5: General chemical composition of HSSL.[4]

Components	Concentration (g/L)
Lignosulphonates	78.2 ± 0.6
Ash	19.8 ± 0.2
D-Xylose	24.6 ± 0.5
D-Mannose	8.5 ± 0.9
Acetic acid	8.2 ± 0.3
L-Arabinose	7.8 ± 0.3
D-Galactose	4.5 ± 0.1
D-Glucose	2.3 ± 0.1
L-Rhamnose	1.6 ± 0.3
L-Fucose	0.4 ± 0.3
Furfural	<0.1

HSSL has already been used as substrate for several biological processes, for example, acidogenic fermentation for VFA production,[6,113] PHA production by MMC,[114] bioethanol production by *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*) fermentation,[4] and single cell protein production by *Paecilomyces variotii.*[115] However, authors refer that one of the major problems associated with bioprocessing of HSSL is the removal of inhibitory compounds, namely phenolics, furfural and hydroxylmethylfurfural and a fraction of LS (polyphenols and low molecular weight derivatives). Thus, a preliminary detoxification step should be considered.[4,115] Nevertheless, HSSL is a prospective substrate for further production of value-added products within the biorefinery concept.[112]

3. Methods and materials

3.1. Acidogenic fermentation of HSSL

3.1.1. Microbial mixed culture

The microbial mixed culture (MMC) used in this worked was collected from the anaerobic tank of the wastewater treatment plant (WWTP) Aveiro Sul (SIMRIA 2013). The MMC was used for the inoculation of the reactor at the beginning of the process and when the reactor was thought to be in wash-out. A pre-treatment was performed by heating the inoculum to 82 ºC for 20 min in order to promote the enrichment of the MMC in acidogenic population over the methanogenic bacteria. The biomass concentration in the reactor and in the WWTP sludge was determined by analysis of the total and volatile suspended solids (TSS and VSS).

3.1.2. Pre-treatment of hardwood sulphite spent liquor

Hardwood sulphite spent liquor (HSSL) from magnesium based acidic sulphite pulping of *Eucalyptus globulus* was supplied by *Caima – Indústria de Celulose S.A.* (Constância, Portugal). The HSSL collected from the factory was subjected to a chemical pre-treatment in order to remove some toxic compounds, such as phenolics, furfural and some lignosulphonates (LS). The pre-treatment of HSSL consisted in pH adjustment to 7.0 with KOH and storage overnight at 4 ºC, followed by aeration with compressed air (2 h/L) to oxidize and precipitate some phenolic compounds . Then, the HSSL was centrifuged for 1 hour at 5000 rpm and the precipitated colloids were filtered off using a 1 μm pore size glass microfiber filter (Filtres Fioroni).[4] The pre-treated HSSL was stored at 4 ºC.

3.1.3. Fermentation medium

Fermentation medium was prepared by weighting the nutrients listed in Table 6 (adapted from Campos 2013), dissolving them in distilled water, adding 78 mL/L of pre-treated HSSL as carbon source, and adjust the pH of the feeding to 7.0. Phosphate salts

were prepared apart, in order to avoid an irreversible precipitate with magnesium salts during sterilization. Both flasks were sterilized in autoclave, and mixed together in a laminar flow hood. The concentration of pre-treated HSSL in the fermentation medium was calculated to obtain a final concentration of COD of approximately 15 g COD/L.[113]

Table 6: Chemical composition of fermentation medium for CSTR adapted from Campos 2013.[113]

Components	Concentration (mg/L)
Nutrients	
$CaSO_4.2H_2O$	80
$FeSO_4.7H_2O$	160
K_2HPO_4	80
KH_2PO_4	160
$MgSO_4.7H_2O$	160
$Na_2MoO_4.2H_2O$	80
NH_4Cl	160
Carbon source	
Pre-treated HSSL	78 mL/L

3.1.4. Reactor configuration and operational conditions

The continuous stirred tank reactor (CSTR) and laboratory equipment used to perform the acidification of chemically pre-treated HSSL under anaerobic conditions are shown in Figure 10. The working volume of the reactor was 1.55 L and the flow rate of the feeding solution was 0.97 L/d (imposed by an Ismatec™ Compact Digital Multichannel Pump) resulting in a hydraulic retention time (HRT) of 1.6 days. The reactor had no system for retaining the biomass; therefore the solids retention time (SRT) was the same as the HRT. The effluent was collected at the outlet of the reactor by overflow. Reactor stirring was performed by a magnetic stirrer and kept constant at 240 rpm. Nitrogen was bubbled occasionally to assure anaerobic conditions. Oxidation-reduction potential (ORP) was monitored with a transmitter M300 2-channel, ORP meter (Mettler-Toledo Thornton, Inc). The system worked with temperature control at 30.1 ± 1.0 ºC using an external serpentine.

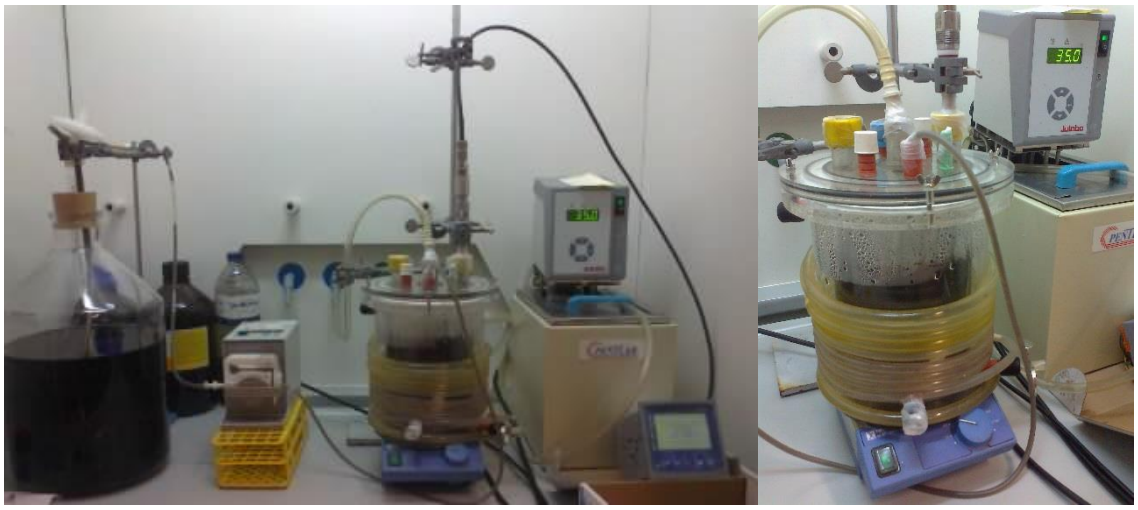

Figure 10: Laboratory setup for the acidification of chemically pre-treated HSSL (left) and detail of the CSTR with water bath (right).

3.1.5. Sampling

Samples were collected three times a day at intervals of 3 hours (sample volume of 5 mL). The ORP and temperature inside the bioreactor were recorded at the time of each sample collection. The samples were further centrifuged at 13000 rpm for 10 minutes (Centrifuge MiniSpin, Eppendorf), the pellet was discarded and the pH of the supernatant was measured. The supernatant was stored in the freezer under –16 °C for later determination of glucose, xylose, ethanol and VFA, COD and LS concentrations. Three times a week 10 mL samples were collected for determination of TSS and VSS.

3.2. Acidogenic fermentation of cheese whey permeate

3.2.1. Microbial mixed cultures

The MMC used as inoculum in this work was aerobic activated sludge collected from a WWTP near Lund, Sweden. The biomass concentration in the reactor and effluent was determined by analysis of total and volatile suspended solids (TSS/VSS).

3.2.2. Fermentation medium

The fermentation medium supplied to the reactors was cheese whey permeate (CWP) resulting from an ultrafiltration process to remove proteins and lipids. CWP is generally composed by water (≈ 94 %) and a dried matter fraction (≈ 6 %), which is composed by 83 % of lactose, 14 % of non-protein nitrogen and 3 % of ash.[116]

However, the CWP used in this work had a percentage of 16 % (w/w) of lactose. CWP was diluted during the feeding period of the reactor by combining the flow of CWP with a separated flow of water, to obtain a COD concentration in the reactor of approximately 2 g COD/L (dilution of about 1:90).

3.2.3. Reactor configuration and operational conditions

For the study of acidogenic fermentation of CWP two SBR with a working volume of 1 L were operated in parallel for 100 days under anaerobic conditions. Both SBRs and all the laboratory apparatus used can be seen in Figure 11. The length of each cycle was 6 hours (4 cycles per day), comprising 15 min of feeding with fresh fermentation medium by combination of CWP flow (Alitea C6 MIDI pump) and water flow (Watson Marlow 120S pump), 5.08 hours of reaction, 20 min of settling (stirring and NaOH pumps switched off), and finally, 20 min of withdrawing (Alitea U1 MIDI pump). All pumps and stirring (performed by a magnetic stirrer at 400 rpm) were controlled with programmable sockets.

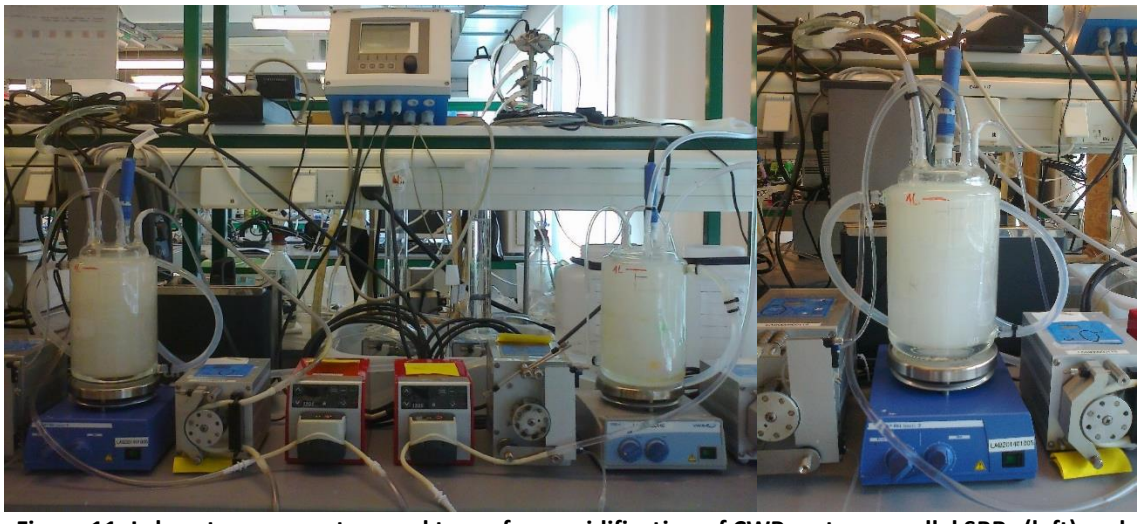

Figure 11: Laboratory apparatus used to perform acidification of CWP on two parallel SBRs (left) and detail of the SBR with two pumps and water bath in the back (right).

The pH was controlled in both reactors using an Endress-Hauser Liquiline CM442 controller, two pH sensors (Endress-Hauser) and two peristaltic pumps for adding 1 M NaOH solution. The reactor 1 (SBR1) was kept at pH 5.5 during the whole process, while reactor 2 (SBR2) was first kept at pH 4.5 and then, on day 75, the pH was changed to 4.0. The reactors were initially operated without a water bath for temperature control (the process was conducted at room temperature of 24 ± 1 ºC), but at day 42 was decided to increase the temperature to 30 ºC in both reactors to improve the conversion of sugars to VFA. Both reactors had an initial volume exchange ratio of 0.75 L/L, which corresponds to a HRT of 8 h, but at day 56 it was decreased to 0.25 L/L (HRT of 24 h) to improve the conversion ratio. Volume exchange ratio of SBR1 was again modified at day 90 to 0.5 L/L (HRT of 12 h) (Table 7).

The SRT was controlled in both reactors during two periods at two different set values (target SRT): 4 days between days 19 and 55, and 8 days from day 75 to day 100. The SRT was controlled by manually purge the reactor. The volume purged was calculated based on the volatile suspended solids (VSS) in the reactor and in the effluent collected daily (see Equation 7 in the chapter 3.4). The median of the SRT calculated during the periods mentioned above is shown in Table 7. The rest of the time the SRT was left uncontrolled to allow the biomass to build-up. The biomass concentration was kept between 1.0 and 3.0 g VSS/L in SBR1, and between 1.0 and 2.0 g VSS/L in SBR2, during most of the fermentation period.

Table 7: Operational conditions during the full period of fermentation of CWP in SBR1 and SBR2.

SBR 1							
Time (d)	**pH**	**T (ºC)**	**Volume exchange ratio (L/L)**	**HRT (h)**	**$HRT_{reaction}$ (h)**	**OLR (g COD/(L·d))**	**SRT (d)**
1-42	5.5	24	0.75	8	6.8	6.0	3.7
44-55	5.5	30	0.75	8	6.8	6.0	
56-89	5.5	30	0.25	24	21.1	2.0	8.3
90-100	5.5	30	0.50	12	10.6	4.0	
SBR 2							
Time (d)	**pH**	**T (ºC)**	**Volume exchange ratio (L/L)**	**HRT (h)**	**$HRT_{reaction}$ (h)**	**OLR (g COD/(L·d))**	**SRT (d)**
1-42	4.5	24	0.75	8	6.8	6.0	4.3
44-55	4.5	30	0.75	8	6.8	6.0	
56-75	4.5	30	0.25	24	21.1	2.0	8.6
76-100	4.0	30	0.25	24	21.1	2.0	

3.2.4. Sampling

Samples were collected daily at the end of the cycle. First, a sample from the reactor was collected immediately before settling, for TCOD, SCOD, VFA and TSS/VSS analysis. Then, after withdraw, a sample of the remaining biomass was taken for TCOD, SCOD and TSS/VSS, and a sample from the effluent was taken for TCOD, SCOD, VFA and TSS/VSS analysis. The internal pH of the reactor and pH of the effluent were measured, as well as the consumption of NaOH. After some time, it was decided to follow the biomass concentration only through the TSS/VSS concentration, so the TCOD of samples from the reactor and effluent was only analysed once a week for confirmation of the results.

For kinetic studies, in addition to the samples normally taken, samples at times 0 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, and at the end of reaction time (5.3 h) were collected for SCOD and VFA analysis, and for TSS/VSS analysis at times 0 h; 3 h and 5.3 h.

Samples for TCOD, SCOD and TSS/VSS were immediately analysed by the methods described in the section 3.3. Samples for VFA analysis were filtrated by 0.45 µm pore syringe filters (Sartorius) and frozen at –20 ºC for posterior analysis.

Twice a week the settled sludge volume (SSV) was measured and the respective sludge volume index (SVI) was calculated.

3.3. Analytical methods

3.3.1. Chemical oxygen demand

COD analysis of the samples taken from the CSTR was performed according to the colorimetric method APHA 5220 D.[117] Test tubes were prepared with 2.8 mL of sulphuric acid solution, 1.2 mL of digestion solution and 2.0 mL of diluted sample (1:20) in order to have a COD concentration within the range of the method (100 – 900 mg/L). A blank was prepared by adding to the test tube 2.0 mL of distilled water instead of sample. Then, the tubes were shaken and placed in a block digester Spectroquant TR620 (Merck Millipore) pre-heated to 150 ºC for 2 h. After cooling down to room temperature, the optical path of

the test tubes was carefully cleaned and the absorbance at 600 nm of each sample and blank was measured in a colorimeter Spectroquant Picco COD/CSB (Merck Millipore). Previously, a calibration curve had been prepared by digesting standard solutions of glucose (Appendix A).

For the COD determination of the samples taken from the SBR, the kit LCK 114 (150 – 1000 mg/L) from Hach Lange was used and the instructions of the manufacturer were followed. Samples were digested in a block heater LT 200 at 148 ºC for 2 h and absorbance was measured in a colorimeter DR 3800 from the same manufacturer.

3.3.2. Gas chromatography

Gas chromatography (GC) was used to quantify the VFAs: acetic acid (HAc), propionic acid (HPr), iso-butyric acid (HiBu), butyric acid (HBu), iso-valeric acid (HiVa), valeric acid (HVa), caproic acid (HCa); and ethanol (EtOH) in the samples from whey permeate fermentation in SBR.

