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Programa de doctorat d'Enginyeria i Ciències Aplicades

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El Dr. JOAN MATA ÁLVAREZ, Catedràtic del Departament d'Enginyeria Química i Química Analítica de la Universitat de Barcelona i el Dr. JOAN DOSTA PARRAS, professor agregat del mateix departament:

CERTIFIQUEN QUE:

El treball de recerca titulat "FOOD WASTE AND WASTE ACTIVATED SLUDGE CONVERSION INTO VOLATILE FATTY ACIDS TO PRODUCE BIOPLASTICS" constitueix la memòria que presenta l'ambientòloga Carme Vidal Antich per a aspirar al grau de Doctor per la Universitat de Barcelona. Aquesta tesi doctoral ha estat realitzada dins del programa de doctorat d'Enginyeria i Ciències Aplicades, en el Departament d'Enginyeria Química i Química Analítica de la Universitat de Barcelona.

I per a que així consti als efectes oportuns, signen el present certificat a Barcelona, 21 de febrer de 2022.

Dr. Joan Mata Álvarez	i	**Dr. Joan Dosta Parras**
Director de la tesi doctoral		*Director i tutor de la tesi doctoral*

Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.

Marie Curie

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Contents

Abstract

Rapid population growth is leading to many environmental problems, among which a large waste generation should be highlighted. It is estimated that more than 40% of these wastes correspond to organic wastes that are mainly treated by composting, anaerobic digestion, incineration, or even landfilled. The European Union has updated its Bioeconomy Strategy aiming to manage the natural resources in a sustainable way and reduce the non-renewable resource dependence through the implementation of new treatment strategies within the circular economy concept. Therefore, organic waste treatment plants should be transformed into biorefineries, where acidogenic fermentation emerges as a key technology to valorise these substrates, since it could produce high value-added products and/or platform chemicals, such as volatile fatty acids (VFAs), that could be further transformed into bioplastics (i.e., polyhydroxyalkanoates or PHA).

Food waste (FW) and waste activated sludge (WAS) are one of the most abundant organic wastes generated in urban areas and are usually treated in a separate way, although their joint treatment could lead to some benefits and synergies. Hence, in this thesis, the acidogenic fermentation and co-fermentation of WAS and FW are studied to achieve a VFA-rich effluent for its subsequent conversion to PHA.

Firstly, the effect of pH on the acidogenic fermentation of FW was studied in batch mode (with pH range from 4 to 11) and in semi-continuous mesophilic fermenters working under acidic and alkaline pH values (near 6 and 9.5-10, respectively). Batch fermentation tests revealed that pH near neutrality or slightly alkaline (8- 9) could lead to a higher VFAs production, with a higher acetic acid content when pH increased within this range. In the semi-continuous fermenter working at alkaline conditions (pH 9.5-10), enhanced solubilization of organic matter was registered with respect to the fermenter working under acidic conditions. In the semi-continuous fermenters, a maximum VFA yield of 503.1 mgCOD/gVS was obtained when working at pH 6 with a VFA profile dominated by acetic, butyric and caproic acids, while at pH 10, a lower yield (315.1 mgCOD/gVS) was obtained, regardless of the improved hydrolysis of FW, with acetic as the main acid.

In order to avoid the consumption of chemical reagents to control the working pH in semi-continuous fermenters, WAS and FW co-fermentation was explored (50%, 70% and 90% WAS on VS basis), demonstrating the benefits to co-ferment these two wastes, since a higher fermentation yield was always obtained in WAS/FW co-fermentation assays compared to mono-fermentation of WAS and FW. In all the mixtures tested, the buffer capacity of WAS was enough to maintain the pH above inhibitory levels without reagents addition. Moreover, when the proportion of FW in the WAS/FW was raised, changes in the fermentation yield and profile were observed. Interestingly, when more proportion of FW was added, more butyric and less propionic acid was produced achieving a maximum fermentation yield of 480 mgCOD/gVS when the mixture was 50%FW+50%WAS (on VS basis). Since the effect of each FW fraction has been barely studied, discontinuous assays using diverse FW fractions were also performed and the principal components analysis (PCA) revealed the relation between each fraction and each fermentation profile. Furthermore, these assays were important to understand the importance of balancing the protein content into the organic matter mixture in order to achieve a maximum VFA yield of about 500 mgCOD/gVS.

Hence, WAS and FW co-fermentation was studied under long-term conditions in semi-continuous fermenters at several organic loading rates (OLR) by increasing the FW influent flowrate (from 0 to 10.86 gVS/(L·d)) while maintaining the WAS feeding (7.14 gVS/(L·d). This study demonstrated the importance of microorganisms' immigration with the feed substrates and their adaptation in semi-continuous processes. In this way, the stages carried out at OLR 9 and 11 gVS/(L·d) obtained lower VFA yields probably due to the methanogenic archaea activity which was favoured at the circumneutral pH obtained. However, when higher OLR were applied (14 and 18 gVS/(L·d)), the pH started to decrease with a concomitant increase on the observed yield until achieving a maximum VFA yield of 475 mgCOD/gVS, with butyric as the main acid. Moreover, this study was important to demonstrate that not only the FW properties and its proportion in the WAS/FW mixture affect the obtained VFA yield and its VFA distribution, but also the WAS characteristics are important, especially when the pH is low, and the buffering capacity of the WAS could avoid a sudden drop in the working pH values.

Finally, the start-up and operation of a sequencing batch reactor (SBR) to select PHA-storing microorganisms was performed using a VFA-rich synthetic feeding with an OLR of 2.0 and 2.8 gCOD/(L·d). This biomass selection was carried out with a double growth limitation strategy (feast/famine and uncoupled carbon and nitrogen feeding). The successful selection of PHA-storing biomass was confirmed in batch accumulations assays, where sludge purge from the selection SBR was treated under aerobic conditions with a VFAs pulse feeding strategy, and a PHA content between 44 and 46% (on suspended solids basis) was reached, being polyhydroxybutyrate (PHB) the main component of the produced bioplastic.

Resumen

El gran crecimiento de la población mundial está comportando muchos problemas medioambientales entre los cuales cabe destacar la gran generación de residuos. Actualmente se estima que más de un 40% de éstos corresponden a los residuos orgánicos y son tratados mediante compostaje, digestión anaerobia, incineración o incluso son depositados en vertederos. La Unión Europea ha actualizado su Estrategia de Bioeconomía con el objetivo de gestionar los recursos naturales de manera sostenible y reducir la dependencia de los recursos no renovables. Para ello, se pretenden implantar nuevas estrategias dentro del concepto de economía circular trasformando las plantas de tratamiento de residuos en biorrefinerías. En este sentido, la fermentación acidogénica emerge como una tecnología clave para valorizar estos sustratos en forma de productos de alto valor añadido como son los ácidos grasos volátiles (AGVs). Éstos, pueden ser finalmente transformados en bioplásticos (polihidroxialcanoatos o PHA), entre otras muchas aplicaciones.

Los residuos alimentarios y los fangos activos de depuradora son algunos de los residuos orgánicos más abundantes en las áreas urbanas y suelen ser tratados por separado. Aun así, se debería contemplar su tratamiento conjunto ya que podrían suponer la obtención de sinergias beneficiosas. Así pues, en la presente tesis se estudia la fermentación acidogénica de estos residuos por separado, así como su co-fermentación, con el fin de conseguir un efluente rico en AGVs para su posterior conversión en forma de PHA.

Inicialmente se llevó a cabo el estudio del efecto del pH en la fermentación acidogénica bajo condiciones mesofílicas de residuos alimentarios de manera discontinua (con un valor de pH entre 4 y 11), y semi-continua (a pH alrededor de 6 y de 9,5-10,0). Los resultados de los ensayos en discontinuo mostraron una mayor producción de AGVs en el pH cercano a la neutralidad o ligeramente alcalino (8-9) con un mayor contenido de ácido acético al incrementarse el pH dentro de este rango. Por otra parte, los ensayos semi-continuos en condiciones alcalinas (pH 9,5-10,0) consiguieron una mejora de la solubilización de la materia orgánica respecto al reactor que trabajaba en condiciones ácidas. Aun así, la máxima producción de AGVs (503,1 mgDQO/gSV) se obtuvo en el reactor que trabajaba a pH 6, con un perfil de AGVs dominado por acético, butírico y

caproico, mientras que a pH 10 se obtuvo una menor producción (315,1 mgDQO/gSV), independientemente de la mejora en la hidrólisis, con el ácido acético como principal AGV.

También se llevó a cabo el estudio de la co-fermentación de los residuos alimentarios y fangos activos (50%, 70% y 90% del fango activo en base SV) con el fin de evitar el consumo de los productos químicos para controlar el pH de los reactores a lo largo del proceso de fermentación acidogénica. En este sentido, para las mezclas estudiadas se demostró que la capacidad tampón del fango activo era suficiente para sostener el pH por encima de niveles inhibitorios sin tener que añadir reactivos al proceso. Asimismo, se observaron importantes cambios en el perfil y en la producción de AGVs cuando se incrementó la cantidad de residuos alimentarios en la mezcla. Cuanta más proporción de residuo alimentario se añadía, más ácido butírico y menos ácido propiónico se producían. Así pues, se obtuvo una máxima producción de 480 mgDQO/gSV en la mezcla 50/50 (%, en base SV). Aun así, el efecto de cada fracción de los residuos alimentarios no está demostrado a día de hoy de modo que se realizaron ensayos en discontinuo usando diferentes fracciones de los residuos alimentarios y se demostró, mediante el análisis de componentes principales, la relación entre cada fracción y la producción de cada tipo de ácido. Además, estos ensayos permitieron entender la importancia de equilibrar el contenido de proteínas en la mezcla de materia orgánica para obtener una máxima producción de AGVs que llegó hasta, aproximadamente, 500 mgDQO/gSV.

Seguidamente, se estudió la co-fermentación del fango activo y el residuo alimentario en modo semi-continuo mediante el incremento de la carga orgánica aumentando la tasa de flujo del residuo alimentario (desde 0 hasta 10,86 gSV/(L·d)) y manteniendo la del fango activo (7,14 gSV/(L·d)). Este estudio fue muy significativo para demostrar la importancia de la inmigración de los microorganismos con el sustrato usado y la adaptación de éstos en el proceso semi-continuo en comparación al proceso discontinuo. En ese sentido, se obtuvo una baja producción de AGVs en las cargas orgánicas de 9 y 11 gSV/(L·d) probablemente debido a la actividad de las arqueas metanogénicas favorecidas por un pH cercano a la neutralidad. Seguidamente, el pH empezó a descender cuando se aplicó una mayor carga orgánica (14 y 18 gSV/(L·d)) incrementando la producción de AGVs hasta llegar a 475 mgDQO/gSV con el ácido

butírico como principal componente. Además, este estudio también fue relevante para demostrar que no sólo las propiedades y la proporción de los residuos alimentarios en la mezcla de co-fermentación son importantes, sino que también lo son las características del fango activo cuando el pH es bajo y se requiere de la capacidad tampón suministrada por el fango para evitar una bajada repentina en los valores de pH.

Finalmente, se llevó a cabo la puesta en marcha y operación de un reactor secuencial por cargas para seleccionar microorganismos con alta capacidad de almacenaje de PHA. Para ello, se utilizó un alimento sintético rico en AGVs y una carga orgánica variable entre 2,0 y 2,8 gCOD/(L·d). Así pues, se utilizó una doble estrategia de limitación del crecimiento (saciedad/hambruna y alimentación por separado de la fuente de carbono y de nitrógeno) para seleccionar esta biomasa. Los posteriores ensayos de acumulación permitieron confirmar la buena selección de los microrganismos ya que poseían una buena capacidad de almacenaje de PHA obtenidos en el proceso anterior. En concreto, estos microorganismos llegaron a obtener un contenido de PHA entre el 44 y 46% de los sólidos suspendidos siendo el polihidroxibutirato (PHB) su principal componente.