All samples were micro-filtered (0.45 µm, Sartorius) and prepared in vials before each run by adding 100 µL of a solution of 25% of formic acid and 3g/L of acrylic acid (internal standard) to 900 µL of sample. Standards were prepared in the range from 0.010 to 1.0 g/L for each compound with the addition of 100 µL of internal standard for a total volume of 1000 µL. The analyses were conducted on a Clarius 400 gas chromatograph equipped with an split injector (injection volume of 0.5 µL and splitting ratio of 1:20), an Elite-FFAP capillary column (30 m × 0.32 mm I.D. × 0.25 µm d_f) and a flame ionization detector (FID), all from PerkinElmer. Helium was used as carrier gas at flow rate of 1.8 mL/min. The injector and detector temperatures were 220 °C and 250 °C, respectively. The column temperature was programmed to be initially 85 °C for 0.5 min and then the column temperature was ramped at 25°C/min to 105 °C, followed by a second ramp at 7 °C/min until 200 °C, and then finally by 20 °C/min to 240 °C and held at 240 °C for 1 min. The analysis time was approximately 20 min.

3.3.3. High pressure liquid chromatography

The concentrations of VFA (HAc, HPr and HBu), sugars (glucose, Glc, and xylose, Xyl) and EtOH in the samples taken from the CSTR were determined by high pressure liquid chromatography (HPLC).

800 μL of each sample were filtered using centrifuge tube filters with a cellulose acetate membrane, 0.45 μm pore size (Corning Costar Spin-X) at 10000 rpm (Centrifuge MiniSpin, Eppendorf) for 15 minutes. The samples were then injected (Auto-sampler HITACHI L-2200) in an ion exchange column Eurokat of 10 mm at 40 ºC (Oven Gecko-2000, CIL CLUZEAU), and analysed by a refractive index detector HITACHI L-2490. The eluent 0.01 N H_2SO_4 was pumped at a flow rate of 0.4 mL/min (HITACHI L-2130 pump) at room temperature. The eluente was prepared with milli-Q water and filtered with a cellulose acetate membrane, 0.2 μm pore size (Whatman).

The concentrations of sugars, VFA and ethanol in g/L were determined by comparison with the calibration curves of each analysed compound obtained using standards of known concentrations.[4] The standards concentrations were within the range of the expected concentrations of the analytes: glucose, propionic acid and butyric acid, from 0.050 to 1.0 g/L; acetic acid and ethanol, from 0.10 to 2.0 g/L; and xylose, from 0.15 to 3.0 g/L.

3.3.4. Microscopy

A ZEISS Axioskop 2 plus microscope with a coupled camera AxioCam HRc (software AxioVision) was used to analyse the biomass from SBR1 and SBR2. The objectives used were Plan-NEOFLUAR 10X and 20X and Plan-APOCHROMAT 63X.

3.3.5. pH

The pH of the supernatant of the samples from the CSTR was analysed with a bench pH meter, HI 9321 Microprocessor from Hanna Instruments, while the pH of the samples from the SBR (external pH) was analysed with a HI991002 pH meter from Hanna Instruments.

3.3.6. Sludge volume index

The sludge volume index (SVI), in mL/g, was obtained by dividing the settled sludge volume (SSV), in mL, by the sample volume, in L, and by the TSS concentration, in g/L (Equation 8). SSV was determined by allowing 100 mL of well-mixed sample of the biomass suspension from the reactor to settle during 30 minutes in a graduated cylinder (adapted from method APHA 2710 D).[117]

3.3.7. Total and volatile suspended solids

TSS and VSS were determined according to the methods APHA 2540 D and APHA 2540 E, respectively.[117] 5 mL samples were filtrated in duplicate through previously ignited (550 ºC for 30 minutes) and weighted 0.7 μm pore size glass microfiber filters (Whatman) using a vacuum pump. The filters were dried in the oven at 105 °C for 24 hours, and then were placed in a desiccator to balance temperature and weigh. After cooling down, the filters were weighted and the biomass concentration was determined in g TSS/L. Then, the filters were ignited in a muffle furnace at 550 ºC for 2 hours. After cooling down in a dessicator, the membranes were weighted and the biomass concentration was determined in g VSS/L. For comparison, the VSS concentration in g/L was converted to g COD/L assuming a typical biomass composition of $C_5H_7NO_2$.[73,114]

In the case of the acidogenic fermentation of cheese whey permeate, the suspended solids were determined in two different ways: the daily measurement of TSS and VSS (described above), and the difference between TCOD and SCOD, corresponding to the suspended COD (XCOD). Since both methods can introduce major errors to the determination of suspended solids, mainly due to sampling and pipetting steps, a comparison between the results from both methods allows verifying if the results are under or overestimated. For comparison, the VSS concentration in g/L was converted to g COD/L. The slope of the regression between VSS and XCOD was 1.006 g COD/g COD (r^2 = 0.929) (see Appendix B), and since the theoretical value of the slope is 1.0 (when the value of VSS is equal to the value of XCOD) the measurements were considered accurate. However, since VSS analysis requires larger sample volumes and wide-bore pipettes can be used the systematic error can be reduced, and so the VSS approach was used to quantify the biomass in both reactors.

3.4. Calculations

For effects of comparison, the concentrations of sugars, ethanol, VFA and VSS in g/L were converted to g COD/L by multiplying the concentration in g/L by the respective conversion factor. It was assumed that the concentration of VSS corresponds to the concentration of biomass and that the chemical formula of biomass was $C_5H_7NO_2$ (cf = 1.4144 gO_2/g).[73,114] The conversion factor was calculated using the following fully oxidation reaction (Equation 1).

$$\boldsymbol{a\ compound_{oxi} + b\ O_2 \rightarrow cCO_2 + dH_2O + eNH_3} \qquad \textbf{Equation 1}$$

Where a, b, c, d and e are the stoichiometric coefficients of the oxidizable compound, oxygen, carbon dioxide, water and ammonia, respectively, after the reaction has been balanced. It was considered that no nitrification occurred. That way, the conversion factor (cf) is given by Equation 2:

$$\boldsymbol{cf(gO_2/g) = \frac{b \times M(O_2)}{a \times M(compound_{oxi})}} \qquad \textbf{Equation 2}$$

For acidogenic fermentation processes both VFA and ethanol were obtained as fermentation products (FP) since ethanol was produced in a significant amount. The degree of acidification (DA) of the effluent was calculated as the percentage of produced fermentation products ($PFP = \sum FP_{out} - \sum FP_{in}$) divided by the influent COD concentration (Equation 3).

$$\boldsymbol{DA(g\ COD/g\ COD) = \frac{PFP}{COD_{in}} \times 100} \qquad \textbf{Equation 3}$$

The percentage of fermentation products in the effluent (FP/SCOD) was calculated by dividing the PFP concentration by the effluent SCOD concentration.

The percentage of each product relatively to the PFP concentration (Equation 4) was calculated to compare the profiles of the fermentation products during periods with different operational conditions.

$$\boldsymbol{Product}(\%) = \frac{\boldsymbol{Product_{out} - Product_{in}}}{\boldsymbol{PFP}} \times \mathbf{100} \qquad \textbf{Equation 4}$$

Product yields reflect the relationship between products formation and substrates consumption and they were calculated for every sample during the continuous periods. In the case of HSSL fermentation, it was calculated the yield of PFP on sugars (glucose and xylose) consumed, $Y_{FP/S}$ (Equation 5). As for the CWP fermentation, the yield of PFP on SCOD consumed, $YF_{FP/SCOD}$ (Equation 6), was calculated.

$$\boldsymbol{Y_{FP/S}(gCOD/gCOD)} = \frac{\boldsymbol{PFP}}{\boldsymbol{S_{in} - S_{out}}} \qquad \textbf{Equation 5}$$

$$\boldsymbol{Y_{FP/SCOD}(gCOD/gCOD)} = \frac{\boldsymbol{PFP}}{(\boldsymbol{SCOD_{in} - FP_{in}}) - (\boldsymbol{SCOD_{out} - FP_{out}})} \qquad \textbf{Equation 6}$$

The volumetric substrates uptake ($-r_S$ in g COD/(L·h)) and volumetric FP production rates (r_{FP} in g COD/(L·h)) were calculated by dividing the substrates consumed ($S_{cons} = S_{in} - S_{out}$) or the PFP, respectively, by the HRT. In batch experiments, $-r_S$ and r_{FP} were calculated by linear regression of the substrates concentration (glucose and xylose) or PFP concentration, respectively, versus time. The specific substrates uptake ($-q_S$ in g COD/(g COD·h)) and specific FP production rates (q_{FP} in g COD/(g COD·h)) were calculated by dividing the respective volumetric rates by the average biomass concentration.

The solids retention time (SRT) and the volume of purge taken daily to maintain a certain SRT were calculated through the expression:

$$SRT\ (d) = \frac{VSS_{reactor}\left(\frac{g}{L}\right) \times V_{reactor}(L)}{VSS_{eff}\left(\frac{g}{L}\right) \times V_{eff}(L) \times \frac{4}{d} + VSS_{purge}\left(\frac{g}{d}\right) \times V_{purge}(L) \times \frac{4}{d}} \qquad \textbf{Equation 7}$$

The sludge volume index (SVI) was calculated based on Equation 8:

$$SVI(mL/g) = \frac{SSV_{30min}(mL)}{0.1\ (L) \times TSS(g/L)} \qquad \textbf{Equation 8}$$

4. Results and discussion

4.1. Acidogenic fermentation of HSSL in a CSTR

A CSTR was operated for 95 days to study the acidogenic fermentation of HSSL. Its initial operational conditions were selected based on the work of Campos (2013), whom has tested hydraulic retention times (HRT) of 1.6 and 4 days, organic loading rates (OLR) from 3.75 to 15 g COD/(L·d) and maintaining the reactor first at 24 ºC and then at 30 ºC.[113] A HRT of 1.6 days, an OLR of 9.4 g COD/L and a temperature of 30 ºC were chosen to start the reactor, since Campos (2013) found that these conditions lead to higher substrate consumption and VFA production.[113]

The initial biomass concentration was also chosen according to Campos (2013).[113] The amount of MMC supplied to the reactor was calculated to obtain a biomass concentration close to 3 g VSS/L.

COD concentration of the feeding was calculated from the HRT and OLR imposed to the reactor, resulting in a desired concentration of 15 g COD/L. The COD concentration of pre-treated HSSL was measured to determine the concentration of pre-treated HSSL in the feeding solution and it was found to be 192.5 g/L. COD concentration of the fermentation medium was on average 15.5 ± 0.40 g/L during the whole fermentation period, accounting for 0.49 ± 0.02 g COD/L of glucose and 2.9 ± 0.06 g COD/L of xylose, corresponding to around 22 % of its COD concentration, and 1.1 ± 0.02 g COD/L of acetic acid.

Figure 12 presents the biomass concentration, pH and oxidation-reduction potential (ORP) values recorded during the fermentation period. The pH and ORP were left uncontrolled since it is advantageous from the industrial point of view, to reduce process costs as, for example, base addition costs (extra equipment and reagents).

Figure 13 shows the evolution of the individual concentrations of the fermentation products, namely, ethanol (EtOH), acetic acid (HAc), propionic acid (HPr) and butyric acid (HBu), the produced fermentation products (PFP) concentration, the xylose (Xyl) concentration, the consumed sugars ($S_{cons.}$), and the SCOD of the effluent measured daily. During the fermentation period, there were some changes between continuous flow mode (green bars) and batch mode (yellow bars) represented in the upper timeline. The process

was also interrupted from day 50 to day 70 during holidays (grey bar). The initial conditions of the CSTR on day 6 were a HRT of 1.6 days and a OLR of 9.4 g COD/(g·L). On day 40, the HRT was changed to 2 days which led to an OLR of 7.7 g COD/(g·L), since the COD concentration of the feeding solution remained the same (approximately 15 g COD/L).

As it can be seen in Figure 13 there were considerable fluctuations on the concentration of consumed sugars and produced fermentation products during the entire fermentation period. Also the products profile showed several shifts, mostly between the propionic and butyric acids production. Along the fermentation process, a gradual and continuous decrease of the biomass concentration was observed, leading to the reinoculation of the reactor several times, as seen in Figure 12. As result, the biomass concentration variation was in the range of 1.9 ± 1.3 g COD/L. In a CSTR, there will be a continuous loss of active microbial biomass due to the absence of a mechanism for retaining the biomass inside the reactor. Thus, the CSTR should be operated with a HRT that exceeds the doubling time of the target population (acidogens). The relation between the populations of acidogens and methanogens and previous results of Campos (2013)[113] led to the decision of choose a short HRT, also associated with higher DA.[95] However, the use of HSSL as substrate source, since it is known to be rich in toxic compounds and inhibitors of bacterial growth, may have decrease the specific growth rate of acidogens and contribute to the reduction of active microbial biomass in the reactor.[109]

The pH was not controlled during this process, but even so, it remained stable around 5.0 ± 0.24 and was never higher than 6.21 or lower than 4.63 (Figure 12). This effect was previously reported by other authors whom referred the buffering effect of HSSL.[113,114] This effect is very advantageous in the present work since the system does not require pH control, thus reducing the expenses with reagents and equipment and contributing for the reduction of costs of the overall process. However, the pH is usually related with the VFA profile, and so, to obtain a specific VFA profile pH adjustments may be needed.

The ORP was also followed since the 70th day of fermentation (Figure 12). ORP was always negative and above –300 mV, which are typical values of an acidogenic fermentation, since methanogenic fermentation usually occurs below –550 mV, and also proved that the fermentation was conducted under anaerobic conditions.[118]

Table 8 resumes the main results obtained during the periods with different operational conditions. For these periods the consumed sugars ($S_{cons.}$) concentration, the PFP concentration, the products profile, the degree of acidification (DA) and the products yield on consumed sugars ($Y_{FP/S}$) were calculated. It's worth noting that all the presented results are the average of the observations during the specified tine range. Two pseudo-steady states (PSS) were chosen based on the stabilization of the concentrations of consumed sugars and PFP to compare the effects of different operational conditions. The first one was between days 13 and 19, when the HRT was 1.6 days and OLR was 9.4 g COD/(g·L) (PSS1); and the second one was between days 82 and 98, when the HRT was 2 days and OLR was 7.7 g COD/(g·L) (PSS2).

Table 8: Results from the acidogenic fermentation of HSSL (mean ± standard deviation).

		Batch1	PSS1	Batch2	CSTR2	CSTR2	Batch3	PSS2	CSTR3
Time (d)		*1-5*	*13-19*	*20-26*	*29-37*	*44-50*	*70-75*	*82-98*	*106-114*
HRT (d)		-	1.6	-	1.6	2.0	-	2.0	2.0
OLR (g COD/(L·d))		-	9.4	-	9.4	7.7	-	7.7	7.7
Sugars Cons.	g COD/L	2.8	2.6 ± 0.039	2.7	2.1 ± 0.41	2.5 ± 0.16	2.5	2.3 ± 0.035	2.4 ± 0.070
	%	83	78 ± 1.2	82	62 ± 12	75 ± 4.7	73	68 ± 1.0	72 ± 2.1
PFP (g COD/L)		0.71	1.1 ± 0.10	0.47	1.2 ± 0.13	0.58 ± 0.072	0.7	1.6 ± 0.11	1.6 ± 0.30
FP profile (%)	**EtOH**	35.8	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0.0	3.3 ± 6.0	0.0 ± 0.0
	HAc	23.4	19 ± 14	23.3	26 ± 3.3	20 ± 7.2	41	17 ± 5.0	49 ± 18
	HPr	0.0	31 ± 4.3	22	46 ± 21	80 ± 7.2	59	77 ± 13	51 ± 18
	HBu	40.8	50 ± 15	55	28 ± 21	0.0 ± 0.0	0.0	2.5 ± 5.5	0.0 ± 0.0
DA (%)		2.9	7.1 ± 0.65	3.1	7.5 ± 0.81	3.8 ± 0.46	4.5	9.8 ± 0.80	10.1 ± 1.9
FP/SCOD (%)		6.3	7.4 ± 0.70	3.5	7.5 ± 2.5	4.2 ± 0.45	5.4	10.3 ± 0.79	n.d.
$Y_{FP/S}$ (g COD/g COD)		0.25	0.42 ± 0.039	0.17	0.58 ± 0.13	0.23 ± 0.046	0.29	0.69 ± 0.053	0.65 ± 0.13

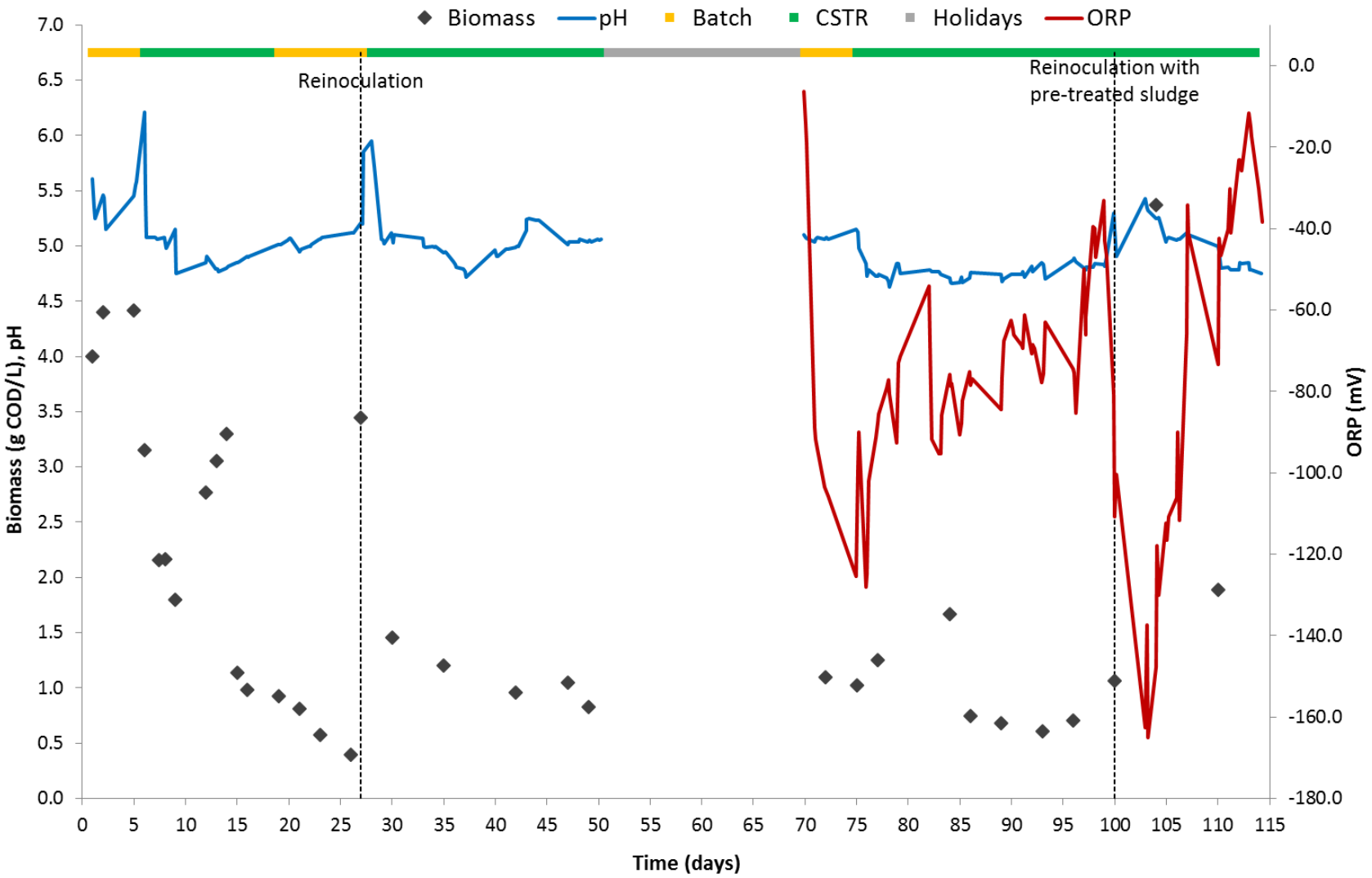

Figure 12: Biomass concentration, pH and ORP during the full length of the fermentation process.

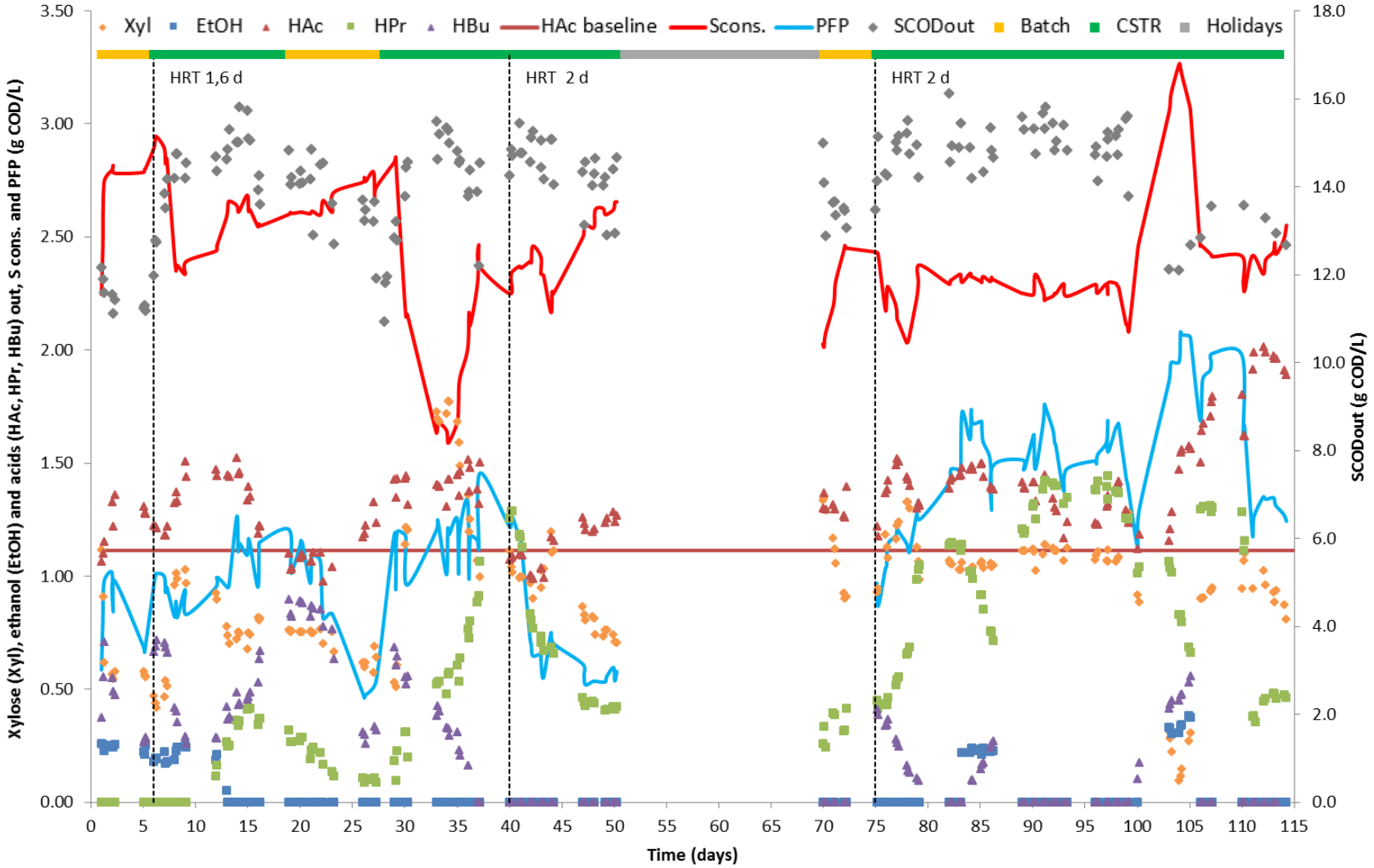

Figure 13: Evolution of fermentation substrates and products during the acidogenic fermentation timeline.

4.1.1. Batch 1 and CSTR 1 periods

The first 5 days of fermentation were performed in batch mode (Batch 1) so the biomass could adapt to the environmental conditions, namely, the high concentration of inhibitors in the HSSL. The concentration of biomass in the beginning of the process was around 4.4 g COD/L. During this period, xylose concentration decreased fast and glucose was not detected in HPLC analysis, which indicated that it was being totally consumed, resulting in a consumption of 83% of the initial sugars concentration.

During Batch 1, the major fermentation product was butyric acid, reaching a maximum production of 0.71 g COD/L. Also acetic acid and ethanol were produced during the first days of fermentation in average concentrations of 0.16 g COD/L and 0.24 g COD/L, respectively. Despite the high percentage of sugars consumed, the $Y_{FP/S}$ was only 0.25 g COD/g COD, which indicates that the consumption of sugars was directed mainly to biomass growth, which was expected since fresh feed was provided to biomass going through starvation. Also, during the first 2 days of operation, the volumetric substrates uptake rate ($-r_S$) was 0.35 g COD/(L·d) and the specific uptake rate ($-q_S$) was 0.083 g COD/(g COD·d), while the volumetric fermentation products production rate (r_{FP}) and the specific fermentation products production rate (q_{FP}) were 0.18 g COD/(L·d) and 0.043 g COD/(g COD·d), respectively.

On the 6th day, the continuous mode was turned on and it was observed an increasing in the xylose concentration until approximately 1.0 g COD/L on days 8 and 9, but the glucose was still not detected in the HPLC since it was readily consumed. After a transition period, the xylose concentration levelled off at 0.75 ± 0.039 g COD/L and the sugars consumption at 2.6 ± 0.039 g COD/L, corresponding to 78 ± 1.2 % of the sugars concentration in the feeding solution. Based on the stabilization of the sugars consumption, a pseudo-stationary period was considered between days 13 and 19 (PSS 1) (see Table 8).

After the beginning of the CSTR 1, it was verified an immediate increasing of produced acetic and decreasing of butyric acid until day 9, with 0.40 g COD/L and 0.26 g COD/L, respectively. The ethanol concentration remained around 0.21 ± 0.029 g COD/L until day 9. After that, acetic acid concentration was stable until day 14, propionic and butyric acids concentrations started to increase and ethanol rapidly decreased to a value under HPLC detection level. Propionic acid concentration reached a maximum of 0.42 g

COD/L on day 15 and started to decrease after that day just like the acetic acid, which was near zero on day 19. Butyric acid concentration continued to increase until day 19, reaching a maximum concentration of 0.90 g COD/L that corresponds to 74 % of the PFP. Although the acids profile suffered several variations during PSS1, the most abundant fermentation product was butyric acid with around 50 ± 15 % of PFP on average, followed by propionic and acetic acids, with 31 ± 4.3 and 19 ± 14 %, respectively. Also, the PFP concentration remained quite stable during PSS1, corresponding to 1.1 ± 0.10 g COD/L. The DA was 7.1 ± 0.65 % and the $Y_{FP/S}$ was 0.42 ± 0.039 g COD/g COD.

During the CSTR 1 period the biomass concentration decreased constantly and it was under 1 g COD/L on days 16 and 19, so the continuous mode was stopped to allow the biomass to build up inside the reactor.

4.1.2. Batch 2 and CSTR 2 periods

During the period corresponding to Batch 2, the consumed sugars concentration increased slightly relatively to the previous period, reaching 82% of the sugars concentration in the feeding solution; the r_S was 0.025 g COD/(L·d) and the q_S was 0.036 g COD/(g COD·d). Also the PFP concentration decreased from around 1.0 g COD/L to 0.5 g COD/L, corresponding to 55 % butyric acid, 23 % of acetic acid and 22 % propionic acid at the end of Batch 2.

On day 26 it was clear that the strategy biomass build-up in batch mode did not worked as planned, since the biomass concentration was 0.39 g COD/L on that day (close to wash-out), and so the reactor was inoculated with new sludge on day 27, leading to a biomass concentration in the reactor of 3.4 g COD/L and a pH of 5.85, quite above the values recorded so far. After the continuous flow was switched on, the biomass concentration decreased to values close to 1.5 g COD/L, but it was always higher than 1.0 g COD/L until the end of the second CSTR period (CSTR 2).

The CSTR mode was restarted on day 28 and short after it was verified an increasing in the xylose concentration until day 34, reaching a maximum xylose concentration of 1.8 g COD/L although glucose was not detected in HPLC analysis. This indicates a reduction in sugars consumption, reaching a minimum of 1.6 g COD/L on day 34, corresponding to approximately 47 % to the measured sugars in the feeding solution.

Despite the decreasing in sugars consumption, PFP concentration increased immediately after the beginning of the CSTR 2 period, mostly due to the increasing of the production of propionic acid at a volumetric production rate of 0.09 g COD/(L·d) until day 40, reaching a maximum concentration of 1.3 g COD/L. On the other hand, despite the increasing in butyric acid at the beginning of the CSTR 2 (0.69 g COD/L on day 29), the butyric acid concentration continued to decrease until it was no longer detected in HPLC analysis on day 37 and until the end of the CSTR 2 period. As for produced acetic acid it was stable around 0.30 ± 0.059 g COD/L until day 37. Thus, the produced fermentation products during this period were acetic, propionic and butyric acids in a relative proportion of 26:46:28 on average. The DA was 7.5 ± 0.81 % and the $Y_{FP/S}$ was 0.58 ± 0.13 g COD/g COD, which represented a slight improvement relatively to the values obtained in PSS1.

On day 40, the HRT was raised to 2 days, by imposing the lowest pump flow rate of 0.79 L/d, to try to increase the biomass concentration by increasing residence time. The OLR was consequently decreased to 7.7 g COD/(g·L), since the COD concentration of the feeding solution was the same as before (15 g COD/L). Despite the increasing of the HRT usually favours the methanogens, since they have a very low growth rate (at the range of days), acidogens can have up to 20 times higher bacteria yields and conversion rates than methanogenic bacteria.[119] Also, it is widely recognized that pH under 5.5 inhibits the methanogenesis and that some compounds can inhibit the methanogenic activity [88,119,120] Thus, the low pH and the presence of toxic compounds of the HSSL are thought to inhibit these bacteria.

After the change in the HRT there was some instability in the substrates consumption and acetic acid production, mostly from day 40 to day 44, since the MMC needed to adapt to the new conditions. However, substrates consumption reached 2.7 g COD/L on day 50, corresponding to 79 % of the measured sugars in the feeding solution. Consequently, acetic acid concentration increased from nearly zero (after the change in HRT) to 0.16 g COD/L on day 50 and propionic acid production dropped until it reached a minimum value of 0.42 g COD/L. Butyric acid and ethanol were not detected in HPLC. PFP concentration was 0.58 ± 0.072 g COD/L, which was considerably lower than in the other CSTR periods with HRT of 1.6 days, which might suggest that the MMC was still adapting to the new conditions imposed to the reactor. Nevertheless, the products profile switched during the CSTR 2 period with a HRT of 2 days (from days 44 to 50) when

compared with a HRT of 1.6 days (from days 29 to 37), being the propionic acid the major fermentation product with 80 ± 7.2 % of PFP, never reaching a percentage below 70 %. Consequently to the low production of fermentation products, the DA and the $Y_{FP/S}$ were very low during this period, only 3.8 ± 0.46 % and 0.23 ± 0.046 g COD/g COD. On day 50 the process was interrupted and the biomass was harvested in the fridge at 4ºC.

4.1.3. Batch 3 and CSTR 3 periods

After the interruption the stored biomass was used to inoculate the reactor at a ratio of 2:1 stored broth to fresh feed and the fermentation process was restarted on batch configuration for 5 days (Batch 3). At the end of Batch 3, biomass concentration was 1.0 g COD/L, consumed substrates concentration was 2.5 g COD/L, corresponding to 73 % of the measured sugars in the feeding solution, r_S was 0.21 g COD/(L·d) and q_S was 0.19 g COD/(g COD·d). Furthermore, PFP concentration was very low, 0.54 ± 0.095 g COD/L on average, and it reached 0.70 g COD/L on day 72, accounting for 41 % of acetic acid and 59 % of propionic acid. The maximum DA and $Y_{FP/S}$ were 4.5 % and 0.29 g COD/ g COD, respectively. The low yield on sugars may indicate a substrate consumption mainly to biomass growth and maintenance. Comparing the batch periods, DA was successively higher and $-q_S$ was higher during Batch 3, what is expected since biomass became better adapted to the HSSL over time.

On day 75, the CSTR configuration was started (CSTR 3) at the same operational conditions than before, namely, HRT of 2 days and OLR of 7.7 g COD/(g·L). Xylose concentration increased inside the reactor during an adaptation period as consequence of the continuous feeding. After that, sugars consumption was found to be stable at 2.3 ± 0.035 g COD/L between days 82 and 98, and so this time lapse was considered a pseudo-stationary state period for the operational conditions mentioned above (PSS2). Also, the biomass concentration increased until 1.7 g COD/L on day 84.

The PFP concentration was 1.6 ± 0.11 g COD/L, mostly from propionic acid with 77 ± 13 % and acetic acid with 17 ± 5.0 %. The ethanol and butyric acid production was lower than 0.5 g COD/L during PSS2 and only during days 83 to 86. Although propionic acid was the most abundant fermentation product, its production was not stable: it presented a trend opposite to butyric acid during days 75 to 86 (see Figure 13), reaching a

maximum concentration of 1.1 g COD/L on day 82. After the disappearance of the butyric acid and ethanol on day 86, the propionic acid concentration raised to values around 1.4 g COD/L until the end of PSS2.