Nomenclature

The used abbreviations, acronyms and symbols are gathered below:

ABS	Absorbance
AD	Anaerobic digestion
ADF	Anaerobic dynamic feeding
Alk_a	Acid alkalinity
Alk_P	Partial alkalinity
Alk_T	Total alkalinity
A/O	Anaerobic/aerobic
BNR	Biological nutrient recovery
COD	Chemical oxygen demand
DF	Dark fermentation
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
EPS	Extracelullar polymeric substances
ES	Excess Sludge
EU	European Union
F/F	Feast and famine
FID	Flame ionised detector
FW	Food waste
GC	Gas chromatography
GHG	Greenhouse gas
HA	Hydroxyalkanoate
HAc	Acetic acid
HB	Hydroxybutyrate
HBu	Butyric acid
HCa	Caproic acid
HHep	Heptanoic acid
HPLC	High-performance liquid chromatography
HPr	Propionic acid

HRT	Hydraulic retention time
HV	Hydroxyvalerate
HVa	Valeric acid
IC	Ionic chromatograph
MBTP	Mechanical biological treatment plant
MBR	Membrane bioreactor
MC	Moisture content
MCFA	Medium-chain fatty acids
MCL	Medium chain lengths
MMC	Mixed microbial cultures
MSW	Municipal solid waste
NLR	Nitrogen loading ratio
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
PAO	Polyphosphate-accumulating organisms
PCA	Principal component analysis
PDLA	Poly(D-lactide)
PDLLA	Poly(DL-lactide)
PF	Photofermentation
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHBV	Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHV	Polyhydroxyvalerate
PLA	Polylactate
PLLA	Poly(L-lactide)
PMC	Pure microbial cultures
PNSB	Purple non-sulphur bacteria
PS	Primary sludge
RRF	Resource recovery facility
SBR	Sequencing batch reactor
SCL	Short chain length
sCOD	Soluble chemical oxygen demand
SDGs	Sustainable development goals

SS	Suspended solid
SRT	Sludge retention time
TAN	Total ammonium nitrogen
TKN	Total kjeldahl nitrogen
TRL	Technological readiness level
TS	Total solids
TSS	Total suspended solids
VFAs	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WWTP	Wastewater treatment plant
XOH	Alcohols

1. Introduction

1.1. World's waste generation

The world population is growing annually achieving 7.9 billion people in 2020 and it is estimated to keep growing reaching 10.9 billion in 2100 (Roser et al., 2013). This population growth with the fast economic and technological development are leading to the generation of huge amounts of wastes. This waste generation is massively increased around the world in recent decades without signs of slowing down. Furthermore, this production directly affects health, environmental, and socio-economic conditions, as well as contributes to climate change (Mor et al., 2006; Uddin et al., 2017). Specifically, in 2016, the annual world generation of municipal solid waste (MSW) was 2.01 billion tonnes and it is expected to increase by 70% achieving the generation of 3.40 billion tonnes in 2050 (Kaza et al., 2018).

Almost 225 million tonnes of MSW were generated in Europe corresponding to an average of 502 kg of MSW per inhabitant in 2019 with a specific generation of 476 kg of MSW per inhabitant in Spain (Eurostat, 2021). The municipal wastes were treated as follows: (i) 30.9 % was recycled, (ii) 26.6 % was incinerated with energy recovery, (iii), 24.3 % was landfilled, (iv) 17.7 % was composted or anaerobically digested, and (v) 0.5 % was incinerated without energy recovery (see Figure 1.1). Henceforth, nowadays, approximately a quarter of the MSW was not treated in an environmental way.

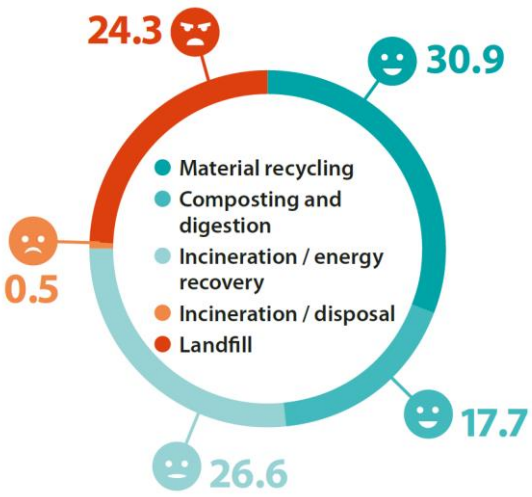

Figure 1.1. Municipal waste treatment methods used in Europe in 2019 (Eurostat, 2021).

The use of each treatment depends, basically, on the waste composition. In Spain, the main composition is: (i) organic fraction (43 %), paper and cardboard (19 %), plastic (14 %), glass (7 %) and other wastes (18 %) (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2012) (see Figure 1.2). Although this composition could vary depending on the geographic region and their social condition, MSW is mainly composed by organic fraction and it needs to be correctly treated.

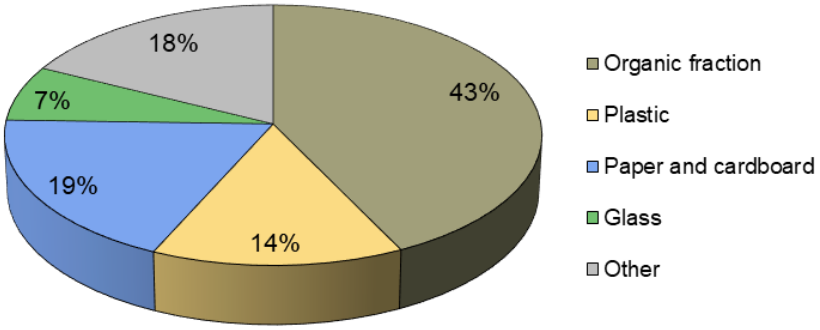

Figure 1.2. Municipal solid waste composition in Spain in 2012 (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2012).

1.2. Organic wastes production and current treatment

Our societies are generating an increasing amount of organic wastes. Specifically, food waste (FW) or organic fraction of municipal solid waste (OFMSW) and sludge from wastewater treatment plants (WWTP) (i.e., primary sludge (PS), waste activated sludge (WAS) and sewage sludge) are the most abundant organic wastes generated in the urban context (Battista et al., 2020). Although FW and sludge are originated from the same area, they usually are treated separately.

1.2.1. Food Waste

FW is defined as the organic material produced for human consumption and discarded, lost, or degraded, primarily at the manufacturing and retail stages (Pfaltzgraff et al., 2013). In Europe, approximately 90 million tonnes of FW were produced annually.

Food and Agriculture Organization (FAO) studied that roughly one-third of the world food production for human consumption is wasted, meaning that vast amounts of the resources used in food production are used in vain (Gustavsson et al., 2011). The FW without treatment can cause severe contamination of air, water and soil by its soul smell, leachate production and quick decomposition due to its high organic (VS/TS: 79-97%) and moisture content (MC) (70-93 %) (Han & Shin, 2004; He et al., 2012). Hereafter, FW is characterised by low pH between 3.7 to 6.5 and by a high carbon-to-nitrogen ratio (C/N) from 9.2 to 63.6 (Zhou et al., 2018). Even so, the characterisation of FW is very heterogenous and could depend on its main composition which changes based on the source and culture.

Regarding this problem, it is important to minimise the FW generation becoming a primary goal or, in case of not being able to avoid it, treat it in the best possible way (Pau et al., 2021). Over past years, FW was reutilised and disposed by feeding animals, landfilling, incinerating or composting (Zhou et al., 2018) although the following disadvantages could arise: (i) animal feeding without previous FW treatment could make animals prone to infection; (ii) landfilling without any treatment produces leachate which leads groundwater, soil and air pollution (greenhouse gases (GHG) emissions), in addition to occupying high quantity of usable land (El-Fadel et al., 2002; Li et al., 2010; Yan et al., 2014); (iii) incineration requires high energy due to the high MC of FW and could lead an unstable combustion with GHG emissions and toxic compounds in the atmosphere (Komemoto et al., 2009; Ren et al., 2018; Shen et al., 2013), (iv) compost is more environmentally friendly due to its fertilizing value, but requires highly energy consumption and occupation of large quantities of land with possible GHG formation and soil pollution by heavy metals and pathogens (Cheng & Hu, 2010; Lawal-Akinlami & Palaniyandi, 2017). Hence, traditional methods are not very feasible and some of them have been forbidden due to public concerns of sustainability and the stringency of environmental standards in some regions (Lim et al., 2008a). For instance, European regulation has been set to strictly limit the disposal of organic waste by landfill (European Council, 2012) and, consequently, FW treatment needs to be improved.

1.2.2. Sludge from wastewater treatment plant

Sewage sludge produced in urban areas reached 10 million tonnes in European Union (EU) on dry matter basis with different characteristics depending on the type of sludge (Battista et al., 2020; Bolzonella et al., 2018) (see Table 1.1). PS consist of settleable solids removed from raw wastewater in the primary settler of WWTPs. Conversely, WAS is produced in the secondary biological process, such as the activated sludge process, and it is formed by the growth of microorganisms and by particles from the treated stream. The main composition is (i) organic carbon compounds from biological origin (i.e., microorganisms either as single cells, filamentous bacteria or microcolonies, fibres and extracellular polymeric substances (EPS)), (ii) inorganic compounds such as salt, sand, silicates or heavy metals, (iii) pathogens and other microbiological pollutants, and, (iv) large amounts of water (which varies from 63% to 99%) (Christensen et al., 2015; Fang et al., 2020; Rulkens, 2008; Wang et al., 2017) (a scheme of typical sludge floc from secondary treatment is presented in Figure 1.3).

Table 1.1. Characteristics and production of primary, biological and sewage sludge (Battista et al., 2020).

Sludge type	Production (L/person·d)	Production (gTS/person·d)	Total solids (%)	Total Nitrogen (% TS)	Total Phosphorus (% TS)
PS	0.9 - 2.2	45 - 60	1.0 – 6.0	1.0 – 5.0	0.6 – 2.8
WAS	1.4 – 7.3	25 - 45	0.5 – 1.5	2.5 – 6.0	1.0 – 6.0[a]
Sewage Sludge	1.9 – 4.3	50 - 70	3.0 – 6.0	4.0 – 6.0	1.0 – 3.0

[a] If phosphorus co-precipitation or biological uptake are applied.

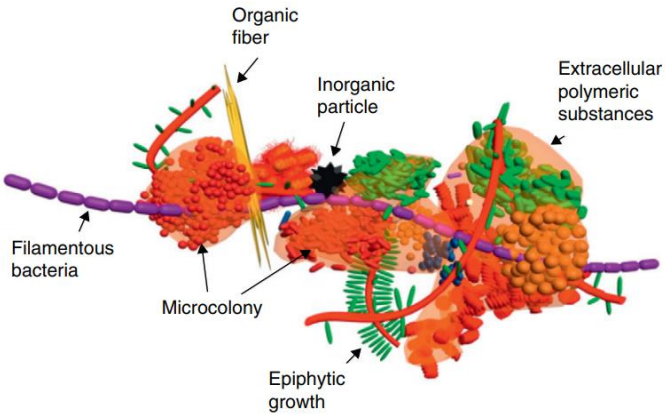

Figure 1.3. Schematic representation of an activated sludge floc model (Nielsen et al., 2012).

WAS is generated by the conversion of soluble organic compounds into biomass during the biological treatment of WWTP. Its worldwide production is estimated to reach 103.0 million tons/year by 2025 (Xu & Dai, 2021). As a general trend, the WAS is characterised by low total solids content (0.5-1.5%), low carbohydrate concentration (<10% of dry weight), low C/N ratio and high buffer capacity (Astals et al., 2013; Battista et al., 2020). Nevertheless, the characteristics vary and highly depend on the influent composition as well as the biological treatment configuration (Wacławek et al., 2019).

WWTPs were designed with the end-of-pipe treatment idea, treating wastewater with the consequent production of wastes that were usually destined to landfill or incineration with the aforementioned disposal problems (see Section 1.2.1). Nowadays, conventional WWTPs usually include sewage sludge treatment through anaerobic digestion (AD) or composting. In most cases, AD is chosen in order to stabilize the organic matter while producing biogas (Mata-Alvarez et al., 2014).

Nevertheless, the low biodegradability of WAS leads to low biogas (methane) yields which are not sufficient to cover the energy demand of the WWTPs. The low biogas yields might be due to: (i) low VS/TS ratio (30-60%), (ii) low C/N which imbalanced the diet of microorganisms and could result in ammonia accumulation that might inhibit methanogenesis, or, (iii) poor biodegradability due to sludge floc structure with recalcitrant products concentrated on the wastewater treatment (Chen et al., 2019; Potdukhe et al., 2021; Wang et al., 2017; Xu et al., 2022; Zou et al., 2018). Consequently, the energy demand for biological wastewater treatment ranged between 20-30 kWh per person equivalent year and the energy recovered by AD of wastewater sludge is only about 15-18 kWh per person equivalent per year (Bodík & Kubaská, 2013; Nghiem et al., 2017). Hence, the WWTPs can achieve up to about 65% energy self-sufficient using their sludge under the optimal conditions (Jenicek et al., 2012). Furthermore, the AD of WAS is less attractive because biogas has a low market value and lower range of applications than other by-products (Dahiya et al., 2018). Hence, new technologies could be implemented towards a more effective and sustainable way of managing these organic wastes.