It is important to point that during PSS2, the highest DA of 9.8 ± 0.80 %, $Y_{FP/S}$ of 0.69 ± 0.053 g COD/g COD, and percentage of PF of 10.3 % were reached. The low DA and conversion indicate that the lignosulphonates (LS), that compose more than 50 % of the COD of HSSL, were not being consumed. HSSL has also phenolic compounds that are microbial inhibitors, resulting in slower kinetics, lower yields and productivities, lower cell growth and/or fermentation products production, and should be removed before using the HSSL as substrate for acidogenic fermentation in order to increase the efficiency of the process.[109] Pereira et al. (2012) has successfully biologically pre-treated HSSL for bioethanol production. The authors indicated that *Paecilomyces variotti* was able to assimilate acetic acid as well as low molecular weight phenolics that inhibit fermentation.[115] Other strategy is to use an inoculum adapted to the degradation of LS and the presence of other inhibitory compounds.

On day 100, a new reinoculation with pre-treated sludge (82 ºC for 20 min) was performed to try to achieve higher sugars consumption and production of VFA. It was possible to reach a biomass concentration of 5.4 g COD/L on day 104, but it rapidly decreased to 1.2 g COD/L on day 110, close to the end of the fermentation process.

After the reinoculation, the reactor entered a period of instability relatively to the production of acids and ethanol: from days 103 to 105 was observed the appearance of butyric acid and ethanol until the maximum concentrations of 0.56 g COD/L and 0.38 g COD/L, respectively. Simultaneously, there was a sudden decreasing of the concentration of propionic acid and increasing of the acetic acid produced concentration. Despite all of this, the sugars consumption and the production of PF were the highest on day 104, reaching concentrations of 3.3 g COD/L, about 97 % of the measured sugars in the feeding solution, and 2.1 g COD/L, respectively. It is also worth to note that the SCOD consumption after the reinoculation with pre-treated sludge was significantly higher when compared with the PSS2 period. This reduction of the SCOD concentration from the average 15.0 g COD/L to 12.1 g COD/L on day 104 was associated with the high sugars consumption and may be due to the high biomass concentration inside the reactor (5.4 g COD/L on day 104). Also, DA was 11 % and $Y_{FP/S}$ was 0.64 g COD/g COD on day 104.

From day 106 until the end of the fermentation process, propionic acid production became very unstable, the butyric acid and ethanol were no longer detected on HPLC analysis and only acetic acid production continued to increase until day 112 when it reached the maximum of 0.90 g COD/L, corresponding to 67 % of FP concentration. Sugars consumed and PFP concentrations were similar to PSS2, but with higher variation (see Table 8). On average, the DA and $Y_{FP/S}$ were 10.1 ± 1.9 % and 0.65 ± 0.13 g COD/g COD, respectively. However, the highest DA and $Y_{FP/S}$ were reached on day 107 with 13 % and 0.80 g COD/g COD, because more acids were being produced on a lower concentration of consumed sugars.

4.1.4. Evaluation of the HSSL fermentation period

The percentage of FP on the effluent and DA achieved in this fermentation process were quite low, reaching a maximum of 10 %.When comparing the two PSS, FP/SCOD and DA were higher in PSS2 (days 83 – 98), 10.3 % and 9.8 %, respectively, than in PSS1 (days 13 – 19), 7.4 % and 7.1 %, respectively.

The difference in the percentage of conversion can be explained in different ways. PSS2 was performed at HRT of 2 days and OLR of 7.7 g COD/(g·L), while PSS1 was performed at HRT of 1.6 days and OLR of 9.4 g COD/(g·L), thus PSS2 had a higher dilution of HSSL, and so a higher dilution of the toxics that inhibit the acidogenic community. Also, PSS2 took place more than 60 days after PSS1, which may also explain the higher conversion from the selection of the bacterial community most resistant and adapted to this severe environment. It is also important to remind that several authors have reported that an increasing in HRT leads to an improvement in conversion of VFA.[73,93,95] FP profile was also different in the two PSS. Butyric acid was the major fermentation product in PSS1, while propionic acid was the major fermentation product in PSS2 (Figure 14).

In general, propionic and butyric acids were the two major fermentation products, but their production seemed to be intercalated instead of cumulative, meaning that the production of one of these acids seemed to inhibit or decrease the production of the other. Butyric acid was relatively more abundant during the first 30 days of fermentation and propionic acid was more abundant during the major part of the rest of the fermentation

period. Despite the pH being uncontrolled and so strong conclusions cannot be taken, it is widely known that the pH strongly affects the fermentation products profile.[73,92,120] Even so, it was noticeable that when the pH was above 5.0 and ORP was below –150 mV, butyric acid was the dominant product (butyric-type fermentation), while when the pH was between 4.5 and 5.0, the main products were propionic and acetic acids (propionic-type fermentation). Ren et al. (2007) studied the effects of ORP and pH on fermentation types and reported similar trends in the production of propionic and butyric acid depending on the pH.[120] The authors reported that the propionic acid maximum production was at pH 5.5 and the butyric acid maximum production was at pH 6.0, when working with fermented molasses obtained from a beet sugar refinery, which may justify the fact that only the trend and not the values were in agreement with the present work that used HSSL from pulp and paper industry.[120] These values have previously been reported for propionic-type and butyric-type fermentation.[121]

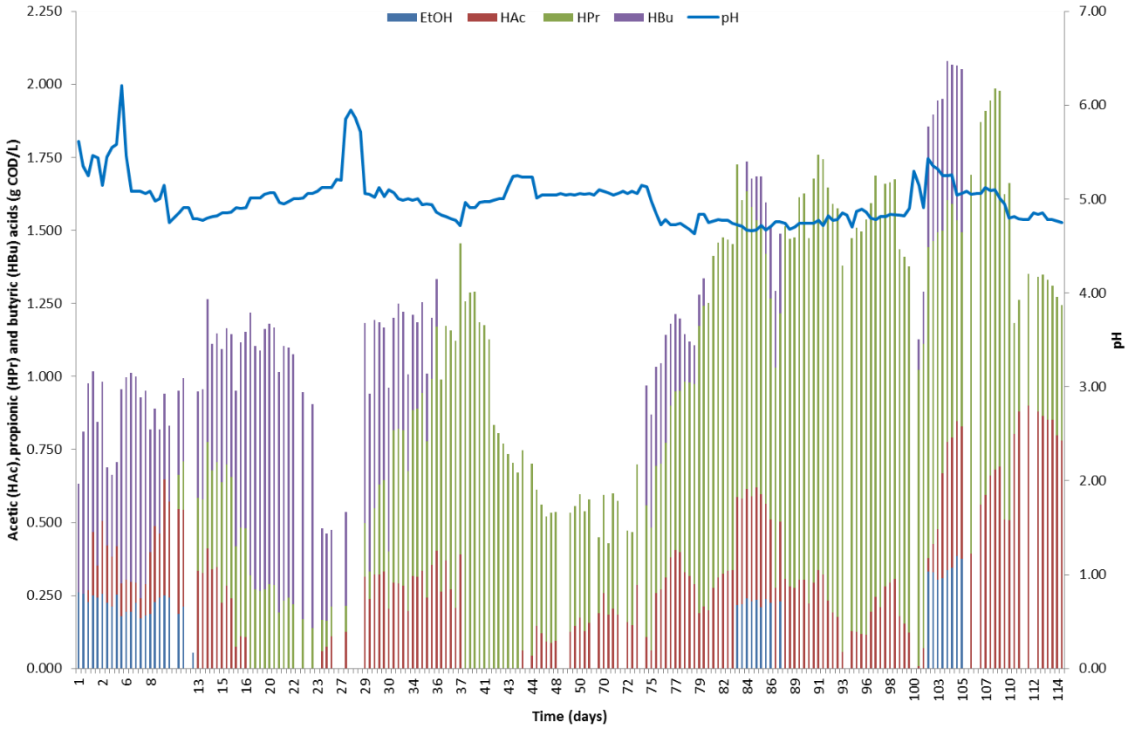

Figure 14: Profile of produced fermentation products and measured pH.

During batch periods, the PFP concentration was always lower than 1.0 g COD/L and it reached always values under 0.5 g COD/L, namely on days 5, 26, 70 e 72. This may be due to the fact that batch periods were usually established after an inoculation when the biomass was still adapting to the new environment, or in the case of day 26, the biomass concentration was too low maybe due to the stress induced by toxic compounds in HSSL.

4.2. Acidogenic fermentation of cheese whey permeate in SBR

Two sequential batch reactors (SBR) were operated in parallel for 100 days to evaluate NaOH consumption during acidogenic fermentation of cheese whey permeate (CWP) diluted to concentrations typical for moderate strength food industrial wastewater (approximately 2 g COD/L). Also, different pH set-points, volume exchange ratios (leading to different HRT and OLR) and SRT (4 and 8 days) were tested.

The SBR was chosen to perform the acidogenic fermentation of CWP because this configuration allows retaining the biomass by gravity settling, improving the fermentation performance by increasing the concentration of biomass at pH lower than optimal. At the same time it is possible to collect the effluent to feed subsequent steps, e.g. PHA production. The SBR were assembled as described in the chapter 3.2.3.

COD concentration of concentrated CWP (approximately 180 g COD/L) and volume of water pumped per cycle were measured regularly so that the amount of SCOD influent could be estimate with accuracy. Also, CWP was analysed by GC and it was concluded that the average acetic acid concentration was 0.17 ± 0.050 g/L. This occurrence can result from the activity of bacteria in the CWP, since it was not kept in sterile conditions, despite of being refrigerated at 4 ºC. However, after the dilution of CWP of about 1:90, the acetic acid concentration in the influent became negligible.

4.2.1. SBR performance during the operation period

Figure 15 and Figure 16 show the evolution of individual concentrations of fermentation products obtained – ethanol (EtOH), acetic acid (HAc) and propionic acid (HPr), their sum (FP), and SCOD and biomass concentrations measured daily in SBR1 and SBR2, respectively. It is worth noting that no butyric acid was detected during the fermentation operational period in both reactors. Over the fermentation period, different operational conditions were imposed, which are summarized in Table 7. The changes on operational conditions in both SBR are represented in the graphs by dotted vertical lines, and the corresponding change is noted at the right side of the line.

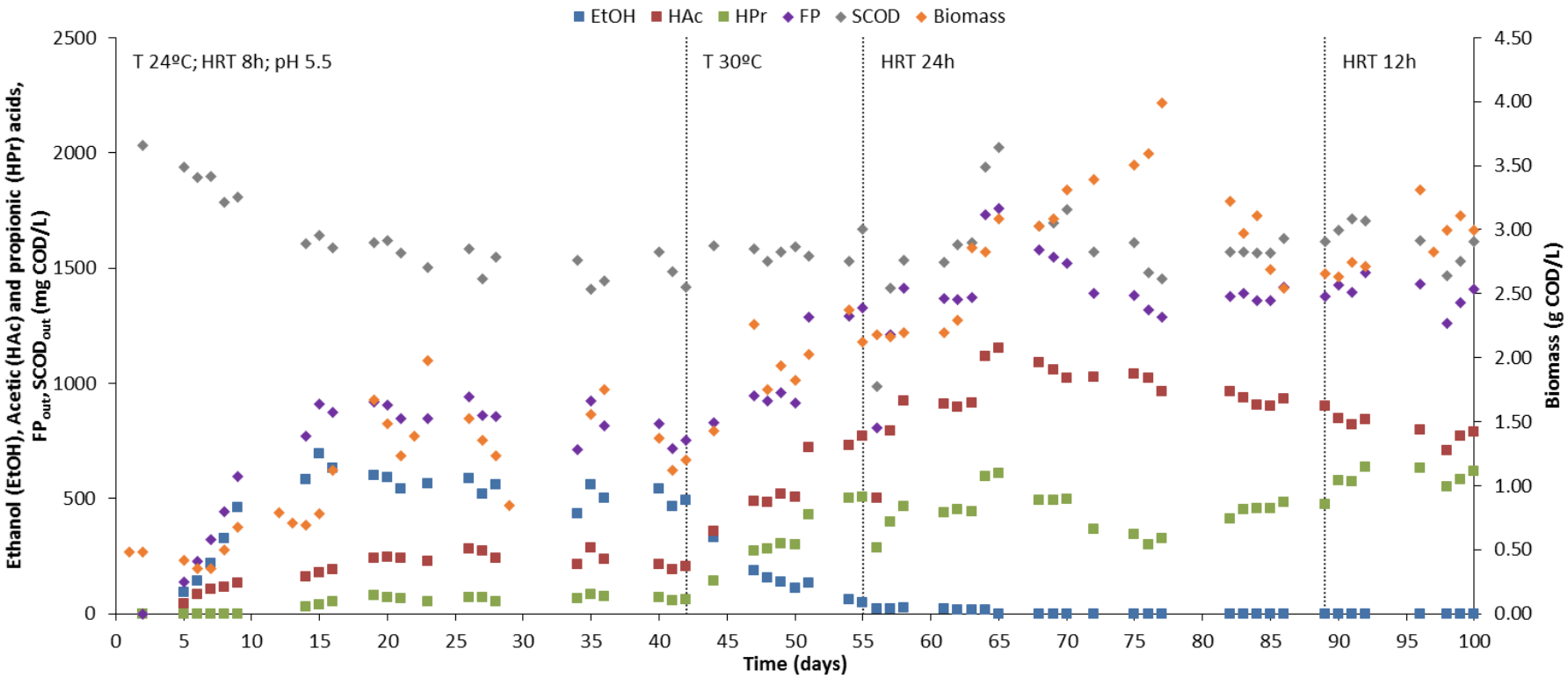

Figure 15: Concentration of ethanol, acetic acid, propionic acid, soluble COD and VSS in SBR1 during its full working period. TFP is also presented to better comparison with SCOD.

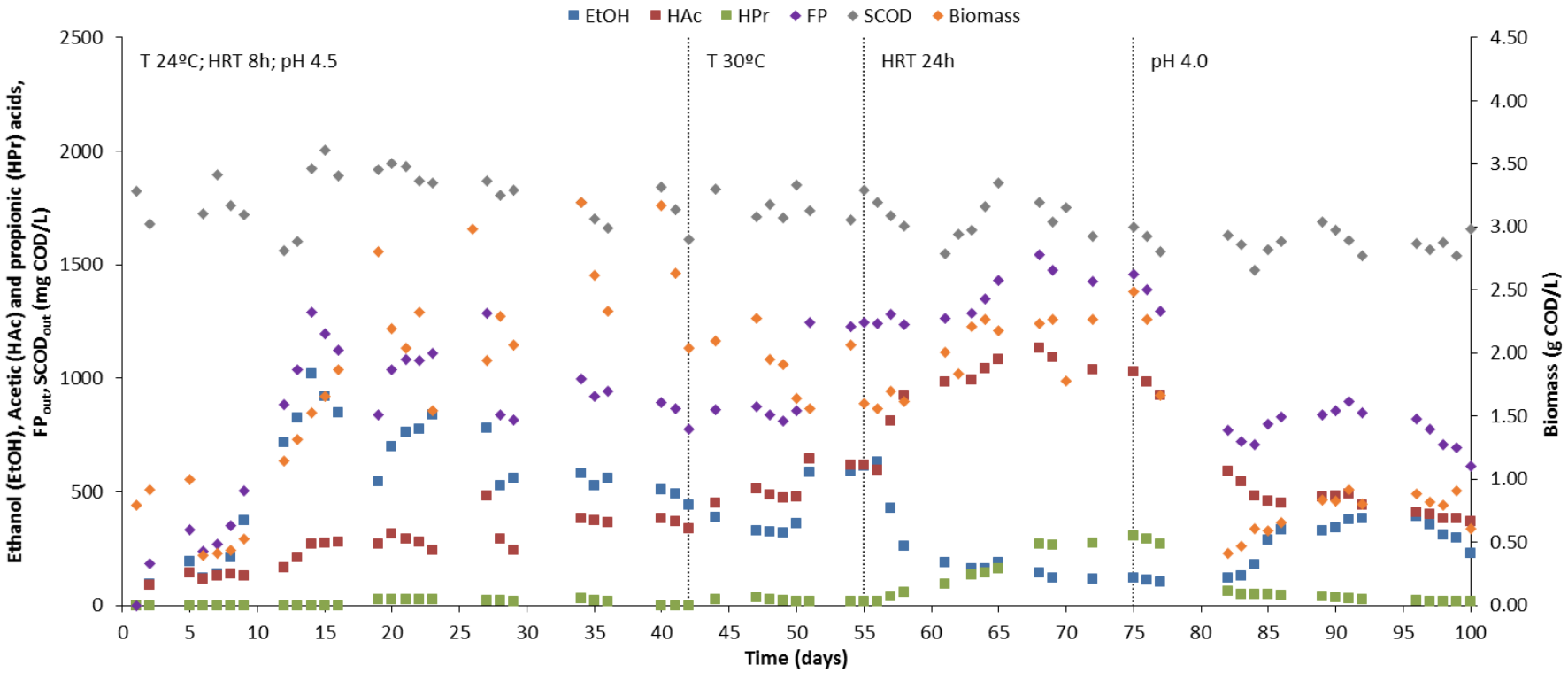

Figure 16: Concentration of ethanol, acetic acid, propionic acid, soluble COD and VSS in SBR2 during its full working period. TFP is also presented to better comparison with SCOD.

Based on the results obtained, periods of stable production were chosen and the average profile of fermentation products, FP concentration, percentage of FP in the effluent (FP/SCOD), DA and yield of fermentation products on SCOD ($Y_{FP/SCOD}$) were calculated and are summarized in Table 9.

Table 9: Parameters calculated during the indicated periods of both SBRs (mean ± standard deviation).

		SBR1				SBR2			
Time (d)		*14-42*	*51-55*	*58-89*	*90-100*	*14-42*	*51-55*	*61-75*	*85-96*
pH		5.5	5.5	5.5	5.5	4.5	4.5	4.5	4
T (ºC)		24	30	30	30	24	30	30	30
HRT (h)		8	8	24	12	8	8	24	24
FP profile (%)	**EtOH**	66 ± 4.9	6.2 ± 3.6	0.34 ± 0.59	0.0 ± 0.0	66 ± 7.9	48 ± 1.1	11 ± 2.7	41 ± 3.9
	HAc	27 ± 3.6	57 ± 1.0	68 ± 3.9	57 ± 1.4	33 ± 7.9	51 ± 1.1	75 ± 2.6	54 ± 2.8
	HPr	7.4 ± 1.6	37 ± 2.9	31 ± 3.7	43 ± 1.4	1.6 ± 1.2	1.4 ± 0.1	14 ± 5.1	4.1 ± 1.3
FP_{out} (g COD/L)		0.84 ± 0.073	1.3 ± 0.023	1.4 ± 0.13	1.4 ± 0.071	1.0 ± 0.16	1.2 ± 0.012	1.4 ± 0.097	0.84 ± 0.031
FP/SCOD (%)		55 ± 4.8	82 ± 2.5	88 ± 2.4	86 ± 2.4	55 ± 7.2	71 ± 2.2	83 ± 5.1	52 ± 2.2
DA (%)		17 ± 6.7	63 ± 9.9	65 ± 4.9	61 ± 9.7	16 ± 3.6	33 ± 1.6	57 ± 8.9	23 ± 2.4
$Y_{FP/SCOD}$ (g COD/ g COD)		0.62 ± 0.060	0.77 ± 0.0075	0.73 ± 0.075	0.78 ± 0.16	0.81 ± 0.086	0.85 ± 0.013	0.73 ± 0.099	0.64 ± 0.19

In SBR1, under the first conditions imposed (pH 5.5, 24 ºC and HRT 8 h), fermentation products were mainly ethanol, which achieved a maximum concentration of 0.69 mg COD/L (76 % of the FP concentration) on day 15, and acetic and propionic acids in minor concentrations. SCOD was very close to 2 g COD/L during the first days of fermentation, when the acidification was very low, since the biomass was still adapting to the new environmental conditions and its concentration was around 0.50 g COD/L, which was rather low. After the acclimatization period (until day 9), biomass built-up to 1 – 2 g COD/L and SCOD decreased to around 1.5 g COD/L. From days 14 to 42, ethanol and VFA production were stable, FP/SCOD of the effluent was 55 ± 4.8 %, accounting for 66 ± 4.9 % of ethanol, 27 ± 3.6 % of acetic acid and 7.4 ± 1.6 % of propionic acid, and DA was 17 ± 6.7 %. On day 42, temperature was started to be controlled at 30 ºC since several

papers reported that increasing the temperature within the mesophilic conditions (20 – 50 ºC) was beneficial to the VFA conversion rate and yield. [58,85–87] Although there was an increasing of the FP concentration from 0.76 g COD/L on day 41 to 0.95 g COD/L on day 47, the greatest improvement of FP production was on day 51, 1.3 g COD/L. This way, the effect of changing the temperature was not immediate, but the biomass needed some time to adapt to the new conditions. However, a more distinct effect was noted: when the temperature was changed there was a shift between ethanol production and VFA production, the concentration of ethanol in the effluent dropped and both acetic and propionic acids concentrations increased. On the second period of set conditions, the VFA concentration was 1.3 ± 0.023 g COD/L, accounting for 57 ± 1.0 % of acetic acid, 37 ± 2.9 % of propionic acid and 1.3 ± 0.023 % of ethanol, the FP/SCOD was 82 ± 2.5 % and the DA was 63 ± 9.9 %. Aiming to achieve full conversion, the volume exchange ratio was changed to 0.25 L/L on day 56, but the concentration of influent COD was the same (around 2 g COD/L), so the HRT increased from 8 to 24 h and the OLR decreased from 6.0 to 2.0 g COD/(L·d). During the third period of tested conditions, ethanol was not detected and the average FP production was 1.4 g COD/L, while the average FP/SCOD and DA were 88 ± 2.4 % and 65 ± 4.9 %, reaching a maximum of 94 % and 70 % on day 68, respectively. Since it was possible to achieve high conversion at a volume exchange ratio of 0.25 L/L, it was decided to increase it to 0.50 L/L to confirm if it was possible to reach the same DA at higher OLR. Thus, from day 90 until the end of the process, the average FP/SCOD was 86 ± 2.4 % and the DA was 61 ± 9.7 %, which were similar to values obtained with previous conditions, but with slightly different relative concentrations of acids: acetic acid decreased from 68 ± 3.9 % to 57 ± 1.4 %, while propionic acid increased from 31 ± 3.7 % to 43 ± 1.4 %. Despite the acetic acid was still the major fermentation product, probably the production of propionic acid will have exceed the production of acetic acid if the fermentation process had been further carried out.

As for SBR2, during the first period of tested conditions (pH 4.5, 24 ºC and HRT 8 h), the major fermentation product was ethanol, which reached a maximum value of 1.0 g COD/L (79% of the FP concentration) on day 14. After that day, the ethanol concentration progressively decreased until 0.44 g COD/L on day 42, but the average relative concentration of ethanol was 66 ± 7.9 % of the FP concentration. Acetic acid was also produced, but in less extent, reaching 33 ± 7.9 % of the FP concentration, while propionic

acid concentration was negligible (1.6 ± 1.2 %). Considering the production between days 14 and 42, the FP production was relatively stable and the FP/SCOD and the DA were 55 ± 7.2 % and 16 ± 3.6 %, respectively.

At day 42, the temperature started to be controlled at 30ºC to improve the conversion of sugars into VFA. During the first days after this change the FP concentration was approximately the same as in the end of the previous period, and the verified tendency of decreasing of ethanol and increasing of acetic acid was confirmed. However, the FP concentration increased from 0.86 g COD/L on day 50 to 1.2 g COD/L on day 51 due to the increasing in both ethanol and acetic acid concentrations. This time lag between the temperature change and the improvement of the conversion was also verified in SBR1 and it is probably related with the adaptation of the biomass to the new operational conditions. Between days 51 and 55, FP concentration was stable around 1.2 ± 0.012 g COD/L (48 ± 1.1 % of ethanol and 51 ± 1.1 % of acetic acid), and FP/SCOD and DA were 71 ± 2.2 % and 33 ± 1.6 %, respectively. On day 56, the volume exchange ratio was changed to 0.25 L/L as it was in SBR1. From that moment on, ethanol concentration dropped and the acetic concentration almost doubled, as it was achieved an average FP/SCOD of 83 ± 5.1 % and a DA of 57 ± 8.9 % in the period between days 61 and 75. Also, the propionic acid concentration, negligible until this moment, started to increase almost immediately and reached its maximum value of 0.31 g COD/L (21 % of the FP concentration) on day 75, when the pH was changed.

In order to reduce the NaOH consumption, the pH set-point was reduced to 4.0 in SBR2, leading to the reduction of the volume of NaOH spent to keep the pH at set-point (see in chapter 4.2.3). It is important to notice that biomass concentration suffered a great decreasing from 2.5 g COD/L on day 75 to 0.41 g COD/L on day 82, mostly due to the loss of settling properties during the first days of adaptation to the new conditions and consequent washout of biomass (increasing of VSS concentration and turbidity of the effluent). Thus, it is not entirely clear if the initial decreasing of the FP production is a direct result of the change of pH or a consequence of the reduction of biomass concentration. After day 82, biomass appeared to overcome the adaptation period and started to increase its concentration. Also the production of ethanol increased and stabilized short after. Acetic acid concentration was still higher than ethanol concentration, but propionic acid concentration decreased to negligible values. Considering the period

between days 85 and 96, the ethanol and acetic acid concentrations were stable and corresponded to 41 ± 3.9 % and 54 ± 2.8 % of the FP concentration, respectively, the FP/SCOD was 52 ± 2.2 % and the DA was 23 ± 2.4 %. It should also be considered that close to the end of the fermentation period, FP decreased, mainly due to the decreasing in ethanol production, while the acetic acid concentration was approximately constant, which could indicate a new shift in the FP profile.

4.2.1. Influence of the operational conditions on the production of ethanol and VFA

Figure 17 presents the FP/SCOD, DA and the relative concentrations of EtOH, HAc, HPr in the effluent collected during the fermentation stable periods for the tested operational conditions, namely pH of 5.5; 4.5 and 4.0, temperature of 24 and 30ºC and HRT of 8, 12 and 24 h. Notice that this graph merge data from both reactors.

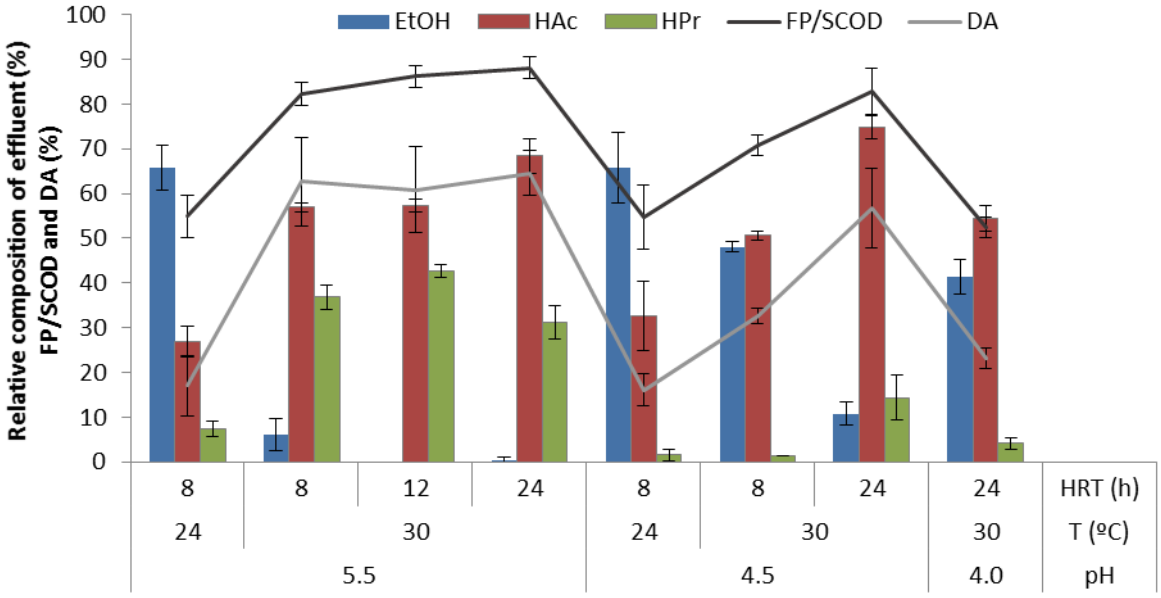

Figure 17: Relative composition of fermentation products in the effluent, FP/SCOD and DA versus operational conditions tested.

The best operational conditions tested were pH 5.5 and 30ºC (all HRT), and pH 4.5, HRT 24 h and 30 ºC, since it was possible to achieve a percentage of conversion higher than 80 % and a DA around 60 %.

Initially, when the temperature was raised from 24 to 30 ºC, it was verified an improvement in the production of FP at both pH 5.5 and 4.5, while maintaining a HRT of 8 h. The average FP/SCOD increased from 55 % to 82 % at pH 5.5 and to 71 % at pH 4.5. The profile of fermentation products also changed. At pH 5.5, the ethanol relative concentration decreased from 66 % to 6.2 %, the acetic acid became the major fermentation product (its concentration was higher than 50 %) and the relative concentration of propionic acid also increased. At pH 4.5 the alterations were similar to those at pH 5.5 although less marked, with the decreasing of ethanol and increasing of acetic acid relative concentrations, resulting in an effluent with relative composition of about 48:51 EtOH:HAc. In general, the increasing temperature seems to benefit the VFA production instead of the ethanol production at both pH 4.5 and 5.5. An explanation for the improvement of conversion with temperature can be related with the substrate uptake. The main substrate in CWP is lactose, a disaccharide of glucose and galactose, which has to be hydrolysed before cells can use monosaccharides to obtain energy and reducing power (resulting in VFA and other products) and biomass. The β-galactosidase (3.2.1.23) catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. The activity of β-galactosidases from mesophilic microrganisms (most common in anaerobic reactors) increases with the temperature until a maximum activity is reached at 40 – 50 ºC.[122,123] Consequently, the temperature increase might have increased enzymatic activity, and thus, improving the conversion of sugars to VFA.

By analysing the Figure 17 it can be seen that both HRT and pH have influence in the conversion and DA and in the FP profile. Regarding to the influence of HRT, the increasing in HRT from 8 h to 24 h at both pH 5.5 and 4.5 favoured the conversion of FP. This effect was clearer at pH 4.5. Relatively to FP profile, the increasing in HRT benefited the production of VFA (acetic and propionic acids) instead the production of ethanol, thus increasing the DA. However, at pH 5.5, propionic acid relative concentration was maximum when HRT was 12 h. Therefore, it would be interesting to evaluate if a similar result occurs at pH 4.5 and HRT of 12 h. Regarding the influence of pH in acidification, the reduction of pH led to the decreasing of the FP/SCOD and DA, when comparing results at the same HRT. pH is also commonly reported to influence the profile of FP.[72,73,88,93]

It is important to notice that there was no ethanol production at pH 5.5 and HRT 12 and 24 h, and it was very low at pH 5.5 and HRT 8, so the fermentation products were

exclusively acetic and propionic acids. These profiles are typical of a propionic-type fermentation, the most common type in acidogenic fermentation of organic wastewater at pH 5 to 6.[121] Propionic-type fermentation produces mainly propionic and acetic acids, and some valeric acid, with no significant gas production, which positions the $NADH/NAD^+$ ratio in a normal physiological rage.[121] Thus, propionic-type fermentation is advantageous since propionic acid is not directly subjected to methanogenesis and accumulates easily in the reactor and the reduced gas production might lead to higher VFA yields.[121]

On the other hand, ethanol was the major fermentation product at pH 4.5, HRT 8 h and temperature of 24 and 30 ºC. Acetic acid was also produced, as it always is to maintain a proper $NADH/NAD^+$ ratio, and no significant propionic acid was produced, in a typical ethanol-type fermentation, in opposition to the previous reported propionic-type fermentation.[121] Inconsistently, at pH 4.5 and HRT 24 h, both ethanol and propionic acid production were low, and the major fermentation product was acetic acid, 75 %, but the FP/SCOD increased to 83 %. Also, propionic and acetic acids production seems to benefit from an increasing in temperature and HRT as was stated by Yang et al. 2004.[124]

When pH set-point was lowered to 4.0, although the high HRT of 24 h (previously associated with high conversion rates), the FP/SCOD dropped to 52 %, the relative concentration of ethanol increased to 41 % (around 4 times higher than at pH 4.5 at same HRT) and acetic and propionic acids decreased from 75 % and 14 % to 54 % and 4 %, respectively. Once again, the results indicated that the production of ethanol was favoured at low pH, while the production of propionic acid was severely affected.[121] Also the FP/SCOD was clearly affected when the pH was below optimal.

The results obtained were in agreement with the study of Bengtsson et al. (2008) about the influence of HRT and pH on VFA yield and VFA composition of fermented cheese whey permeate in a chemostat.[73] The authors have found that the DA increased with an increase of HRT at pH 6. At a HRT of 48 h, the DA was maximum at pH 5.25, when acetic and butyric acids production were maximum. Furthermore, when the pH was controlled at 6.0, the DA was insensitive to variation of HRT between 8 and 24 h (the values tested in the present study), however looking to the overall process, DA seems to increase with the HRT, which was also a tendency in the present study. In addition, the acetic and propionic acids profile found in the present study when the HRT was 24 h was in agreement with the profile found by Bengtsson et al (2008).[73]

Another controlled condition was the SRT. In SBR1 (pH 5.5), the SRT was controlled at 4 days from day 19 to day 55, when working at a HRT of 8 h. As it can be seen in Figure 15, the biomass concentration was 1.5 – 2 g COD/L and FP/SCOD was between 50 and 60 % during this period. However, from days 75 to 100, the SRT was increased to 8 days, increasing the biomass concentration to 2.5 – 3.5 g COD/L, and consequently, the volumetric rates and FP/SCOD ratio, even when HRT was reduced from 24 h to 12 h. In SBR2, the pH was lower, first 4.5, and after day 75, 4.0, which affected the biomass yield and overall bioactivity.[125] In this way, the effect of SRT was not clear. Still, when the pH was reduced to 4.0 on day 75, the biomass concentration dropped to 0.4 g COD/L, but slowly increased to almost 1 g COD/L. During this period, the average SRT was 8.6 days but no purge was being done. This might indicate that the MMC selected at pH 4.0 was favoured by a SRT of 8 days.