1.3. Circular economy

To overcome the current situation, an update of the original Bioeconomy Strategy that emerged in 2012 is necessary to accelerate the deployment of a sustainable European bioeconomy maximising its contribution towards Sustainable Development Goals (SDGs). Hence, the EU Bioeconomy strategy aims to manage natural resources sustainably and to reduce the dependence on non-renewable and unsustainable resources (European Commission, 2018a). The implementation of this strategy is performed through a Bioeconomy Strategy Action Plan which highlights the importance of facilitating the development of new sustainable biorefineries substituting the fossil-based materials for bio-based, recyclable and biodegradable materials using organic wastes, residues and side streams (European Commission, 2018b).

The linear economy has been employed in our society for years with the "take-make-use-dispose" model where raw materials are collected and transformed into products that are used until they are finally discarded as wastes leading to a scarcity of resources and the environmental damage. This economic model prioritizes the economic benefit over sustainability. Fortunately, challenges call for a paradigm shift in resource and environmental management resulting in the emergence of the circular economy concept. According to the European Commission (2015), the circular economy is defined as an economic system where the value of products, materials and resources is maintained in the economy as long as possible with waste minimisation (see Figure 1.4). Furthermore, the products at the end of their service life or waste materials are conceived as valuable resources for another purpose, closing loops and minimising wastes in the circular economy (Battista et al., 2020; Nghiem et al., 2017).

The conversion of low value products into higher-value products, fully consistent with the EU approach to the circular economy by closing the loop, can be realized with the application of biorefinery concept which is a key element to enhance this transition (Maina et al., 2017; Moretto et al., 2020).

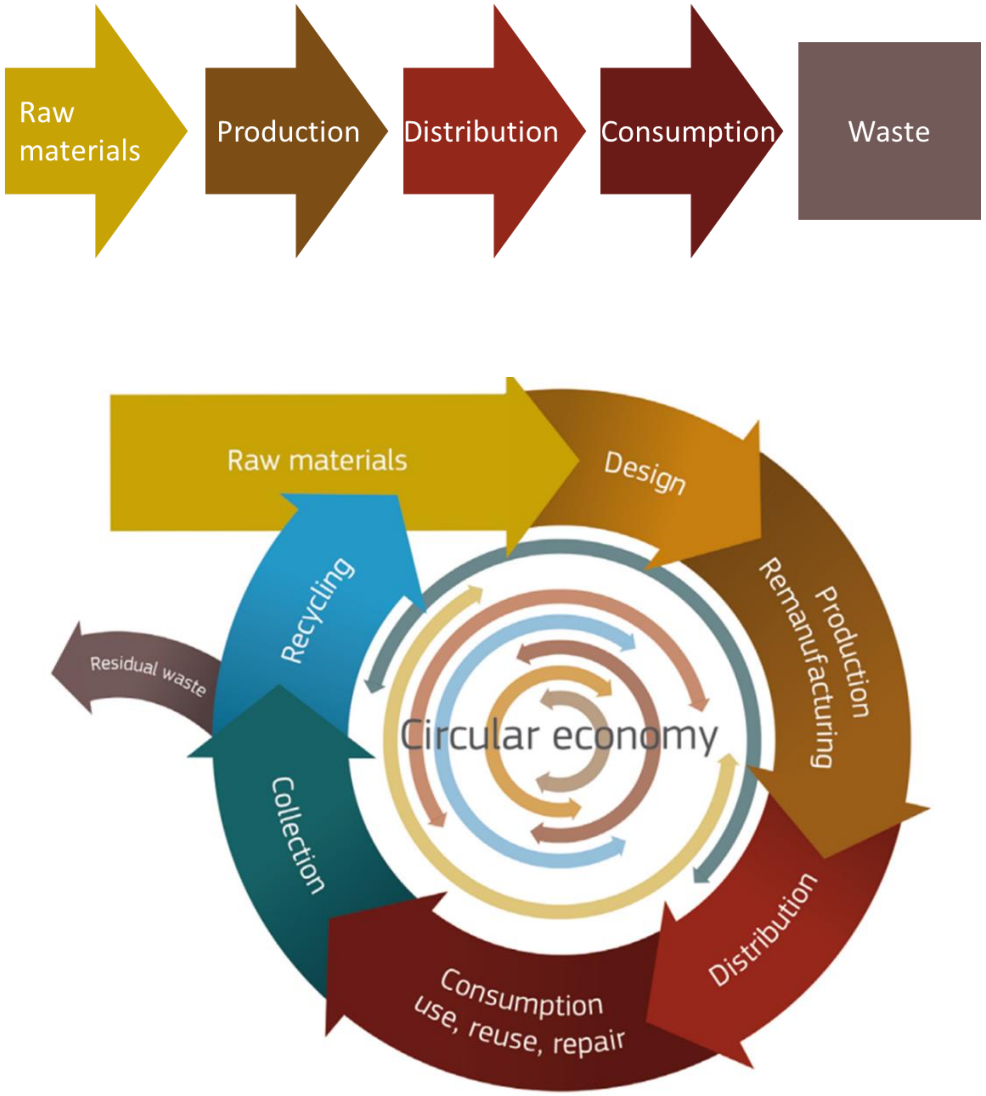

Figure 1.4. Linear economy model (top) versus circular economy (bottom). (Adapted from European Commission (2015)).

1.4. Biorefineries as valorisation facilities of organic wastes

The biorefinery could be defined as a facility where several types of bio-wastes are converted into valuable bio-based products minimising any residual or consequent waste to be disposed of. Depending on the technology used, bioenergy, biofuels chemicals, and high value-added compounds could be obtained (Kıran et al., 2015; Stephen & Periyasamy, 2018; Valentino et al., 2017). In this way, the bio-wastes conversion into value-added products turns the problem into an opportunity by reducing the generation of wastes and the dependency on fossil fuels resources achieving high value-added products (Awasthi et al., 2020).

The first article about biorefinery was published in the 1980s (Marchessault et al., 1988), so it is not a completely new concept but is gaining attention in the last decades. Over the years, different biorefineries generations have been established with increasing sustainable characteristics. Consequently, classifications of the biorefineries are diverse and depend on the basis used. The most commons are in terms of the origin of biomass source (i.e., agriculture, forestry, industries and household and aquaculture), the generation of feed-stock (i.e., first-generation, second-generation, and third-generation), and the processing technologies used (i.e., carbohydrate-based, lipid-based, lignocellulosic-based, and waste and residue-based) (Thongchul et al., 2022). The most used classification is based on terms of the generation of the feedstocks.

As Figure 1.5 shown, the biorefineries have been divided into three generations of development. The first generation of biorefineries employed a simple feedstock and technology to produce limited products. The feedstock consists of high-sugar crops, maize stovers, or straw to produce biofuels (i.e., biodiesel, bioethanol and biogas) (Karp & Shield, 2008). Nevertheless, high amounts of these cereals are needed and are considered in competition with food supply leading to huge disagreements (i.e., "food vs. fuel" debate), because these fuels are produced from feedstocks usually used for human consumption involving food shortages and price rises (Ghosh et al., 2019).

Consequently, the rise of the second generation of biorefinery was promoted. The feedstock used was based on the use of raw material derived from non-food crops including lignocellulosic material to produce biofuels and chemicals with high added value (Scoma et al., 2016). Although the second generation was more sustainable and did not compete with food production, they are still far from the circular economy approach due to resource consumption (e.g., water), huge land needed and forest clearance adding an adverse impact on the environment.

	SUSTAINABILITY →			
	Conventional refinery	**1st generation biorefinery**	**2nd generation biorefinery**	**3rd generation biorefinery**
Feedstock sources	Fossil reserves	Edible crops	Non-edible crops	Waste biomass / marine biomass
Land usage for cultivation	High land usage	Grows on arable land	Grows on arable and marginal land	Independently produced (no land usage)
Impact on environment	High contribution to CO_2 emissions	Low contribution to the mitigation of CO_2	Medium contribution to mitigation of CO_2	High contribution to mitigation of CO_2
Main advantage	Well-stablished technologies	Relatively simple conversion process	No competition with food resources	High amount of feedstock and wide spectrum of products
Main disadvantage	Finite reserves	Food vs. fuel debate	Recalcitrant structures of the feedstock	Difficulties in integration

Figure 1.5. Comparison among conventional refinery and 1st, 2nd, and 3rd generation biorefineries (Adapted from Jambo et al., (2016)) and Strazzera (2020)).

Finally, the best choice from a sustainable point of view and economic cost is a novel third generation biorefinery based on residual biomass (multi-feedstock and multi-purpose biorefinery). Specifically, a huge spectrum of wastes such as agricultural residues, municipal solid wastes, manure, FW and so on, along with marine biomass (i.e., microalgae and macroalgae) could be used to obtain a wide range of valuable products reducing environmental problems and saving resources simultaneously (Chang et al., 2010). This third generation biorefinery is very promising since combining multiple feedstocks and processes to produce a high amount of chemicals, energy and materials using bio-wastes. Henceforth, the third generation biorefineries achieve the greatest environmental benefits and lowest economic cost than first and second generation biorefineries. Furthermore, the use of bio-wastes does not have an impact on the land or on the food competition since this raw material is produced regardless of whether it is used in biorefineries or not.

The integration of these biorefineries could be realised in the existents WWTPs or mechanical-biological treatment plants (MBTPs) converting them into resource recovery facilities (RRFs) where waste streams are conceived as valuable sources of energy, chemicals, nutrients, and water (Nghiem et al., 2017; Pikaar et al., 2020;

Strazzera et al., 2018). Regarding organic waste biorefinery technology, acidogenic fermentation has drawn increased attention due to the potential to synthesise value-added bio-based products from bio-wastes (Cerdán et al., 2021). This type of biorefinery represents the carboxylate platforms. The carboxylate platforms are based on the transformation of organic feedstocks to short-chain carboxylates as intermediate feedstocks using hydrolysis and fermentation processes throughout mixed cultures under anaerobic conditions (Agler et al., 2011). Hence, the carboxylates obtained are themselves valuables and can be future converted into a spectrum of products such as bioplastics, pharmaceuticals and agrochemicals (Chavan et al., 2022).

1.5. Acidogenic fermentation

Acidogenic fermentation is a key biotechnology process in most biorefineries due to its capacity to transform organic wastes into easily assimilable organic compounds such as volatile fatty acids (VFAs, i.e., acetic, propionic, butyric, valeric and caproic acids), lactic acid and alcohols (Gianico et al., 2021; Puyol et al., 2017).

The VFAs are linear short-chain mono-carboxylate compounds that contain from two (acetic acid) to six (caproic acid) carbon atoms (Bergman, 1990). Nowadays, commercial VFAs production is mostly accomplished by chemical routes through oxidation or carboxylation of chemical precursors as aldehydes and alkenes needing a large amount of non-renewable petrochemicals as raw material (Huang et al., 2002; Shen et al., 2017). Nevertheless, the use of non-renewable petrochemicals and the increasing price of oil is leading interest in biological VFAs production through circular economy. Although pure sugars have been employed as the main carbon source (Kondo & Kondo, 1996), the utilisation of organic-rich wastes for VFAs production using microorganisms is gaining attention since it is an opportunity to recycle organic wastes producing valuable-added products while reducing the increasing amount of waste generated.

To carry out biological valorisation, the acidogenic fermentation contemplates the utilisation of pure microbial cultures (PMCs) for a targeted acid or mixed microbial cultures (MCCs) for mixed VFAs (Dai et al., 2017). MMCs are preferred because can metabolize a wide range of organic molecules and types of substrates as agricultural

waste, food waste or wastewater sludge, among others, making the process more economically favourable and cost-competitive (Domingos et al., 2017; Jankowska et al., 2015; Lee et al., 2014; Strazzera et al., 2018). The VFAs production through MMC is possible using different metabolic pathways which depend on the combined effects and the interaction of the microbial population of the inoculum, the substrate composition (i.e., carbohydrates, lipids and proteins), processes parameters and operational conditions (e.g., hydraulic retention time (HRT), organic loading rate (OLR), temperature, pH, operation mode, headspace H_2 pressure, etc.) during the set-up and throughout the experiment (Ramos-Suarez et al., 2021; Zhou et al., 2018). In fact, all these variables affect the VFA yield and VFA profile (i.e., acids distribution in the VFAs mix). Depending on the final VFA use, it is important to produce one type of VFAs or another because each one has different properties. Table 1.2 summarises the chemical properties of VFAs with some current market data.