Considering the results described above, the ability of decouple the HRT and SRT seems extremely advantageous for acidogenic fermentation, since it is possible to select the most adapted and desired MMC, by manipulating the SRT, and, at the same time, select an optimal HRT for consumption of the provided substrate and VFA production. So, the SBR configuration should be increasingly investigated for acidogenic fermentation, since it does not need a settler (while the CSTR does) and avoids recirculation, reducing equipment costs and preventing the stress of high flow rates of recirculation of biomass.

In conclusion, HRT and pH are strongly correlated with DA and FP profile, and it seemed most likely that a great part of the variance from acidogenic fermentation data can only be explained by the interaction between these two factors and others, which makes it very complex to analyse and interpret.

4.2.2. SBR cycle studies

Cycle studies were performed to assess the variation of ethanol and VFA concentrations and consumption of SCOD and NaOH over the length of reaction cycles at different operational conditions. Cycles had the duration of 6 h in both reactors. During cycle studies, samples were collected every hour and more frequently in the beginning of the cycle since the FP production may under some conditions occur at higher rates during that period.

Figure 18 shows the concentrations of SCOD, EtOH, HAc, HPr and FP during the studied SBR cycles. At 6 h the sample was collected from the effluent, since the reaction time ended at 5.5 h followed by 30 min of settling and withdraw. Fermentation parameters as the specific production rates of each product and FP, calculated for the periods when the production was linear (correlation coefficients higher than 0.90), the yield of products on substrate, the percentage of FP on effluent and the DA were calculated and are summarized in Table 10. It is also presented the average biomass concentration during the cycle.

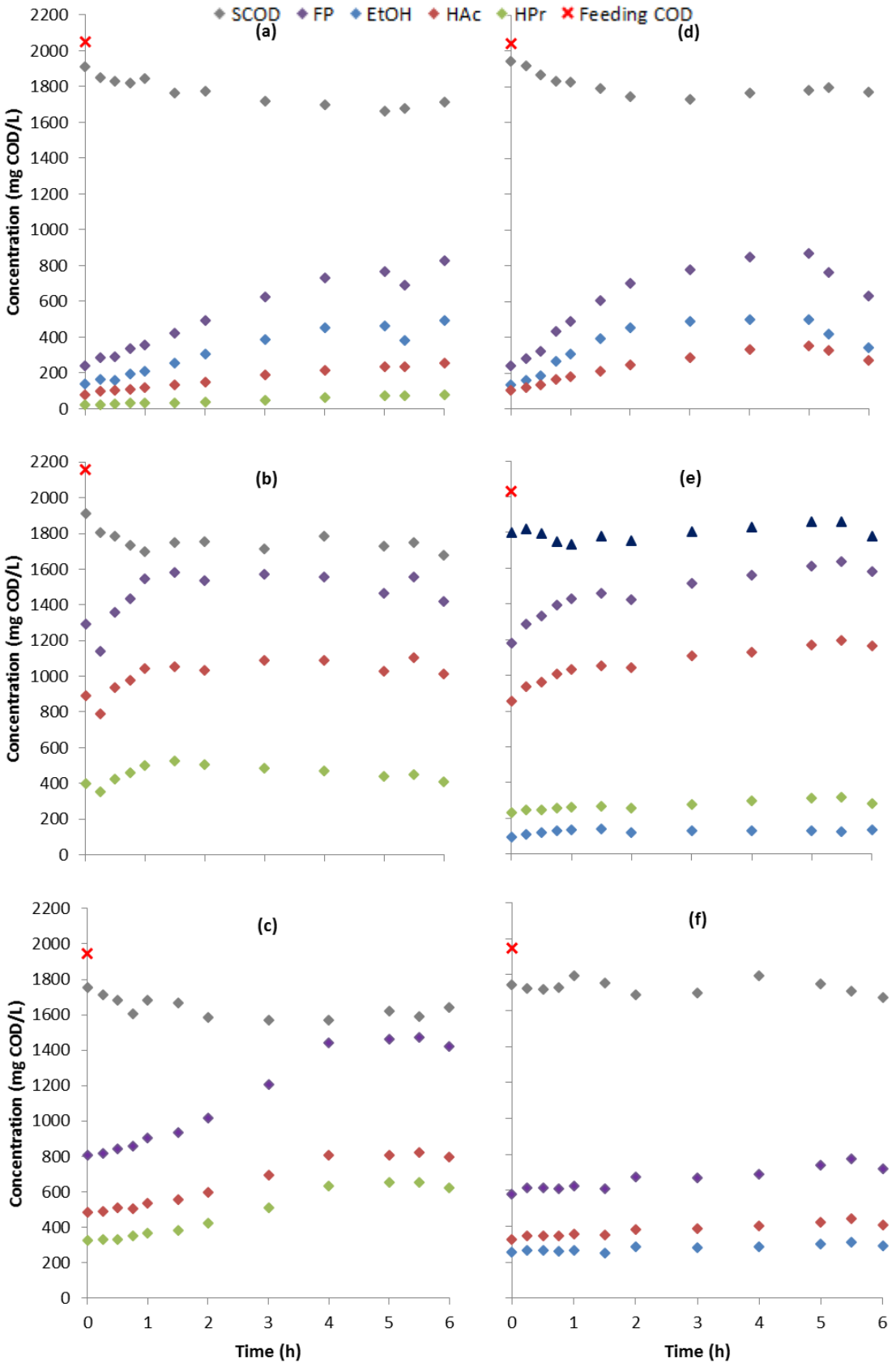

Figure 18: Fermentation products and SCOD concentration over time during the cycle studies performed at different operational conditions.

Table 10: Rates, yields, DA and productivity calculated for the cycle studies performed in SBR1 and SBR2.

	SBR1			SBR2		
Time	*35th day*	*70th day*	*99th day*	*35th day*	*70th day*	*99th day*
Graph	*(a)*	*(b)*	*(c)*	*(d)*	*(e)*	*(f)*
pH	5.5	5.5	5.5	4.5	4.5	4.0
T (ºC)	24	30	30	24	30	30
HRT (h)	8	24	12	8	24	24
$\mathbf{X_{av.}}$ (g COD/L)	1.83	3.34	3.08	2.48	2.19	0.658
$\mathbf{SCOD_{in}}$ (g COD/L)	2.05	2.16	1.95	2.04	2.03	1.95
$\mathbf{SCOD_{out}}$ (g COD/L)	1.68	1.75	1.59	1.78	1.86	1.71
$\mathbf{FP_{out}}$ (g COD/L)	0.766	1.56	1.47	0.872	1.64	0.776
$\mathbf{q_{EtOH}}$ (g COD/(g COD.h))	0.045	0.0	0.0	0.069	0.018	0.014
$\mathbf{q_{HAc}}$ (g COD/(g COD.h))	0.018	0.097	0.026	0.028	0.079	0.028
$\mathbf{q_{HPr}}$ (g COD/(g COD.h))	0.0051	0.058	0.024	0.0	0.013	0.0
$\mathbf{q_{FP}}$ (g COD/(g COD.h))	0.068	0.16	0.051	0.098	0.11	0.046
FP/SCOD (%)	45.6	89.1	92.7	49.0	88.0	45.4
DA (%)	15.0	72.1	75.6	18.1	74.4	23.7
$\mathbf{Y_{FP/SCOD}}$ (g COD/g COD)	0.669	0.791	0.804	0.763	0.905	0.764
Productivity (g COD/(L.h))	1.44	2.83	2.68	1.64	2.98	1.41
$\mathbf{Prod_{max}}$ (g COD/(L.h))	1.70	4.39	1.59	2.58	4.39	1.25
Time of $\mathbf{Prod_{max}}$	15 min	15 min	4 h	45 min	15 min	15 min

In Figure 18 (a) and (d), FP production increased until 5 h of reaction time, reaching 0.766 and 0.872 g COD/L, respectively, corresponding to only 46 and 49 % of SCOD. So, a high amount of substrate was still available at the end of the cycle and it was not converted to target fermentation products. This suggests that acidification could benefit from a longer time of contact between substrate and biomass, which means increasing the HRT. To increase the HRT two options are possible: to increase the duration of the cycle, and consequently reduce the number of cycles per day and the frequency of fresh feed; or to reduce the volume exchange ratio, that was set at 0.75 L/L. The latter option allows maintaining a practical duration of the cycle, reducing the OLR, and at the same time, increasing the residence time of the substrate.

Therefore, the volume exchange ratio was reduced to 0.25 L/L and the HRT increased to 24 h. An immediate consequence was that 75 % of the working volume of the reactor was retained between cycles, thus the initial concentration of FP of the next cycle was higher that it was at volume exchange ratio of 0.75 L/L because the dilution factor is lower. This tendency can be seen in Figure 18 (b) and (e). In the same way, the initial SCOD concentration in each cycle was much lower when the reactors were operated under these conditions. In the cycle study performed at conditions (b), corresponding to SBR1 on the 70th day of fermentation, a FP/SCOD of 91.0 % was reached at 1 h of fermentation and it was stable until the end of reaction time (5.5 h). In Figure 18 (e), corresponding to SBR2 on the 70th day of fermentation, the major production of FP also occurred in the first hour of reaction at a r_{FP} of 0.24 g COD/(L·h), reaching a FP/SCOD of 82.3 %. However, after the first hour the r_{FP} decreased to 0.048 g COD/(L·h), and the concentration of FP/SCOD only increased to 88.0 % at 5.5 h of reaction. Note that in Figure 18 (b) the concentrations of FP, HAc and HPr at time 0 h were higher than at time 0.25 h. This is most likely due to a mixing problem, which means that the sample was collected before a perfect mixing has been achieved after feeding of diluted CWP at time 0 h. For that reason, this sample has been ruled out of calculations. Although Figure 18 (b) and (e) have similar profiles, the difference in the pH may justify the lower q_{HPr} and the production of ethanol (even though low) in SBR2 (e). As it has been discussed, the pH strongly affects the production of propionic acid, which is commonly triggered at pH between lower than 5.5.[120,125]

Since a high percentage of FP on SCOD basis was obtained in a short time at volume exchange ratio of 0.25 L/L, a higher volume exchange ratio of 0.5 L/L was tested in SBR1. The results are presented in Figure 18 (c). FP concentration curve presents the shape of a sigmoid since the volumetric production rate is lower at the beginning of the reaction time, higher in half the reaction time and FP concentration tends to a limit that is close to the SCOD in the end of reaction phase. In fact, FP/SCOD was higher than 90 % from 4 to 5.5 h, without significant alteration.

Relatively to Figure 18 (f), r_{FP} and FP/SCOD at 5.5 h were very low, 0.030 g COD/(L·h) and 45.4 %, respectively. This showed that a reduction of pH below a certain threshold, even at the highest HRT tested, had a drastic consequence in VFA and ethanol production and profile.[88,125]

4.2.3. Evaluation of consumption of NaOH during the fermentation period

Another aspect in focus in this work was the consumption of NaOH for maintenance of the pH set-point. Controlling the pH is very important in the case of using substrates with low buffering capacity, as it is the case of CWP, since the production of acids leads to a decrease of the pH below a fermentation-limiting threshold.

The volume of 1M NaOH solution pumped into the reactor was measured in graduated cylinders to follow the consumption of base in different operational conditions during the full working period of SBR1 and SBR2. The results are presented in Figure 19 in g NaOH per volume exchanged to eliminate the effect of the volume exchange ratio in NaOH consumption, since the higher the volume exchange ratio, the higher the volume of acidic CWP pumped into the reactor, and the higher the amount of NaOH consumed just to raise the pH of the feeding to the set-point. Titration curves from CWP, diluted CWP and fermented CWP (effluents from SBR1 and SBR2 on day 100, pH 4.5 and 4.0, respectively) were obtained and are presented in Appendix C.

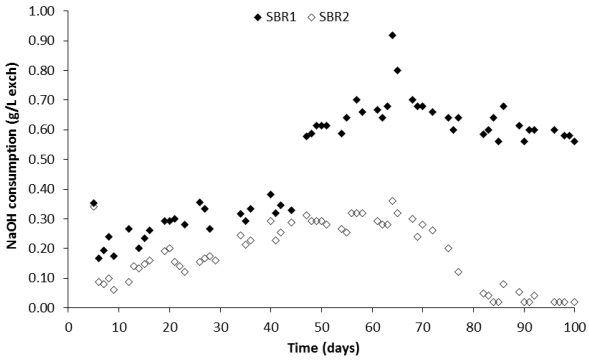

Figure 19: Consumption of NaOH in g per volume exchanged of SBR1 and SBR2.

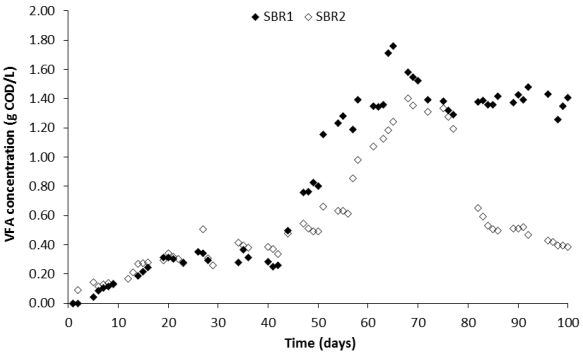

Figure 20: VFA concentration in g COD/L in the effluent of SBR1 and SBR2.

During the first set of tested conditions (24 ºC, HRT 8 h and pH 5.5 and 4.5, respectively), the NaOH consumption increased gradually over time until day 44 in both SBR1 and SBR2. On day 42, temperature was increased to 30 ºC. On day 47, the consumption of NaOH in SBR1 increased to 0.58 g/L (almost the double than on day 44), and it was always between 0.55 and 0.70 g/L until the end of the fermentation period. The NaOH consumption had a similar distribution to the VFA concentration (Figure 20), as it

can be seen above, proving that the increasing of the production of VFA corresponded to an increasing of NaOH consumption.

In SBR2, NaOH consumption did not increased substantially and it was 0.29 ± 0.029 g/L between days 47 and 72. However, during the period between days 56 and 72, the production of VFA increased from 0.61 to 1.4 g COD/L. In the last period, between days 82 and 100, the NaOH consumption was between 0.020 and 0.080 g/L. This major decreasing was due to the reduction of the pH to 4.0, but it was followed by a drop in the production of VFA.

Also, it was calculated the amount of NaOH spent by amount of produced VFA (Figure 21) and by amount of produced FP (Figure 22). The differences between Figure 21 and Figure 22 reflect the concentration of ethanol produced in the fermentation process.

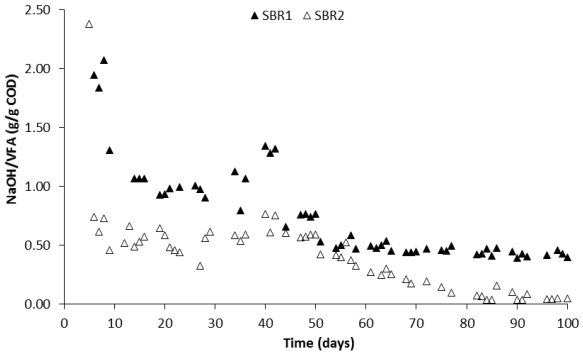

Figure 21: Amount of NaOH spent by amount of produced VFA in SBR1 and SBR2.

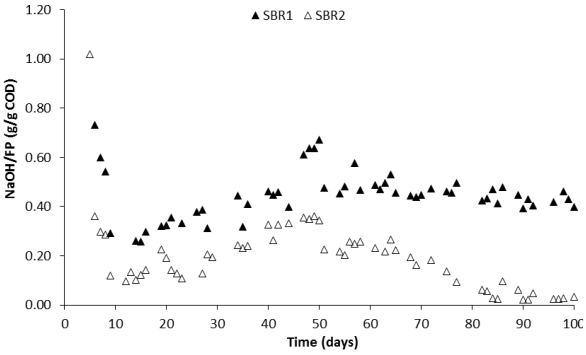

Figure 22: Amount of NaOH spent by amount of produced FP in SBR1 and SBR2.

For SBR1, after an initial adaptation period, NaOH/VFA ratio was stable from day 14 to day 28 and it was 0.99 ± 0.060 g/g COD. From day 34 to day 42, it was verified an increasing in NaOH/VFA resulting from a slightly decreasing in the VFA concentration, and so these values were not considered in the stable period. From day 47 to day 50, after increasing the temperature to 30 ºC, the NaOH/VFA decreased to 0.76 ± 0.010 g/g COD, corresponding to a decreasing of around 25 %. After day 50, the NaOH/VFA was stable until the end of the fermentation period, around 0.46 ± 0.069 g/g COD.

As for SBR2, NaOH/VFA was stable until day 50 and corresponded to around 0.58 ± 0.099 g/g COD. After day 50, the NaOH/VFA ratio started to decrease as a consequence of the increasing in VFA production that occurred until day 75. In the last set of tested

conditions, the pH was reduced to 4.0, and both NaOH consumption and production of VFA dropped, leading to a very low NaOH/VFA ratio (0.073 ± 0.047 g/g COD).

On the other hand, NaOH/FP ratio was much lower than NaOH/VFA, because it included the produced ethanol. Besides, the major difference between the two ratios was that the profiles until day 50 were different: while NaOH/VFA was almost constant in both reactors, NaOH/FP increased gradually, since the concentration of ethanol decreased during that period. After day 50 there was no production of ethanol in SBR1, and very low production in SBR2, so the VFA and FP concentrations were the same, and consequently, NaOH/VFA and NaOH/FP were also approximately the same during this period.

Figure 23 shows the average consumption of NaOH in g/L and the average NaOH/VFA and NaOH/FP in g/g COD during the periods considered as stable. To reduce the costs of the CWP acidogenic fermentation, the main approach in this work was to try to minimize the NaOH consumption and the amount of NaOH added per g COD of VFA. However, it is important not to forget that NaOH consumption and VFA concentration are not independent factors, and so, although NaOH consumption can be lowered, if NaOH/VFA is quite high, it means that the production of VFA in those conditions is rather low, and so those conditions are not optimal for VFA production (Figure 23, pH 5.5 and 4.5 at 24ºC and HRT of 8 h). Thus, a compromise between them should be found.

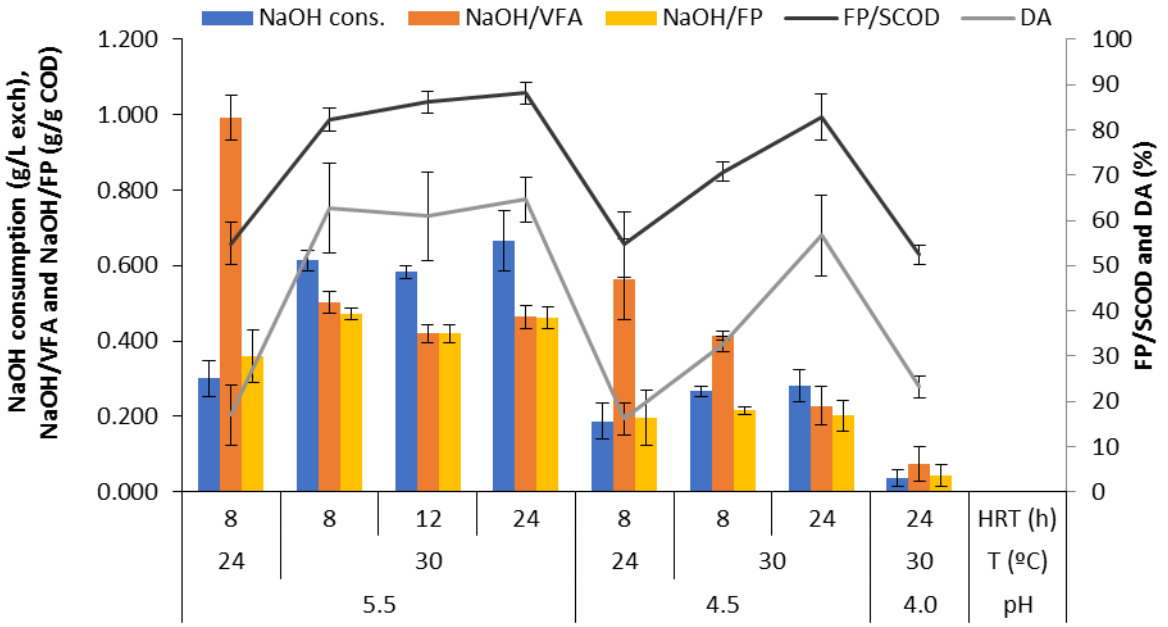

Figure 23: NaOH consumption per volume exchanged, per VFA concentration and per FP concentration, FP/SCOD and DA versus operational conditions tested.

Previously, it was proposed to reduce NaOH consumption by lowering pH. As expected, both NaOH consumption and NaOH/VFA were lower at pH 4.5 than at pH 5.5 when comparing the periods at the same temperature and HRT.

Considering consumption of NaOH at pH 5.5 and 30 ºC, the three HRT tested presented similar NaOH consumption around 0.6 g/L. Also NaOH/VFA and NaOH/FP were very similar between the three HRT (0.4 – 0.5 g/g COD) and to each other. However, at HRT 12 h, the NaOH consumption was slightly lower (0.58 ± 0.018 g/L) and both NaOH/VFAand NaOH/FP were also lower (0.42 ± 0.023 g/g COD), and corresponded to a TP/SCOD of 86 ± 2.4 % and DA of 61 ± 9.7 %. Thus, the best conditions to minimize the NaOH consumption without compromise the VFA production were pH 5.5, HRT 12 h at 30 ºC. At pH 4.5 the effect of the temperature and the HRT in the NaOH consumption was similar to that at pH 5.5: the NaOH consumption increased with the increasing of temperature and HRT and the NaOH/VFA decreased with the increasing of temperature and HRT. Therefore, the best tested conditions to reduce the NaOH consumption during the acidogenic fermentation of CWP with low compromise of FP conversion were pH 4.5, HRT 24 h at 30 ºC, since those conditions corresponded to the second lowest NaOH/VFA of 0.23 ± 0.052 g/g COD and to a FP/SCOD of 83 ± 5.1 % and a DA of 57 ±8.9 % that were still high. In addition, it could be interesting to ascertain the consumption of NaOH at pH 4.5, 30 ºC and HRT of 12 h, since at pH 5.5 those were the best temperature and HRT to spare NaOH.

Furthermore, when the pH decreased to 4.0 the NaOH consumption and the NaOH/VFA ratio decreased substantially to 0.036 ± 0.023 g/L and 0.073 ± 0.047 g/g COD, respectively, considerably lowest than all of the other conditions tested. Also, at pH 4.0 was still possible to perform acidogenesis with FP/SCOD similar to those achieve at 24 ºC (52 ± 2.2 %), a slightly higher DA of 23 ± 2.4 %, and much lower NaOH consumption. In this case, the pH of the feeding solution was higher than the pH set-point, so the NaOH consumption was reduced drastically, while the reduction of NaOH/VFA was due to the dropping of VFA production and the reduction of the addition of NaOH to the reactor. Therefore, must be considered if the economic advantages of performing acidogenic fermentation of CWP at pH much lower that optimal make up for the disadvantages of performing the acidogenic fermentation at pH 4.0, e.g., higher production of by-products as hydrogen and carbon dioxide from the metabolic pathway of ethanol and acetic acid

(main products at pH 4.0), reduction of specific enzymatic activity and overall bioactivity.[121,125]

The effects reported before were also verified during the cycle studies. Figure 24 shows the NaOH consumption in g per L exchanged and Figure 25 shows the NaOH/VFA ratio in g/g COD in different operational conditions tested. The main conclusion to retain was that the major NaOH consumption occurred mostly during the first hour of the cycle, being approximately stable during the rest of the cycle time, which was previously stated to be the period of major VFA production (see Figure 18). Thus, there was a clear correlation between NaOH consumption and VFA production during the cycle, since the profiles were very similar. Based on Figure 24, the conditions that implied a greater NaOH consumption were, in descending order, (b), (c), (a), (d), (e) and (f) (see conditions description in Table 10). However, when considering the amount of NaOH spent per amount of VFA, the conditions that implied a greater NaOH consumption were slightly different: (a), (d), (b), (c), (e) and (f), in descending order (Figure 25). So, the worst conditions to save NaOH were pH 5.5 and 4.5, HRT 8 h at 24ºC and the best conditions to save NaOH were pH of 4.5, HRT 24 h at 30 ºC, when excluding the period at pH 4.0 due to reduced VFA production.

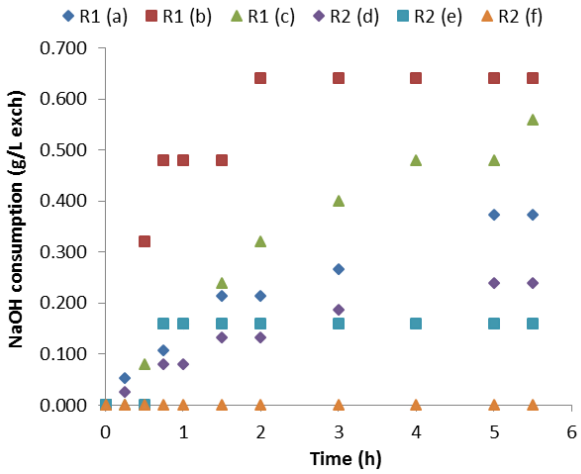

Figure 24: NaOH consumption in g per L exchanged during the full length of the cycle time.

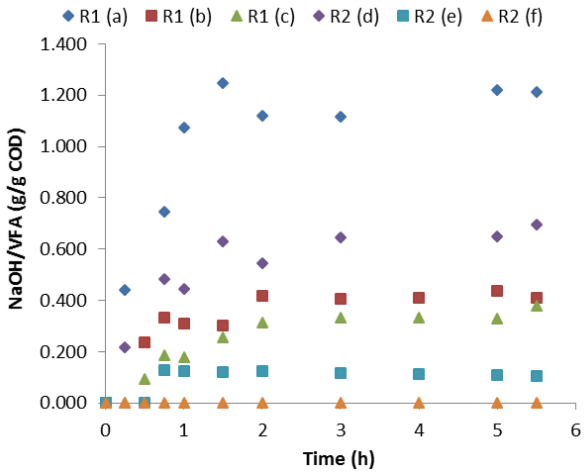

Figure 25: NaOH consumption in g per g COD of VFA during the full length of the cycle time.

4.2.4. Assessment of biomass settling properties and morphological characteristics

To assess the biomass quality, the SSV and SVI were measured from day 23 to the end of the fermentation time around 2 times per week. One of the reasons to start measuring the SVI was the instability of the biomass in SBR1 after day 23 as it can be seen in Figure 15. From day 26 to day 40, the SVI in SBR1 was above 200 mL/g, which indicated that the sludge settled very slowly and compacted poorly due to light and not dense flocs. These characteristics are not desired in a SBR where the whole operation depends on the settling properties of the sludge. In addition, sludge that settles slowly requires a longer settling phase, and consequently, a shorter reaction phase. However, it is advantageous to maximize the length of the reaction phase of the SBR cycle. Then, after day 49, the SVI decreased to lower and more suitable values, and it was stable and under 60 mL/g until the end of the fermentation period.

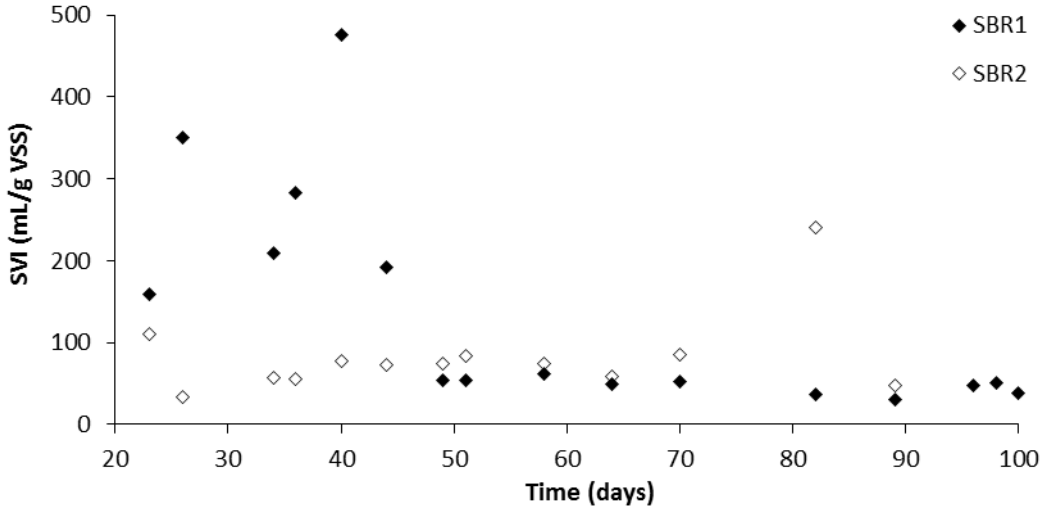

Figure 26: SVI calculated during the fermentation period for SBR1 and SBR2.

The sludge was observed by phase contrast (PC) or differential interference contrast (DIC) microscopy after the instability period on the 54th day (Figure 27) and at the end of the fermentation period (100th day) (Figure 28). On day 54, the flocs were moderately abundant, had an irregular shape and medium to high compactness (Figure 27 (a) and (b)). They were big and dense, mostly between 300-500 µm with some flocs larger than 500 µm. The sludge presented rapid settling characteristics so it was possible to obtain a fairly

clear, high-quality effluent. On day 100, the flocs presented similar morphologic characteristics to those observed on day 54, except for the size, since on day 100 were observed much bigger flocs. Figure 28 (a) shows an open floc with 1mm of diameter with a smaller floc by its side. Despite the presence of very large flocs, visible with the naked eye, there was a great dispersion of floc size. The filament index was 2 (filaments commonly observed, but not present in all flocs) on day 54, and 1 (filaments present, but only observed in an occasional floc) on day 100. Filaments were exclusively inside the flocs, but do not open them. Instead, filaments were connecting different flocs (Figure 27 (b, d) and Figure 28 (b, c)). In small amounts, filamentous bacteria form a backbone or rigid support that strengthens the floc structure. This allows the floc particles to increase in size, helping to reduce the amount of pinpoint flocs and improving the settling of sludge, since larger flocs are more readily settled, and producing an even higher quality effluent.[126] It was possible to observe free-living bacteria in moderate abundance in both days (Figure 27 (c) and Figure 28 (b)), which influenced the turbidity of the effluent. The main reason of a high amount of free-living bacteria was a lack of filter-feeding micro animals due to oxygen deficiency.

Relatively to SBR2, the SVI was under 100 mL/g from day 26 to day 70, with good settling properties. On day 54 the sludge presented quite abundant flocs with an irregular shape and medium compactness and size between 300 and 500 µm (Figure 27 (d)). The filament index was 1, and the filaments observed had the same characteristics as in SBR1: they were inside compact flocs (without opening up them) and also connecting different flocs (Figure 27 (d)). Free-living bacteria were moderately abundant. At this point, the biomass in SBR2 was very similar to biomass in SBR1, but slightly smaller and less compact. One of the explanations was the pH, since it was the only difference between the reactors on day 54. The low pH is a stress-inducing condition, so it can affect the ability of biomass to form flocs. On day 76, the pH was changed to 4.0 which shocked the biomass. On the 77th day was impossible to measure the SSV because no interface between settled biomass and clear effluent could be seen. As a consequence, the concentration of biomass in the effluent increased and the concentration of biomass inside the reactor decreased to only 0.29 g VSS/L on day 82, while SVI was 241 mL/g. The same happened from day 96 to day 100. When observed on day 100, the biomass in SBR2 presented poor floc formation, with mainly pinpoint flocs (less than 50 µm) and small flocs (50-150 µm)

(Figure 28 (d) and (e)) with irregular shape and medium compactness. This observation explained the high turbidity of the effluent during this period and the absence of settled biomass during the measurement of SSV. It was not observed filamentous bacteria, whose absence was associated with the occurrence of pinpoint flocs. Free-living bacteria abundance was low to moderate (Figure 28 (f)).

Summarizing, there was loss of settling properties during adaptation periods, and when observing directly the biomass, its settling properties could be related to its morphological characteristics. Also, the settling properties of biomass clearly affected the concentration of produced VFA, since less biomass produced less acids. Thus, the settling properties and characteristics of biomass should be tightly monitored and controlled during a fermentation process.