Table 1.2. VFAs acids chemical properties and market data (Adapted from Ramos-Suarez et al. (2021)).

Acid	**Chemical formula**	**Molecular weight**[a] (g/mol)	**Density**[a] (g/cm^3) at 20 ºC	**Boiling point**[a] (ºC)	**pKa**[a] at 25 ºC	**Average price** (USD/kg)
Acetic (HAc)	CH_3COOH	60.05	1.05	117.9	4.76	0.89
Propionic (HPr)	CH_3CH_2COOH	74.08	0.99	141.1	4.88	2.20
Isobutyric (i-HBu)	$(CH_3)_2CHCOOH$	88.11	0.95	154.4	4.84	2.75
Butyric (n-HBu)	$CH_3CH_2CH_2COOH$	88.11	0.96	163.7	4.82	2.55
Isovaleric (i-HVa)	$(CH_3)_2CHCH_2COOH$	102.13	0.93	176.5	4.77	-
Valeric (n-HVa)	$CH_3(CH_2)_3COOH$	102.13	0.94	186.1	4.84	4.63
Isocaproic (i-HCa)	$(CH_3)_2CHCH_2CH_2COOH$	116.16	0.92	200.5	5.09	-
Caproic (n-HCa)	$CH_3(CH_2)_4COOH$	116.16	0.93pka	205.2	4.88	-

[a]Physical properties taken from PubChem Compound Database (Information National Center for Biotechnology, 2018).

The VFAs production from bio-wastes using MMCs is based on a set of bioreactions carried out in different steps with distinct groups of microorganisms as explained above. Hence, it can be summarised that VFAs are produced during the acidogenesis and acetogenesis steps of the conventional AD processes when methanogenesis is successfully inhibited (Lee et al., 2014) (see Figure 1.6).

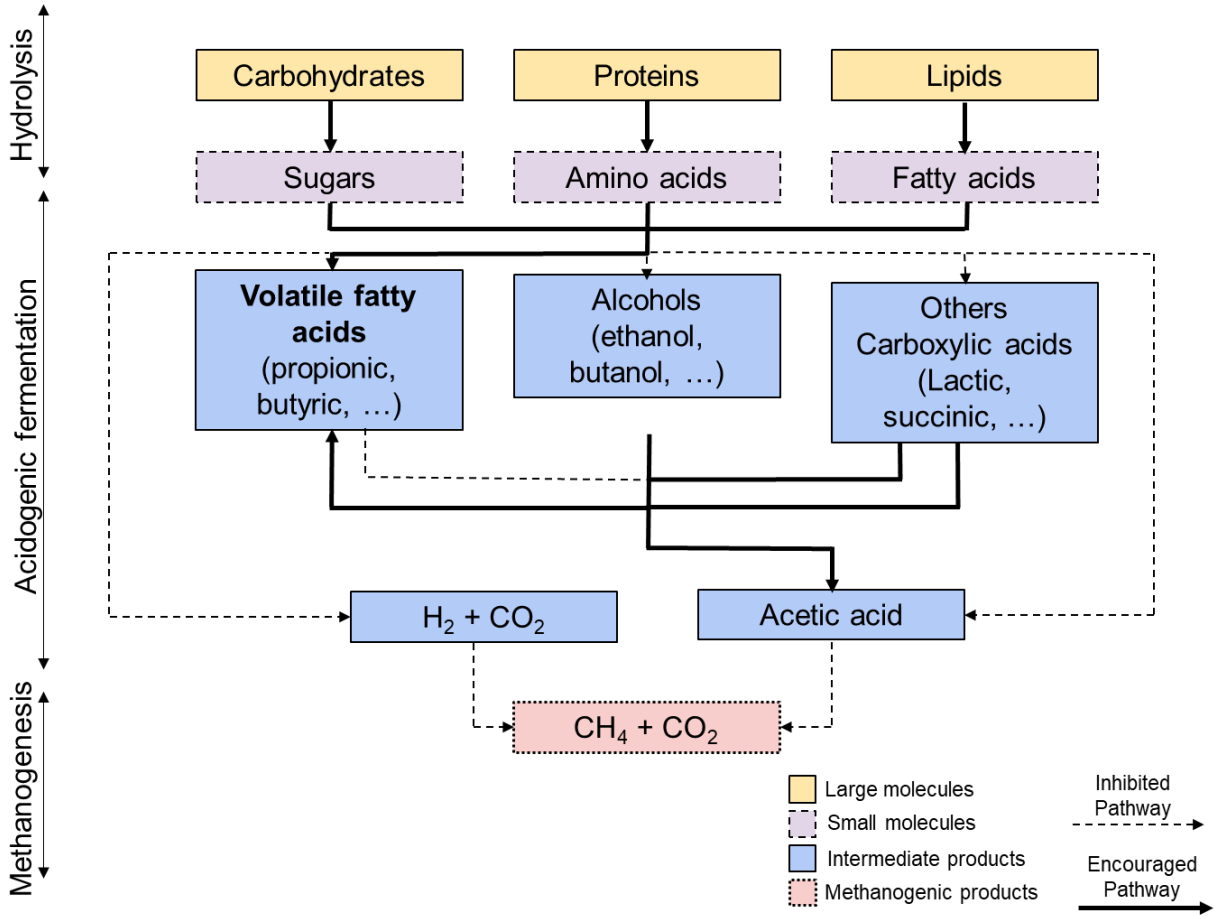

Figure 1.6. Anaerobic digestion steps and pathways: encouraged pathways show the preferred metabolic routes for acidogenic fermentation towards VFAs production (Adapted from Ramos-Suarez et al. (2021)).

The first step is hydrolysis which includes non-biological and extra-cellular biological processes to breakdown and solubilise the complex matter that cannot be directly used by bacteria in form of soluble compounds. Firstly, the complex organic matter is disintegrated into macromolecules (i.e., carbohydrates, proteins, and lipids) that are further hydrolysed to soluble compounds as monomers (i.e., monosaccharides, amino acids, and long chain fatty acids), which can be directly used for VFAs production. This process is carried out by the hydrolytic enzymes (cellulases, proteases and lipases) excreted from the hydrolytic microorganisms.

Hydrolysis is the rate-limiting step of degradation of particulate organic matter (i.e., solid and semi-solid wastes) and, specifically, on the FW acidogenic fermentation (Kim et al., 2005). The improvement of the hydrolysis step could increase the readily

available substrate carbon for future VFAs conversion (Zhou et al., 2018). The enhancement of the hydrolysis step is affected by several parameters such as particle size, pH, temperature, biomass characteristics or the intrinsic substrate characteristics (Zhou et al., 2018). Moreover, pre-treatments are also used to boost the solubilisation of the organic matter (Fdez.-Güelfo et al., 2011). The most popular pre-treatments are (i) chemical pre-treatments (acid and alkaline pre-treatment), (ii) physical pre-treatments (thermal, microwave and ultrasound), (iii) biological pre-treatment (enzymes), or (iv) the combination of various of them (Li et al., 2019; Zhou et al., 2018).

Acidogenesis, also known as fermentation, is the next step where a heterogeneous population of facultative and obligate anaerobic bacteria are capable to transform the hydrolysis products by a series of oxidation-reduction reactions into VFAs (i.e., acetic acid, propionic acid, butyric acid, valeric acid and caproic), lactic acid, succinic acid, alcohols (i.e., ethanol and butanol), pyruvate, ammonia, sulphide, hydrogen and carbon dioxide (Dai et al., 2017). This stage is affected by the characteristics of substrate and inoculum, and operational parameters as pH, temperature, HRT, OLR, and headspace (Zhou et al., 2018).

Finally, the VFAs, excluding acetic acid, and other products derived from the acidogenesis step are converted by hydrogen-producing acetogens to acetate, hydrogen, and carbon dioxide in the acetogenesis step. Homoacetogenesis can also lead to the production of acetate using hydrogen as the electron donor to reduce carbon dioxide (Siriwongrungson et al., 2007). Moreover, syntrophic acetogenesis involves the anaerobic oxidation of propionate and butyrate to acetate and hydrogen, which is inhibited with a high presence of hydrogen (Angelidaki et al., 2011).

Finally, as explained before, the methanogenesis needs to be inhibited to accumulate large VFAs quantities avoiding the conversion of the end products of the acetogenesis into biogas by methanogenic archaea. The methanogenic activity can be prevented using low HRT (HRT<10 days), low (pH<6.5) or high pH (pH>7.5), high OLR (OLR>4 gVS/(L·d)) or a combination of the different approaches (Braguglia et al., 2018; Reis et al., 2011). Moreover, operating parameters of acidogenic fermentation could be tuned to maximize the VFAs conversion yield and to control the composition of the VFAs mixture produced.

1.5.1. Substrates of acidogenic fermentation

Organic substances can be used as feedstock for anaerobic fermentation. Hence, the use of bio-wastes is preferred to other organic substances to avoid the food vs fuel debate and contribute to waste management strategies (Ramos-Suarez et al., 2021). The most abundant solid wastes used for anaerobic fermentation were: animal manure (Lian et al., 2021; Saritpongteeraka et al., 2014), agricultural residues (Guo et al., 2015; Potdukhe et al., 2021), sewage sludge (Fang et al., 2020; Garcia-Aguirre et al., 2017), WAS (Chen et al., 2007; Pang et al., 2020; Tong & Chen, 2007), OFMSW (Cheah et al., 2019; Colombo et al., 2017; Moretto et al., 2020) or FW (Cao et al., 2019; Dahiya et al., 2018; Strazzera et al., 2018). In this thesis, the acidogenic fermentation of FW and WAS was studied and, therefore, the next sections are focused in the acidogenic fermentation and co-fermentation of these wastes.

1.5.1.1. Food waste acidogenic fermentation

FW stands as a good substrate for acidogenic fermentation. As explained before (section 1.2.1), FW generation from households and the assimilable sectors (canteen, restaurants, catering, vegetables and fruit residues from markets, supermarkets, and food factories) is increasing leading to huge environmental and management problems. All these wastes present characteristics that, although variable and often seasonal, are in some way similar. FW, in general, is characterised by high MC, high organic matter content, high carbon/nitrogen ratio and acidic pH (see Table 1.3). These characteristics make FW an ideal feedstock for acidogenic fermentation with reported yields between 50 and 400 mgCOD/gVS depending on the fermentation parameters and inherent FW composition and characteristics (Jiang et al., 2013; Li et al., 2018; Lim et al., 2008a). Acidogenic fermentation is preferred to AD or composting because the fermentation products have a higher potential market value and a wider range of applications than biogas and compost (Dahiya et al., 2018; Fernández-Domínguez et al., 2020). Although FW acidogenic fermentation is well studied, is constrained by (i) its particular nature with complex polymers such as lignocellulosic materials, lipids and proteins which makes hydrolysis the rate-limiting step and (ii) the lack of buffer capacity that requires chemical dosage to prevent the inhibition of hydrolytic-

fermentative microorganisms by its low pH (Cheah et al., 2019; Kim et al., 2003; Marin et al., 2010).

To improve hydrolysis, different strategies would be carried out. Firstly, process parameters such as temperature, pH, HRT and OLR, among others can be modified to improve the hydrolysis (Chen et al., 2017; Garcia-Aguirre et al., 2017; Jiang et al., 2013; Moretto et al., 2019). Furthermore, diverse pre-treatments could be applied to overcome the lower hydrolysis. These pre-treatments can be classified as physical, chemical, and biological. Physical pre-treatments (thermal, microwave, mechanical including ultrasound) are based on reducing the substrate dimensions and disintegrating the cell membrane leading to better contact between substrate and microorganisms increasing the solubilisation (Bougrier et al., 2007). The chemical pre-treatments use the addition of chemicals such as acids, alkalis, organic aqueous solvent mixtures at different concentrations to increase the solubilisation of FW and alter the lignin structure (Strazzera et al., 2018). Finally, the biological pre-treatments are based on enzymatic addition to enhancing hydrolytic activity without using reagents and without requiring high-energy demands make them more economically attractive and environmentally friendly compared to the physical and chemical pre-treatments. These pre-treatments are chosen depending on several factors. Even so, each pre-treatment could have its intrinsic disadvantages and limitations which lead to combining various pre-treatments increasing the synergistic effects that are still missing and unclear (Fang et al., 2020). However, it is worth mentioning that pre-treatments require an environmental and techno-economic feasibility study because the improvements could not be enough to justify the capital and operational costs (Bolzonella et al., 2018; Strazzera et al., 2018). Even so, except for alkaline pre-treatments, the pre-treatments do not solve the constraints related to the FW lack of buffer capacity.

Table 1.3. Physical and chemical characterizations of FWs (Adapted from Strazzera et al. (2018)).

Characteristics												References
Origin	**pH**	**TS**	**VS**	**TKN**	**tCOD**	**C/N**	**TAN**	**Carbohydrates**	**Proteins**	**Lipids**	**VFAs**	
University canteen	3.9	12.9 (%)	12.5 (%)	3.3 (g/L)	166.2 (g/L)	49.9	< 10.0 (mg/L)	69.3 (%TS)	16.1 (%TS)	10.6 (%TS)	3.6 (g/L)	Zhang et al. (2005)
University canteen	5.9	874.0 (g/kg)	724.1 (g/Kg)	14.9 (g/L)	472.0 (g/L)	37.0	0.13 (g/L)	-	-	-	0.5 (gHAc/L)	Forster-Carneiro et al (2008)
Synthetic	4.7	97.8 (g/L)	91.8 (g/L)	2.5 (g/L)	108.4 (g/L)	-	54.0 (mg/L)	45.3 (g glucose/L)	16.2 (g albumin/L)	-	-	Lee et al. (2008)
Synthetic	4.9	1.0 (%)	94 (%)	-	-	-	-	-	-	-	816.3 (mg/Kg)	Komemoto et al. (2009)
Synthetic	6.7	16.5 (%)	15.5 (%)	-	250.5 (g/L)	-	-	-	-	-	-	Izumi et al. (2010)
University canteen	-	18.3 (%)	87.5 (%)	-	-	63.6	-	35.5 (%)	14.4 (%)	24.1 (%)	-	He et al. (2012)
Garbage collection	3.8	10.3 (%)	9.2 (%)	-	152.0 (g/L)	-	-	-	-	-	-	Nagao et al. (2012)
Synthetic	4.6	20.5 (%)	20.0 (%)	-	-	13.4	760.6 (mg/L)	-	-	-	829.5 (mg/L)	Jiang et al. (2013)
University canteen	-	22.6 (%)	17.9 (%)	2.6 (%)	30.3 (%)	11.5	-	-	-		-	Shen et al. (2013)
University canteen	3.9	20.0 (%)	19.0 (%)	-	-	24.0	-	70.0 (%TS)	13.0 (%TS)	10.0 (%TS)	-	Bo and Pin-jing (2014)
Catering premises	4.8	31.5 (%)	91.2 (%)	-	-	14.4	-	34.7 (%TS)	29.6 (%TS)	26.7 (%TS)	-	Browne and Murphy(2014)
University canteen	5.6	150.0 (g/L)	90.0 (%TS)	-	-	34.0	0.1 (g/L)	-	-	-	0.8 (g/L)	Gou et al. (2014)
University canteen	6.1	24.0 (%)	-	1.8 (%)	-	-	-	39.5 (%)	11.0 (%)	-	-	Wang et al. (2014)
Waste treatment plant	-	23.6 (%)	22.0 (%)	7.4 (g/Kg)	998 .0 (g/kg)	-	-	-	-	-	-	Yirong et al. (2015)
University canteen	4.7	80.7 (g/L)	78.8 (g/L)	6.3 (g/L)	233.0 (g/L)	-	-	-	-	-	3.0 (g/L)	Ratanatamskul and Saleart (2016)
University canteen	4.5	20.0 (%)	19.2 (%)	-	257.9 (g/L)	-	-	-	-	-	-	Tang et al. (2016)
University canteen	-	25.7 (%)	98.2 (%)	-	1.1 (%)	-	-	0.6 (%)	15.1 (%)	0.1 (%)	-	Yin et al. (2016)

Table 1.3. Physical and chemical characterizations of FWs (Adapted from Strazzera et al. (2018)). *(Continued)*

Characteristics Origin	pH	TS	VS	TKN	tCOD	C/N	TAN	Carbohydrates	Proteins	Lipids	VFAs	References
University canteen	-	17.4 (%)	16.1 (%)	2.0 (%TS)	229.7 (g/L)	-	-	61.5 (%TS)	9.8 (%TS)	9.2 (%TS)	-	Zhang et al. (2016)
University canteen	4.8	3.9 (%)	90.2 (%TS)	-	124.2 (g/L)	-	-	8.9 (%TS)	5.3 (%TS)	-	-	Zhen et al. (2016)
Synthetic	5.1	35.1 (%)	32.9 (%)	-	1.5 (g/gTS)	-	10.5 (mg/gTS)	0.3 (g/gVS)	-	0.6 (g/gVS)	27.9 (mg/gVS)	Zhao et al. (2016)
University canteen	5.3	33.0 (%)	31.7 (%)	-	-	21.5	-	-	-	-	-	Zhang et al. (2017a)
Synthetic	-	21.6 (%)	20.8 (%)	27.1 (g/KgTS)	1.4 (g/gTS)	16.3	-	687.0 (g/KgTS)	169.0 (g/KgTS)	72.3 (g/KgTS)	-	Capson-Tojo et al. (2018)
Synthetic	5.2	17.0 (%)	16.2 (%)	-	58.3 (g/L)	21.3	20.0 (mg/L)	-	-	-	-	Feng et al. (2018)
Garbage collection	5.0	26.3 (%)	25.0 (%)	-	-	-	621.0 (mg/L)	-	-	-	4657.0 (mg/L)	Jiang et al. (2018)
University canteen	4.7	27.5 (%)	25.8 (%)	4.2 (g/Kg)	173.3 (g/Kg)	23.7	-	-	-	-	-	Li et al. (2018)
University canteen	-	16.5 (%)	15.5 (%)	-	264.0 (g/L)	-	-	-	-	-	-	Xiong et al. (2019)
Synthetic	5.7	257.3 (g/Kg)	250.9 (g/Kg)	9.9 (g/Kg)	292.4 (g/Kg)	29.0	-	23.9 (%TS)	21.1 (%TS)	15.6 (%TS)	-	Strazzera et al. (2021)
University canteen	3.4	-	-	-	78.3 (g/L)	-	91.2 (mg/L)	3.6 (g/L)	1.0 (g/L)	21.5 (mg/L)	-	Yu et al. (2021)

1.5.1.2. Waste activated sludge acidogenic fermentation

WAS is generated in huge amounts on the biological treatment of the WWTPs accounting for around 50% of the total operational costs (Appels et al., 2008; Fang et al., 2020). Conventionally, WAS is treated by AD achieving low biogas yields by its characteristics (see Section 1.2.2 for more information). To take advantage of the organic matter that WAS contains, acidogenic fermentation has been studied by several authors (Chen et al., 2007; Fang et al., 2020; Gonzalez et al., 2018; Guo et al., 2015; Luo et al., 2020a; Zhao et al., 2018). However, low VFA yields are obtained (10-250 mgCOD/gVS) due to low hydrolysis rates and biodegradation of WAS (Chen et al., 2007; Gonzalez et al., 2018; Gou et al., 2014; Ma et al., 2017; Peces et al., 2020; Xu et al., 2020a). To overcome these problems, different process parameters and pre-treatment could be applied as occurs with FW (Feng et al., 2009; Liu et al., 2009; Moretto et al., 2019; Morgan-Sagastume et al., 2011; Xiong et al., 2012; Yan et al., 2010). Notwithstanding, the enhancements probably are not enough to justify the associated costs as commented before. Alternative, co-fermentation stands as an interesting approach to increase VFAs production as commented in the next section.

1.5.2. Co-fermentation

Co-fermentation is defined as the simultaneous fermentation of two or more substrates. This strategy is a feasible alternative to enhance FW anaerobic fermentation yields and to overcome the limitations of single substrate fermentation (mono-fermentation). The increased FW fermentation performance due to co-fermentation has been associated with: (i) an increase in the organic matter content, (ii) an improvement in the buffer capacity which prevents pH drops and alkali consumption, (iii) the balance of micronutrients, macronutrients (e.g., C/N ratio) and MC, (iv) the dilution of inhibitory and/or toxic compounds and, (v) the diversification of the hydrolytic and fermentative bacteria (Banerjee, 1999; Fang et al., 2020; Feng et al., 2011; Peces et al., 2020; Perez-Esteban et al., 2021; Wu et al., 2016). In this way, co-fermentation stands as an opportunity to enhance higher VFA yields treating two different waste streams in the same facility without incurring major capital and operating costs.

Among different organic wastes, WAS stands out as the most researched co-substrate for FW fermentation due to its high availability and its buffer capacity that allows sustaining the pH above inhibitory levels (pH>5.0) without the necessity of external chemicals addition (Wu et al., 2020; Zhao et al., 2016). The co-fermentation of FW and WAS was carried out successfully by several authors studying the impact of (i) pH (Chen et al., 2013; Feng et al., 2009, 2011; Moretto et al., 2019), (ii) the mixing ratio of FW and WAS (Ma et al., 2017; Wu et al., 2020; Zhao et al., 2016), (iii) the FW composition (Bevilacqua et al., 2022; Li & Li, 2017; Peces et al., 2020), among others variables or the combination of them (Chen et al., 2013; Garcia-Aguirre et al., 2019; Hong & Haiyun, 2010; Li et al., 2014; Xu et al., 2020a).

1.5.3. Use of volatile fatty acids

Once the methanogens are inhibited, the intermediate fermentation products such as VFAs, alcohols, lactic acid, hydrogen, etc. are accumulated in the fermenter and can be directly used to provide the carbon source needed to sustain other microbially-mediated units in the biorefinery such as bioenergy production (i.e., biogas and biohydrogen), biological nutrient recovery, chain elongation and biopolymer production, among others (Abreu et al., 2019; Dahiya et al., 2015; Duber et al., 2020; Frison et al., 2013; Serrano et al., 2020; Valentino et al., 2018).

1.5.3.1. Bioenergy (biogas and biohydrogen) production

The VFAs were firstly studied due to their key role in AD for biogas production. AD is one of the most extended, robust and well-known technologies in Europe which is used to treat a huge amount of organic wastes and the combination of them (Astals et al., 2014; Mata-Alvarez et al., 2014; Romero-Güiza et al., 2016). This biological process is based on hydrolysis, acidogenesis, acetogenesis and methanogenesis steps to produce biogas as an end-product. This biogas consists of 60-70% CH_4, 30-40% CO_2, and traces of other gases as H_2 or H_2S. After CO_2 and impurities removal, biogas can be used as vehicle fuel, for power generation or be injected into the natural gas grid (Shen et al., 2017a). To carry out all these steps, various microbial communities are involved with

different tolerance. The VFAs production led to a pH decreasing which directly affects methanogenesis processes (Braguglia et al., 2018). Hence, the AD pathways could be divided into two main routes from the metabolic point of view: (i) acidogenic fermentation and (ii) methanogenesis pathways. In this way, VFAs and hydrogen can be produced from the first stage and methane from the second one dividing the process in a two-stage AD configuration (Micolucci et al., 2020) connecting better with the biorefinery concept obtaining biogas and added-value streams (Valentino et al., 2021).

Another profitable gas studied during the acidogenic fermentation step is hydrogen. Hydrogen is one of the most desirable forms of renewable energy, and it is considered to be one of the future fuels due to its social, economic and environmental benefits and its high combustion energy per unit mass (i.e., 142 kJ/g) (Meher Kotay & Das, 2008; Xia et al., 2015). Many researchers have studied an environmentally friendly manner to produce H_2 from residual biomass by dark fermentation (DF) and photofermentation (PF) (Pandey et al., 2021; Policastro et al., 2021; Rai et al., 2014; Tawfik et al., 2014).

DF is based on the acidogenic fermentation of heterotrophic bacteria in absence of light and oxygen which are capable to convert organic compounds in bio-hydrogen (H_2), VFAs, alcohols and other by-products (Policastro et al., 2021; Valdez-Vazquez & Poggi-Varaldo, 2009). In DF, the hydrogenase is the key enzyme that catalyses the hydrogen formation combining protons and electrons in dark fermentation (Nicolet et al., 2010; Trohalaki & Pachter, 2010).