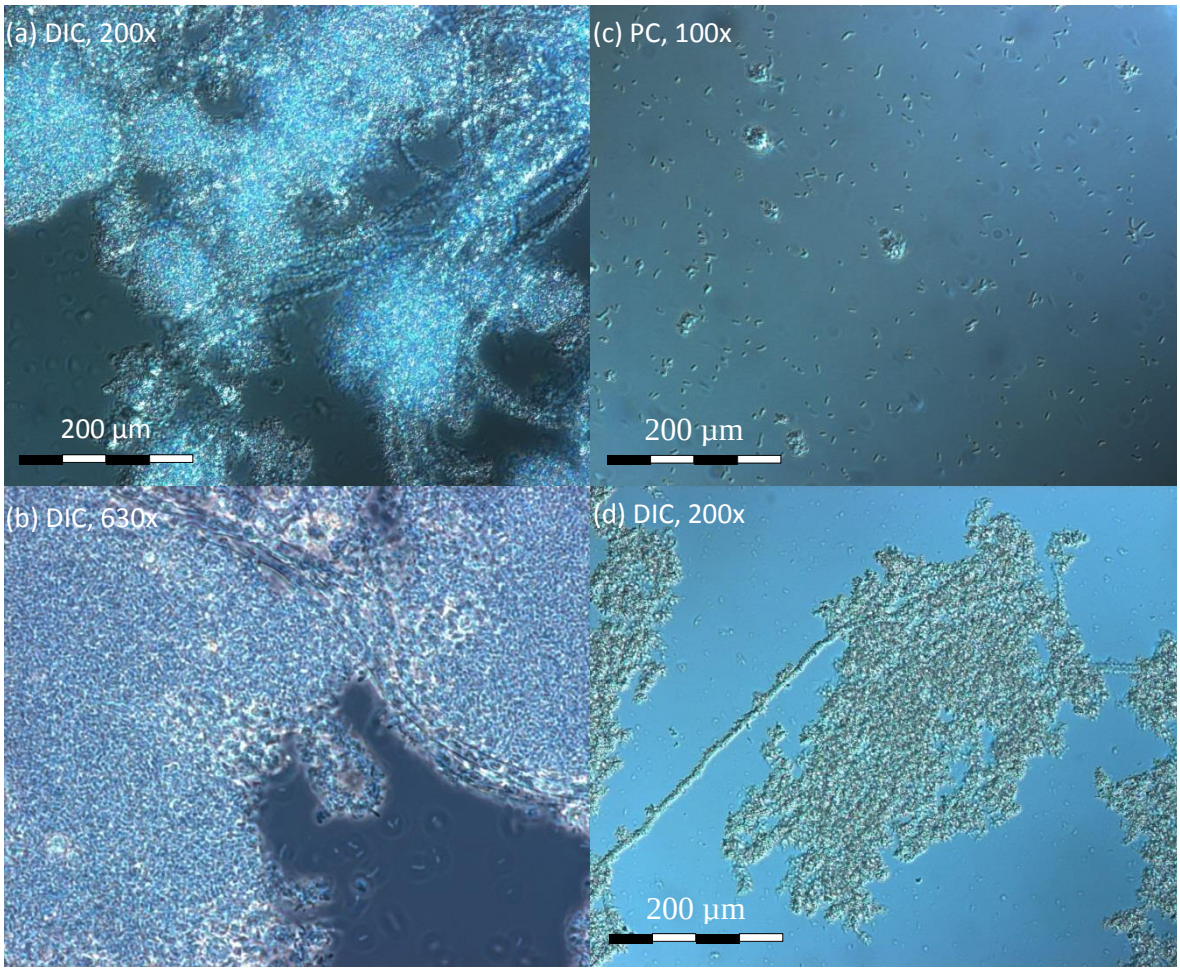

Figure 27: Microscopy images of the biomass in SBR1 (a, b, c) and SBR2 (d) taken on the 54th day of fermentation period.

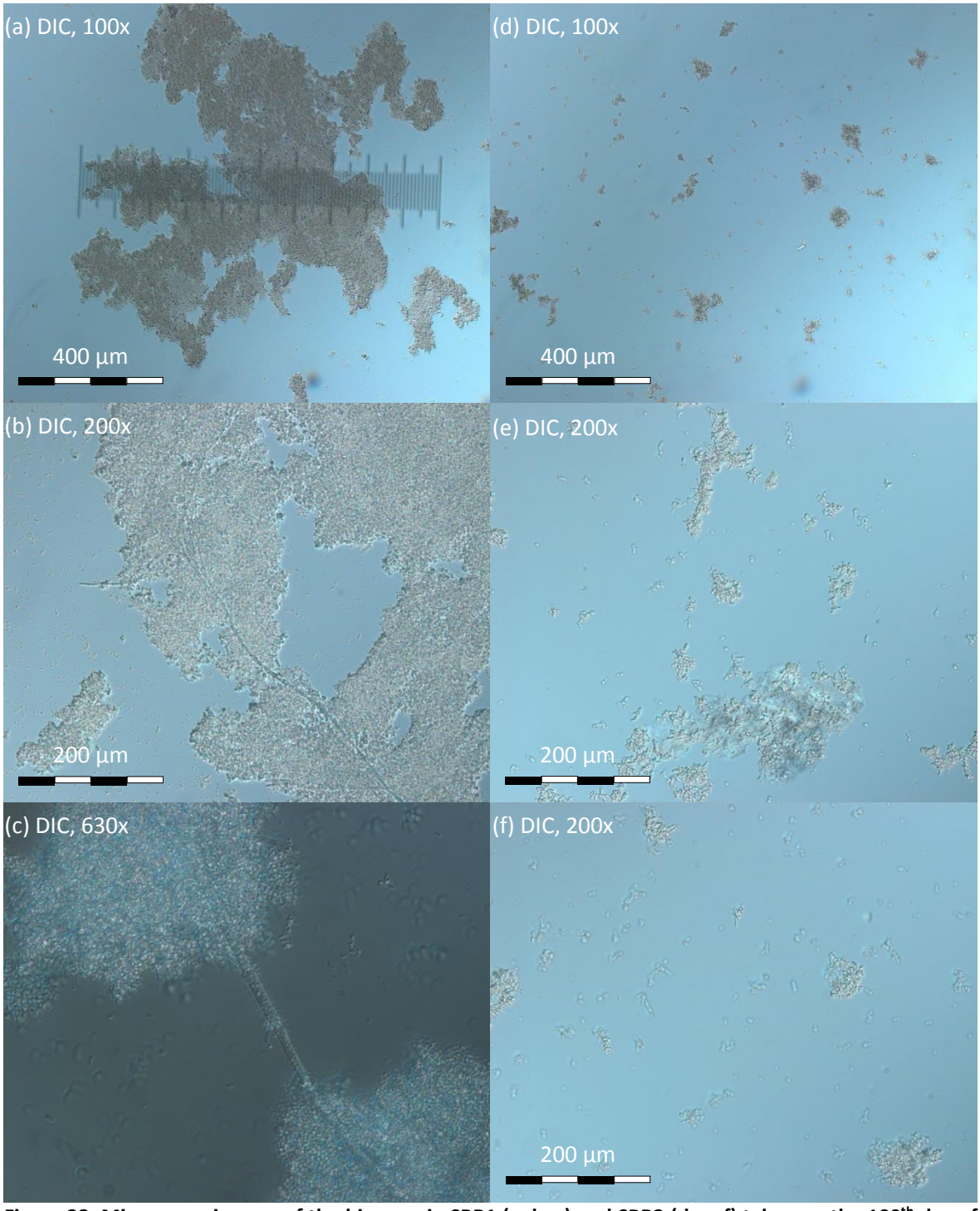

Figure 28: Microscopy images of the biomass in SBR1 (a, b, c) and SBR2 (d, e, f) taken on the 100th day of fermentation period.

5. Final remarks

The objective of this work was to study the acidogenic fermentation of industrial by-products for the production of VFA. Industrial wastewaters and by-products with no commercial interested are usually discarded and have an associated cost of treatment. Thus, the valorisation of these streams could be a solution for both environmental and economic problems. VFA are valuable products with a broad range of applications, mostly for energy production (biogas, electricity) and intermediates for chemicals and substrates for PHA production. The focus of this work was the acidification of HSSL, a by-product of pulp and paper industry, in a CSTR, and cheese whey permeate in a SBR to obtain VFA for PHA production.

The main products of HSSL acidogenic fermentation were propionic, butyric and acetic acids. Nevertheless, the production of propionic and butyric acids suffered several productions shifts and their relative proportion was not stable. Thus, the operational conditions seemed to select different populations and metabolic pathways, for propionic or butyric acids production. However, the highest DA and conversion obtained were quite low, around 10 %, when the reactor was operated at HRT of 2 days, OLR of 7.7 g COD/L and temperature of 30 ºC. During this period the sugars consumption was 68 ± 1.0 %. The consumption of sugars, specially xylose, and other carbon sources present in HSSL should be further investigated in order to achieve full consumption of the sugars content, improve the percentage of COD readily available by hydrolysis of LS and increase the conversion on VFA. Since HSSL is a very complex carbon source, increasing the HRT at the same time as increasing the biomass concentration in the reactor by controlling the SRT (decouple the SRT from the HRT) could contribute to achieve full fermentation. The OLR tested was in the range stated by the literature as optimal for acidogenic fermentation of complex real substrates. In the case of HSSL, increasing the OLR can cause loss of microbial activity due to high concentrations of inhibitory compounds (e.g. phenolics) in HSSL.

The SBR system allowed studying the effect of pH, HRT, SRT on the acidogenic fermentation of CWP. The fermentation products obtained were ethanol, acetic and propionic acids. No butyric acid was produced. The optimal value of pH for VFA production was 5.5 (FP/SCOD higher than 80 % and DA higher than 60 % for at all HRT

tested). The reduction of pH to 4.5, and then to 4.0, resulted in a decrease of FP/SCOD and DA. The increasing of HRT was beneficial within the range tested at pH 5.5 and 4.5. Both HRT and pH had an effect in the FP profile: pH 5.5 was associated with propionic-type fermentation, while pH below 5.5 was associated with ethanol-type fermentation, while an increase in HRT led to the increase of acetic acid concentration. Also, a higher and controlled SRT could help to increase the biomass concentration and select a population for VFA production, increasing the volumetric rates, and consequently the VFA production, especially at low pH. NaOH consumption was also evaluated during CWP fermentation with the purpose of reducing the amount of NaOH spent, and consequently, reducing costs. The best tested conditions to reduce the NaOH consumption with low compromise of FP conversion were pH 4.5, HRT 24 h at 30 ºC, since those conditions corresponded to the second lowest NaOH/VFA of 0.23 ± 0.052 g/g COD, a FP/SCOD of 83 ± 5.1 % and a DA of 57 ± 8.9 %. Further reduction of the pH resulted in a drastic reduction of NaOH consumption and NaOH/VFA ratio, but the FP/SCOD was much lower that at pH 4.5 (only 52 ± 2.2 %). Thus, a compromise should be found.

SBR configuration was proven to be suitable for acidogenic fermentation of CWP, but the success of this strategy depends on the settling properties of biomass. During the fermentation period there was loss of settling properties during adaptation periods, and it was clear that the settling properties of biomass were related to its morphological characteristics.

This work reinforces the general idea that operational conditions, namely pH, HRT, SRT, OLR, etc, play an enormous role in the conversion efficiency, DA and FP profile. Since the acidogenic fermentation of complex real wastewaters and by-products with MMC is a very complex process, it is difficult to assess the individual contribution and interactions between different environment and intrinsic factors. Regarding the previously stated, strategies as the response surface methodology and other factorial analysis could be interesting to evaluate the optimal conditions for acidogenic fermentation of a certain substrate. In the case of real wastewaters and by-products, as for example HSSL, their composition has a great influence in the fermentation, either by the type of SCOD or its availability for ready fermentation, or by the presence of toxic compounds that can inhibit acidogens. Therefore, the construction of a universal model for acidogenic fermentation is still in construction and further investigation is needed on this subject.

6. Future prospects

Regarding the acidogenic fermentation processes tested in this research work, further research is needed to understand and improve the conversion of the carbon substrates from complex wastewaters and industrial by-products to VFA by MMC.

Respecting to HSSL, the consumption of sugars and other carbon compounds (e.g. lignosulphonates) present should be investigated in detail, since they can be hydrolysed and consumed by the microorganisms under certain circumstances. Also, the inhibitory compounds (e.g. phenolics) of the HSSL should be characterized and its effect in the biomass activity should be evaluated.

Since the decreasing of biomass concentration was a problem verified along the CSTR operation period, it would be interesting to try to retain the biomass in the system using a settler, or to try a different configuration, for example a SBR, since it was proven in this work with CWP that this configuration can be used for acidogenic fermentation.

In order to achieve full conversion, it should be determined the optimal range of operational conditions, such as, substrate composition, pH, temperature, HRT, SRT, biomass concentration, OLR, that are not yet described for acidogenic fermentation of HSSL. In addition, longer fermentation periods should be considered to obtain well-selected MMC and to determine its behaviour on a long-term fermentation.

Regarding to the SBR, it achieved good results at pH 5.5, the optimal pH for acidogenic fermentation of CWP. pH 4.5 and 4.0 could be further investigated to increase the FP production at these pHs, resulting in better NaOH/FP ratios and greater reduction costs. Also, protocols for improving the settling properties of biomass should be investigated.

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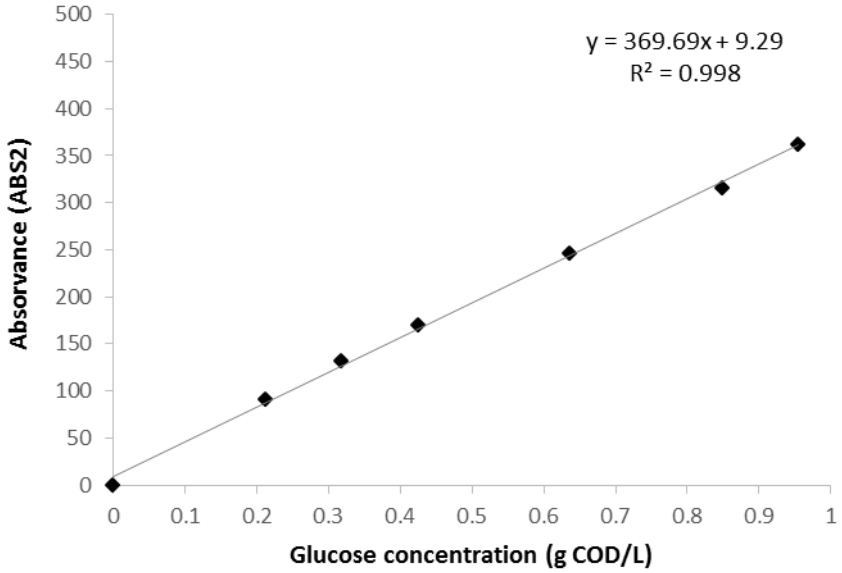
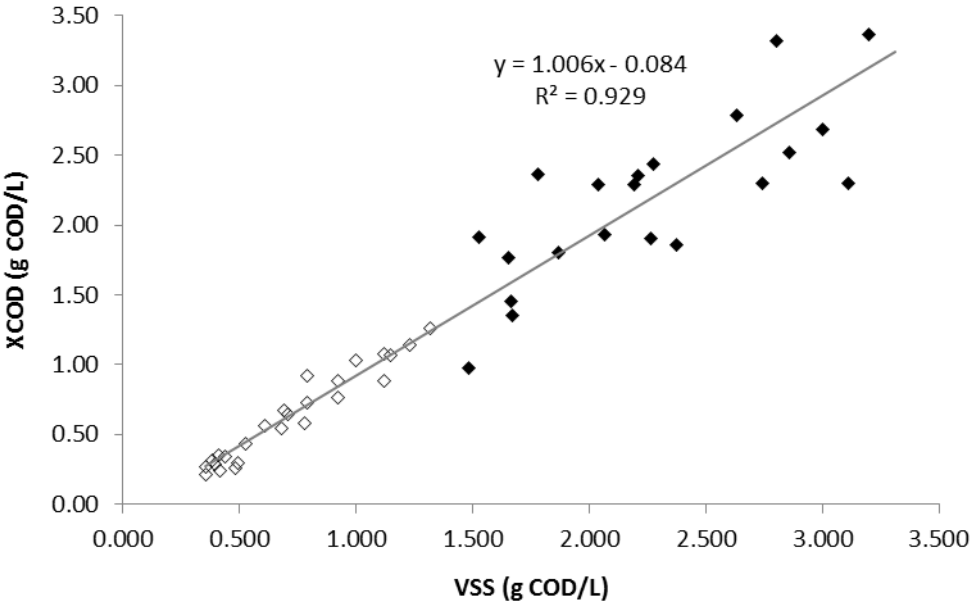
8. Appendices

8.1. Appendix A: Glucose standard for COD quantification



8.2. Appendix B: Correlation between biomass quantification by VSS and XCOD calculation



8.3. Appendix C: Titration curves from CWP, diluted CWP and fermented CWP