PF is a biochemical route of purple non-sulphur bacteria (PNSB) to obtain H_2 using energy driven from the light because the reaction is not spontaneous. PNSB bacteria have the capacity to use carbon sources like VFAs for hydrogen production in presence of light (Argun & Kargi, 2011). This process is carried out by means of nitrogenase enzyme that catalyses molecular hydrogen formation as a by-product during nitrogen fixation to ammonia, consuming ATP produced by photosynthesis, and the electrons produced from the metabolism of organic compounds (Morsy et al., 2019; Xia et al., 2015).

Hence, the DF process suffers from low hydrogen yields by the VFAs accumulation on the medium and, at the same time, the PNSB microorganisms could use these VFAs (produced by DF) to generate hydrogen. Henceforth, DF and PF can be operated

simultaneously obtaining higher hydrogen yield. Moreover, this combination is receiving increased attention due to its cost-effectiveness, environmentally friendly and carbon-neutral characteristics (Argun & Kargi, 2011)

1.5.3.2. Biological nutrient removal

In biological nutrient removal processes of municipal WWTPs, the organic carbon present in the wastewater is often quite limited and a large amount of external carbon addition could be needed to complete nitrate conversion to dinitrogen gas in the denitrification step. Namely, methanol, ethanol and sodium acetate are typically used in industries to increase the denitrification efficiency (Guerrero et al., 2011; Hwang et al., 2016). VFAs are a viable alternative source of carbon for heterotrophic denitrification (Lim et al., 2008b; Peces et al., 2016). Their use reduces the overall costs of the process and increases the denitrification rate limiting nitrite accumulation compared to other compounds (Kim et al., 2016; Tong & Chen, 2007). VFAs show different employment patterns depending on the type of VFAs used. Acetic acid was preferred obtaining a more than double greater denitrification rate with respect to butyrate and propionate (Elefsiniotis & Wareham, 2007; Galí et al., 2006).

Regarding phosphorus removal, an alternation of anaerobic and aerobic conditions is required to carry out the enhanced biological phosphorus removal (EBPR) promoting the growth of polyphosphate-accumulating organisms (PAOs). As nitrogen removal, a carbon source is needed during the anaerobic stage of EBPR with a C/P ratio of 5 or higher, and VFAs are a suitable class of compounds for EPBR itself (Rashed & Massoud, 2015). PAOs take up the VFAs converting them into intracellular polyhydroxyalkanoates (PHAs). The energy used for these anaerobic transformations is generated by hydrolysis of their internally stored polyphosphate (poly-P) and glycogen, releasing phosphates (P). Under aerobic conditions, PAOs oxidise the PHA stored anaerobically to produce energy phosphate uptake, glycogen replacement, and cell growth (Oehmen et al., 2007; Yuan et al., 2012) (see Figure 1.7). The EBPR typically requires an acetate to propionate ratio ranging from 0.25 to 0.75 (Broughton et al., 2008; Yuan et al., 2012). Hence, different VFAs profiles had different impacts on the EBPR process (Long et al., 2021).

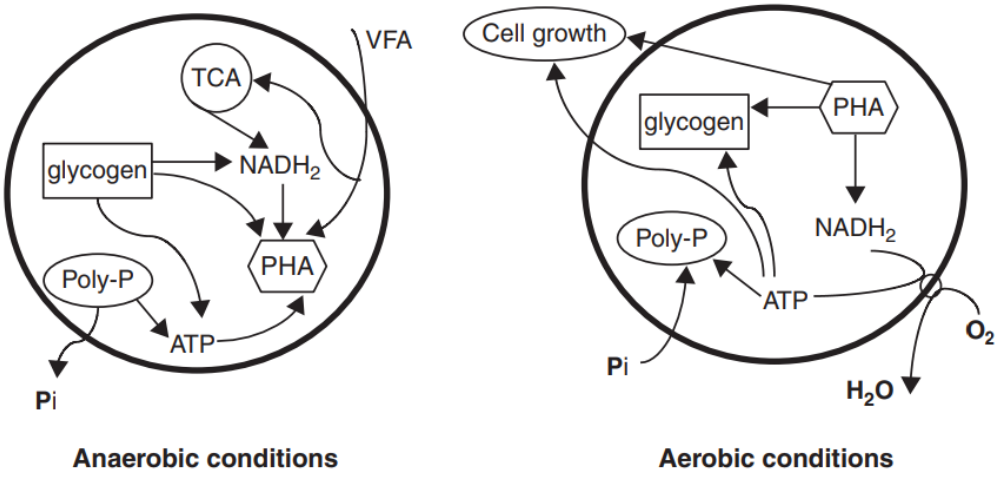

Figure 1.7. Schematic diagrams of the anaerobic and aerobic PAO metabolism. (Yuan et al., 2012).

1.5.3.3. Chain elongation

Chain elongation is an anaerobic bioprocess that converts VFAs with an electron donor into more valuable medium-chain fatty acids (MCFAs) (Bevilacqua et al., 2022). MCFAs are saturated fatty acids that contain from six to twelve carbons with one carboxylic group (e.g., n-caproate (C6), n-heptanoate (C7), n-caprylate (C8), etc.) (Angenent et al., 2016). MCFAs have higher energy densities and lower solubility in water compared to ethanol to their undissociated acid form that allows selective removal. This fact is due to its longer hydrophobic carbon chain and lower oxygen/carbon ratio compared to VFAs, that increases the energy density and makes the separation of the fermentation liquor easier (Steinbusch et al., 2011). The development of the MCFA continuous process is desirable since its potential as valuable platform chemicals (Roghair et al., 2018).

Caproate is one of the most attractive products from the carboxylate platform. The chain elongation of VFAs into n-caproate occurs via the reverse β oxidation pathway using ethanol as a carbon source, energy, and reducing equivalents (Agler et al., 2012). The reverse β oxidation pathway consists of a cyclic process where an acetyl-CoA molecule derived from the oxidation of ethanol is added to a carboxylate, elongating its carbon chain length with two carbons at a time (i.e., acetate to n-butyrate (C4),

propionate (C3) to n-valerate (C5), n-butyrate (C4) to n-caproate, n-valerate to n-heptanoate (C7), n-caproate (C6) to n-caprylate (C8), etc.) (Angenent et al., 2016).

Even so, not only the ethanol can be used as an electron donor but also hydrogen (Steinbusch et al., 2011), methanol (Chen et al., 2016; de Leeuw et al., 2020), and lactic acid (Kucek et al., 2016; Zhu et al., 2015), among others, that can be produced from organic residues too. Moreover, as occurred in AD or bio-hydrogen technologies, the MCFA production from bio-wastes by hydrolysis, acidogenesis and chain elongation can be realised in a single-stage system (Agler et al., 2012) as well as in two stage-systems, where hydrolysis and acidogenesis could be performed in one stage and chain-elongation in a subsequent stage, optimizing each stage independently (Grootscholten et al., 2014).

1.5.3.4. Biopolymers

The production of biopolymers using VFAs, or lactic acid produced in acidogenic fermentation units is a possibility with growing interest also driven by the establishment of the novel circular economy approach. The acidogenic fermentation end-products can be used to produce biopolymers such as polylactates (PLAs) or PHAs, among others.

PLA is a biodegradable aliphatic thermoplastic polyester derived from lactic acid which is a fermented product of bio-resources (Ebnesajjad, 2012; Lee et al., 2021). PLA is one of the most extensively researched and utilised bioplastic due to its mechanical properties, renewability, biodegradability and relatively low production cost (Auras et al., 2004). Since PLA is produced from lactic acid that is a chiral molecule, it exists in the form of poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), and poly(DL-lactide) (PDLLA). The properties of PLA depend on the selection of stereoisomer inside the polymer chain (see Table 1.4).

Table 1.4. Chemical and physical properties of stereo isomers of PLA (Munim & Raza, 2019).

Properties	**PDLA**	**PLLA**	**PDLLA**
Crystalline structure	Crystalline	Hemi crystalline	Amorphous
Solubility	PLLA solvents and acetone	Chloroform, furan, dioxane and dioxolane	Tetrahydrofuran, ethyl acetate, dimethyl sulfoxide and dimethyl formamide
Melting temperature (ºC)	120 - 150	173 - 178	230 - 240
Glass transition temperature (ºC)	40 - 50	55 - 80	43 - 53
Elongation at break (%)	20 - 30	20 - 30	30 - 35
Half-life in 37 ºC normal saline (months)	4 - 6	4 - 6	2 - 3
Density (g/cm^3)	1.25	1.29	1.25

On the other hand, PHAs are other biodegradable polymers with great potential due to their variable properties and their inherent biocompatibility and biodegradability. Due to their high importance, the characteristics and production of these biopolymers will be detailed in Section 1.6.

1.6. Polyhydroxyalkanoates

PHAs are a new generation of biopolymers very promising as substitutes of petroleum-based plastics since they are produced from renewable resources and are natural, recyclable, biocompatible, and completely biodegradable under aerobic and anaerobic conditions. These bioplastics are synthesised in the form of intracellular granules by some microorganisms from the degradation of organic matter as carbon and energy storage mechanisms under growth-limiting conditions (see Figure 1.8). Furthermore, PHAs are characterised by a high range of adjustable properties which are similar to those found in thermoplastics (Lemos et al., 1998; Morgan-Sagastume et al., 2010; Reis et al., 2011; Senior et al., 1972; Serafim et al., 2004). The diversity of their properties is mainly affected by the monomer type, monomer proportion and molecular weight of PHA (Mannina et al., 2020; Pagliano et al., 2017).

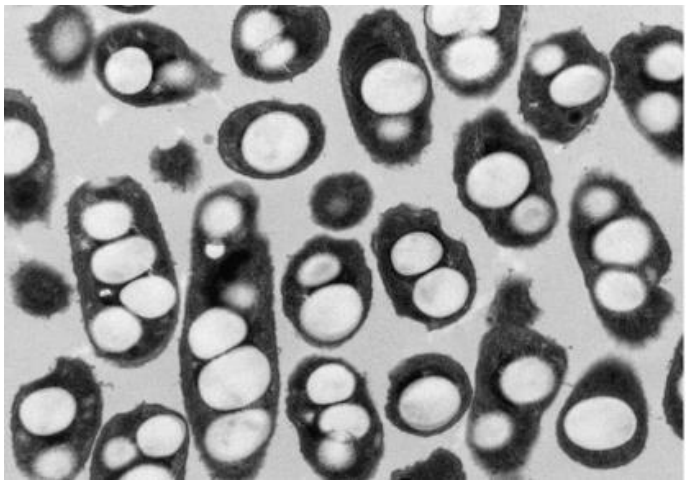

Figure 1.8. Morphology of PHA granules in the cells of *Cupriavidus necator* (Obruca et al., 2020).

1.6.1. PHA structure and properties

PHAs are linear polyesters of hydroxyalkanoate (HA) monomers connected by an ester bond with the general structure presented in Figure 1.9. This structure is characterised by: (i) n, which represents the amount of HA in the polymer chain which could variate from 100 to 30,000, (ii) x, which represents the number of methylene groups with a value between 1-4, and (iii) R, which represents side-chain alkyl groups, which range from methyl (C_1) to tridecyl (C_{13}) (Akaraonye et al., 2010; Wei & Fang, 2022). The molecular weight of each PHA varies in the range of $2x10^5$ to $3x10^6$ Da depending on the microorganism and the growth conditions used (Lee, 1996).

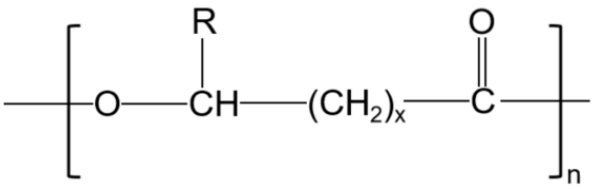

Figure 1.9. The generic structural formula of PHA (Wei & Fang, 2022).

Over 150 different types of HA monomers have been identified giving an enormous possible variation in the PHA composition. Hence, depending on the monomers, the PHA can be classified into two main groups: (i) short chain lengths (SCL) which consist of monomers composed of 3-5 carbon atoms, and (ii) medium chain length (MCL)

which consist of monomers with 6-14 carbon atoms (Anderson & Dawes, 1990). The SCL-PHAs are characterised by high crystallinity degree, fragility and rigidity with a high melting point and low glass transition temperature. Contrariwise, the MCL-PHAs are characterised by more elastomeric properties and low crystallinity which turn the biopolymer more flexible and soft (Mannina et al., 2020; Pagliano et al., 2017; Reddy et al., 2003; Wei & Fang, 2022). PHAs have gained attention as plastic materials due to their biodegradability and their remarkable similarities in physical properties with synthetic polymers such as polypropylene (see Table 1.5).

Table 1.5. Comparison of the physical properties of SCL-PHAs and MCL-PHAs with propylene (Adapted from Akaraonye et al. (2010) and Zinn & Hany (2005)).

Properties	**SCL-PHAs**	**MCL-PHAs**	**Polypropylene**
Crystallinity (%)	40 - 80	20 - 40	70
Melting point (ºC)	80 - 180	30 - 80	176
Density (g/cm^3)	1.25	1.05	0.91
Glass transition temperature (ºC)	−148 - 4	−40 - 150	−10
Extension to break (%)	6 - 10	300 - 450	400
UV light resistant	Good	Good	Poor
Solvent resistant	Poor	Poor	Good
Biodegradability	Good	Good	None

Both SCL-PHAs and MCL-PHAs can be found as homopolymers (with the same monomer unit) or as copolymers (with more than one type of monomer units) depending on the bacteria type, the process conditions and the substrate used for feeding bacteria (Akaraonye et al., 2010; Reddy et al., 2003). The two most common HA monomer types on PHA production are hydroxybutyrate (HB) and hydroxyvalrate (HV) which form the polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) homopolymers. On the other hand, the most studied copolymer is the poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) which is suited for various applications by its chemical-physical characteristics. The PHB has very strong hardness and water resistance by its high crystallinity, stiffy and high brittleness, but its low thermal stability is not conducive to ductile processing (Bugnicourt et al., 2014; Dias et al., 2006; Wei & Fang, 2022). To enhance this ductility, a certain proportion of HV monomer could be added. Consequently, the higher the HV content, the stronger

the toughness of the copolymer improving the flexibility and wider thermal processing (Bengtsson et al., 2008a; Jankowska et al., 2015; Kourmentza & Kornaros, 2016). Hence, it is clear that the proportion of HB and HV would affect the PHA-polymer characteristics that will be very distinct in terms of heat resistance, elasticity, durability, and transparency, among others (Bugnicourt et al., 2014; Chanprateep et al., 2010; Chee et al., 2010). The enormous possible variations on the PHAs make them suitable for an array of potential applications such as packaging, agricultural films and fibres, compost bags, manufacturing disposable everyday articles, bulk commodity plastics, pharmaceutical and medical uses, etc. (Akaraonye et al., 2010; Bucci et al., 2005; Reddy et al., 2003).

1.6.2. Carbon sources

The overall cost of PHA production is ranged about 5.0 and 8.0 €/kg using the conventional production method, which limits their use in the commodities industry and makes that its production is only economically feasible for high-value applications in the medical and pharmaceutical sectors (Valentino et al., 2020). The carbon source represents near 25-45 % of the total production costs (Lee, 1996; Lee & Choi, 1999; Nath et al., 2008). Consequently, the substrate choice could be important to reduce the overall costs. The different carbon sources used for PHA production are sugars, starches, alcohols, or other sources derived from waste materials which reduce the cost of the polymer but also for waste management (Akaraonye et al., 2010; Serafim et al., 2004).

VFAs produced by acidogenic fermentation of organic wastes are considered one of the most suitable carbon source for PHA production (Albuquerque et al., 2011; Mengmeng et al., 2009; Wei & Fang, 2022). The PHA composition and characteristics depend on the percentage of each VFA in the feedstock. The presence of even VFAs (acetic and butyric acid) tend to form HB monomers which turn the material highly crystalline, stiff and brittle; whereas the odd VFAs (propionic and valeric acid) tend to HV monomers which become the biopolymer more flexible and broader to thermal processing (Bengtsson et al., 2008a; Jankowska et al., 2015; Kourmentza & Kornaros, 2016). Consequently, the VFAs profile will be important to obtain the copolymer PHBV

enriched in HB or HV with different characteristics as explained in Section 1.6.1. In other words, the PHA production could be tuned to produce copolymers with different compositions controlling the VFA profile on the acidogenic reactor (Albuquerque et al., 2007; Jayakrishnan et al., 2021; Wei & Fang, 2022). Hence, driving the acidogenic fermentation towards odd VFAs need to be considered for the industrial application of the PHA compounds.

1.6.3. Microbial cultures used for PHA production

The PHAs are synthesized by more than 300 different microorganisms which can be aerobic and anaerobic bacteria, of both Gram-positive and Gram-negative groups (Dias et al., 2006).

Pure microbial cultures. Industrial processes are based on the use of PMCs. The PHA production using PMC is already commercially available but not as a real alternative to conventional plastics by their high production costs (Oliveira et al., 2017). Many bacteria such as *Cupriavadus necator* formally known as *Ralstonia eutropha* or *Alcaligenes eutrophus* (Cavalheiro et al., 2009; Koutinas et al., 2007), different *Pseudomonas* (Cromwick et al., 1996; Jiang et al., 2008), strains of *Azotobacter* species (Kim, 2000; Page et al., 1992), *Bacillus* spp. (Halami, 2008; Law et al., 2003), recombinant *Escherichia coli* (Lee et al., 1997; Liu et al., 1998) and *Burkholdeira* spp. (Nonato et al., 2001; Silva et al., 2004), among others, are capable of synthesise PHAs as intracellular carbon and energy source in form of granules in the cytoplasm (Reddy et al., 2003).

The production of PHA by PMCs is constrained by the need for well-defined feedstock and aseptic process conditions that requires equipment for sterilization, the use of complex and expensive equipment and the control devices when the PHA production is compared with open mixed cultures (Duque et al., 2014; Johnson et al., 2009; Mannina et al., 2020; Sabapathy et al., 2020).

Mixed microbial cultures. The production cost of PHAs could be lowered using MMCs eliminating costs associated with maintaining aseptic conditions and allowing the use of organic wastes or by-products as feedstock to produce a wide range of PHA copolymers (Mannina et al., 2020; Reis et al., 2011). Hence, the PHA production by MMCs is cheaper, more environmentally-friendly, and more sustainable (Wei & Fang, 2022).

To carry out the PHA production using wastes/by-products with MMCs, three stages need to be performed: (i) acidogenic fermentation of the organic carbon to produce VFAs which are the precursors of PHAs, (ii) selection of microorganisms with PHA storage ability, where selective pressure is applied (namely, feast and famine regime) to enrich the MMCs with PHA-storing microorganisms, and (iii) the PHA accumulation, where MMCs selected on the previous stage ii are fed with organic-rich stream (VFAs obtained on the first step) to achieve the maximum PHA content (Duque et al., 2014; Oliveira et al., 2017; Reis et al., 2011) (see Figure 1.10).

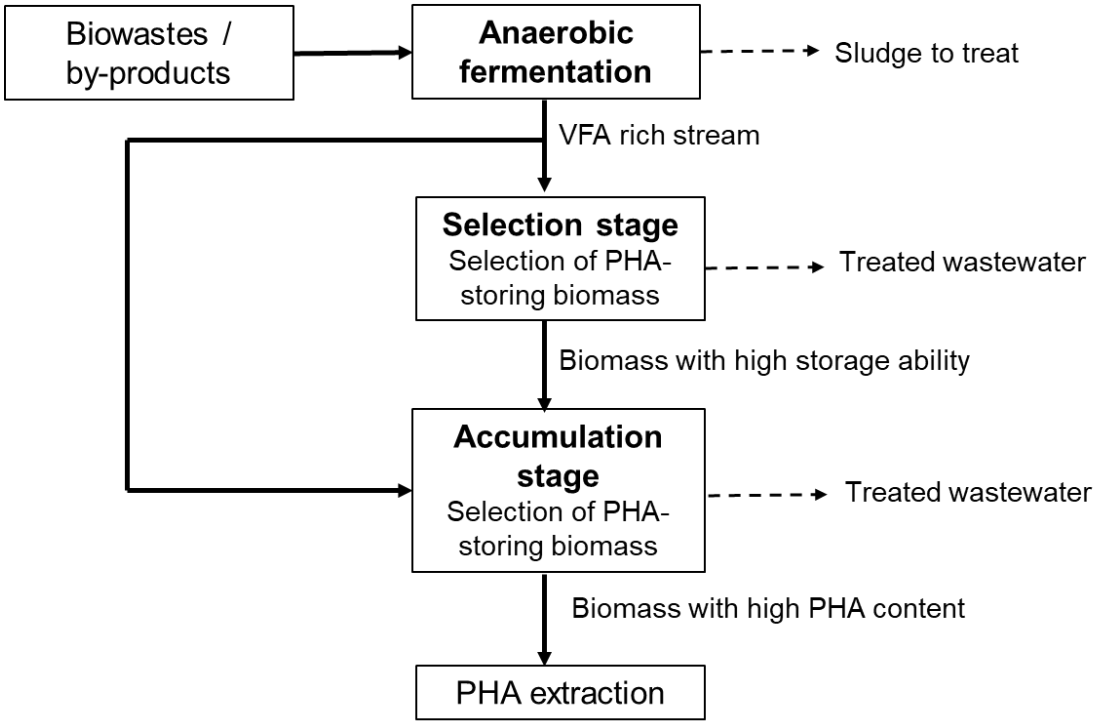

Figure 1.10. Schematic representation of the widely used 3 step process for PHA production from biowaste feeds using MMC (Adapted from Reis et al. (2011)).

1.6.4. Strategies for PHA production using mixed microbial cultures

Alternative processes are studied to lower the production cost using substrates based on wastes and MMCs that requires lower investment and operating costs. Consequently, various strategies are developed to enhance PHA production.

1.6.4.1. Anaerobic dynamic feeding

PHA storage using activated sludge as MMCs under aerobic conditions is carried out by consecutive periods of external substrate excess (feast) and limitation (famine) enhancing the capacity to store PHA (Majone et al., 1996). This process is known as Feast and Famine (F/F) or Anaerobic dynamic feeding (ADF) strategy. The feasibility of this strategy relies on the PHA synthesis under restricted growth conditions. Henceforth, the alternation of substrate availability compelled the organisms to a physiological adaptation (Albuquerque, et al., 2010a). The microorganisms capable to store PHA on the famine stage have an advantage over the rest of the microbial population because they will use the PHA accumulated as a carbon and energy source on the famine phase when the external carbon source is completely depleted (Mannina et al., 2020). The famine phase needs to be long enough to ensure an internal limitation, then the culture will be less fit to grow when external carbon sources were added, accumulating more PHA (Albuquerque et al. 2010a). The microorganisms not allowed to store PHA, are affected by long famine periods and are eliminated from the reactor. Hence, the internal limitation produced by the long famine stage is the main selective pressure to enrich the reactor with PHA-storage microorganisms (Oliveira et al., 2017). Consequently, most studies tested the F/F ratio, concluding that an F/F ratio lower than 0.2-0.3 achieves the enrichment in PHA-storage microorganisms (Albuquerque et al., 2010a; Beccari et al., 2009; Bengtsson et al., 2008b; Dionisi et al., 2007; Duque et al., 2014; Johnson et al., 2009). However, the feast stage must be large enough for complete substrate depletion and PHA storage, and the length of the famine phase must be enough to ensure a significant PHA consumption accumulated previously (Hao et al., 2018; Mannina et al., 2020).

1.6.4.2. Uncoupled carbon and nitrogen feeding strategy

Nutrients are essential in the selection stage to enrich the MMCs in PHA-storing microorganisms and to sustain the high growth of the enriched MMCs (Reis et al., 2011). Even so, most wastes used for PHA production are rich in carbon but poor in nutrients (sugarcane, molasses, paper mill wastewater, olive oil mill water), and nutrient supplementation is required during the selection stage (Albuquerque et al., 2010a; Beccari et al., 2009; Bengtsson et al., 2008b). The production of PHA is favoured when the growth is limited by the lack of another nutrient. Then, when the limited nutrient is added, the PHAs are degraded intracellularly and metabolized as a carbon and energy source increasing rapidly the number of bacteria (Pagliano et al., 2017).

Generally, nutrient-deficient strategy is applied to maximize the PHA accumulation (Johnson et al., 2010; Montiel-Jarillo et al., 2017; Venkateswar Reddy & Venkata Mohan, 2012). This strategy consists of a carbon source feeding without nitrogen content on the famine stage. Once the carbon source is depleted, the nitrogen source was added (Ahmadi et al., 2020). Hence, when C and N feeding are decoupled, the PHA accumulation is favoured on the feast stage while microbial growth is carried out on the famine stage (Lorini et al., 2020; Silva et al., 2017). Consequently, under nitrogen and phosphorus limited concentrations, the biomass grows towards PHAs accumulation rather than protein synthesis (Jayakrishnan et al., 2021; Oliveira et al., 2017; Venkata Mohan & Venkateswar Reddy, 2013). Moreover, the nitrogen limitation also prevents the growth of non PHA-accumulation bacteria, while increasing the PHA storage yield and PHA final content (Lorini et al., 2020; Oliveira et al., 2017; Silva et al., 2017). Henceforth, double growth limitation is applied on the microorganisms (internal and external) using ADF and C and N uncoupling strategies (Oliveira et al., 2017).

Finally, it is important to consider that the PHA storage versus a growth response is also regulated by using different reactor operation conditions such as sludge retention time (SRT), OLR, carbon substrate concentration, nutrient concentration, temperature, etc (Albuquerque et al., 2007; Kourmentza & Kornaros, 2016; Sabapathy et al., 2020; Serafim et al., 2004; Valentino et al., 2015). Moreover, the feedstocks used also have an important effect (Albuquerque et al., 2007; Bengtsson et al., 2008b; Liu et al., 2008; Salmiati et al., 2007).

1.6.5. Integration of PHA production on wastewater treatment plants

From its origin, the WWTPs are aimed to remove contaminants from the water to preserve human health. However, the current situation with the scarcity of resources, demands to consider wastewater as a renewable resource to recover water, materials and/or energy. In this way, the WWTPs have started to be conceived as RRFs or biorefineries as stated in Section 1.3.

The PHA production using MMCs and waste substrate is being demonstrated by several European projects at pilot scale as RES URBIS project (RESources from URban Bio-waSte). RES URBIS project aimed to integrate into a single facility and to use the main technology chain for the bioconversion of several types of urban bio-wastes into value bio-based products, while also minimizing any residual or consequent waste (RESources from Urban BIo-waSte project, Grant agreement ID: 730349). In this way, the integration of co-fermentation in WWTPs using WAS and other co-substrate to produce PHA has been proposed by some authors (Moretto et al., 2019, 2020; Perez-Esteban et al., 2021; Valentino 2019a, 2019b). Hence, more than one waste will be treated in the same facility to enhance a VFA-rich stream to produce PHA.

Figure 1.11 shows a possible WWTP configuration to integrate PHA production and it is based on the three steps needed for PHA production using organic wastes and MMC: (i) acidogenic fermentation, (ii) selection reactor, and (iii) accumulation reactor. After the PHA accumulation stage, the PHA-rich biomass will be processed for PHA extraction and purification. To carry out this process, a pre-conditioning of VFA-rich stream is required to remove the solids which could reduce the purity of the recovered PHA (Moretto et al., 2020). Moreover, in this process not only PHA is produced, but also biogas and fertilizer are achieved valorising all waste streams being a promising configuration.

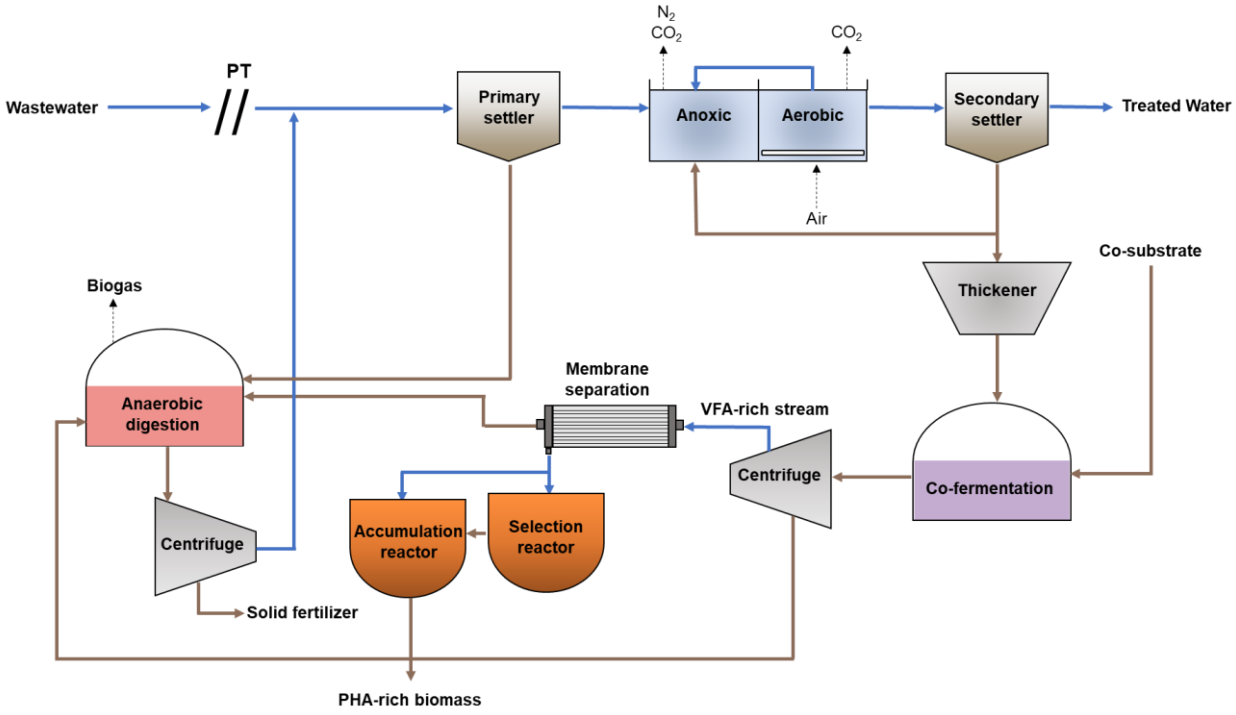

Figure 1.11. Schematic representation of configuration proposed to integrate the co-fermentation for PHA production in a WWTP (Adapted from Perez-Esteban et al. (2022)).

2. Objectives and thesis structure

In this chapter, the motivation and objectives of the thesis will be presented as well as the thesis structure.

2.1. Motivation and objectives

As stated in Chapter 1, the rapid population growth is resulting in huge environmental problems with a massive waste generation and a high amount of organic wastes that should be properly treated. This current situation leads to the integration of biorefineries to valorise organic wastes into value-added products throughout the circular economy concept. Within this scenario, the acidogenic fermentation emerges as a key process to produce VFAs, building blocks in the so-called carboxylate platform, from organic wastes.

Although FW is a highly heterogeneous feedstock and its VFA yield and profile could be strongly affected not only by the operating conditions but also by its composition, it is well known that FW acidogenic fermentation is generally limited by hydrolysis and its low alkalinity content. On the other hand, WAS fermentation is limited by its low hydrolysis rates and biodegradation. Consequently, the co-fermentation of FW and WAS stands as a new opportunity to overcome the limitations of both substrates on acidogenic fermentation treating two urban wastes in the same facility. However, there is little knowledge about the effect of the co-fermentation mixture without pH adjustment. Moreover, the effect of the WAS and FW mixture, the FW composition and the study in the semi-continuous mode are still limited.

Moreover, the produced VFAs could be used for the production of PHAs using MMCs under the alternation of feast/famine conditions and carbon and nitrogen uncoupled feeding strategies to select PHA-storing biomass and subsequently increase its PHA content by the pulse-feeding of a VFA-rich fermentation stream.

These considerations are the motivation of this thesis, which deals with the enhancement of FW fermentation, as well as WAS and FW co-fermentation, to

maximize VFA yields and/or to tune the VFA profiles for PHA production using MMCs. To reach this general objective, the following specific goals are proposed:

- To study the effect of pH on the acidogenic fermentation of FW (collected in a university canteen) in batch and semi-continuous fermenters.
- To test the impact of WAS and FW co-fermentation at different proportions on the VFA yield and profile with and without external control of pH or alkalinity.
- To determine the feasibility of WAS auto-hydrolysis pre-treatment to improve the fermentation yields.
- To test the results reproducibility throughout the batch test assays.
- To understand how FW composition could influence the VFA yield and profile during its co-fermentation with WAS.
- To study the effect of OLR and HRT on semi-continuous co-fermentation of FW and WAS.
- To determine the role of WAS of different origins on WAS and FW co-fermentation.
- To start-up and operate a selection reactor of PHA-storing microorganisms in a SBR fed with a synthetic VFA solution at different OLR
- To evaluate the increase of PHA content in biomass purged in the selection SBR using a synthetic VFA solution pulse feeding.

2.2. Thesis structure

This thesis is divided in nine chapters:

Chapter 1: Introduction

This first chapter provides a general overview of the main concepts studied throughout the thesis. Hence, the current problems in organic waste management as well as the typical treatments used in the European Union are explained. Then, the main features of the acidogenic fermentation process are discussed to understand why it stands as key technology to convert conventional organic wastes treatment plants into

biorefineries. Finally, PHA production using VFA-rich fermentation effluents are briefly explained.

Chapter 2: Objectives and thesis structure

In chapter 2, the justification and objectives of this study are presented, as well as the structure followed throughout the thesis.

Chapter 3: Materials and methods

In this chapter, the biological reactors for acidogenic fermentation (in batch mode and continuous mode) and for selection and accumulation of PHA-storing biomass are presented. Moreover, the inoculum and substrates used, and all the analytical methods applied to perform the experimental research of the thesis are detailed.

Chapter 4: Volatile fatty acids production from food waste under different working pH

The study of canteen FW fermentation in batch mode and semi-continuous reactors at mesophilic temperature is presented in this chapter, considering the heterogeneous composition of the FW. The effect of the pH on the fermentation process is studied firstly in short-term assays (pH 4.0, 6.0, 7.5, 9.0, 10.0, 11.0 and uncontrolled) to determine the best conditions to ferment FW. Hence, the operation of two reactors working at acidic (pH 6.0) and alkaline conditions (pH 9.5-10.0) is discussed to evaluate the pH effect and the microorganisms' adaptation to pH changes on medium-term operation.

Chapter 5: Assessing the potential of waste activated sludge and food waste co-fermentation for carboxylic acids production

In this chapter, the co-fermentation of WAS and FW is studied throughout three experiments in batch mode to produce carboxylic acids using different feedstock

mixtures. Hence, three mixtures were used in all the experiments: (i) 50%WAS +50% FW, (ii) 70%WAS+30%, and (iii) 90% WAS + 10% FW (on VS basis). All the tests were performed at mesophilic conditions without pH adjustment and without inoculum addition. Moreover, the effect of alkalinity addition and WAS auto-hydrolysis pre-treatment were studied too.

Chapter 6: Impact of food waste composition on acidogenic co-fermentation with waste activated sludge

In this study, WAS and FW co-fermentation batch tests were performed to elucidate how FW composition influences the fermentation yield and profile. To this end, various batch tests at mesophilic conditions were performed using WAS as the main co-substrate (70% on VS basis) with various components of FW (i.e., rice, pasta, meat, fish, fruit, vegetables, and cellulose) to study the influence of the FW composition on WAS and FW co-fermentation.

Chapter 7: Effect of the organic loading rate on the acidogenic co-fermentation of waste activated sludge and food waste

In this chapter, WAS and FW co-fermentation is studied in a semi-continuous fermenter by progressively increasing the OLR to prove the long-term influence of the operational parameters. The co-fermentation was carried out in a jacketed 5L reactor for 160 days without pH control and without an initial inoculum addition. Specifically, four phases were performed increasing the OLR (9, 11, 14 and 18 gVS/(L·d)) increasing the FW influent flowrate while feeding a constant WAS flowrate. Moreover, another identical reactor was run using only WAS as control.

Chapter 8: Study of a sequencing batch reactor for the selection of polyhydroxyalkanoates accumulating cultures

The start-up and performance of sequencing batch reactor to select PHA-storing microorganisms using synthetic feeding is performed in this chapter. The selection was based on feast/famine and uncoupled carbon and nitrogen feeding strategies.

Moreover, three periods were operated by increasing the OLR and the nitrogen loading rate. Finally, the PHA-storing microorganisms were fed to an accumulation reactor, aimed to maximize the PHA content on the biomass.

Chapter 9: General conclusions and recommendations

In this chapter, the general conclusions of the experimentation carried out in this thesis are compiled and recommendations for further studies are proposed.

3. Materials and methods

3.1. Experimental set-up

In this study, several experimental devices have been used related to the steps of PHA production from organic wastes: (i) acidogenic fermentation and (ii) PHA-storing biomass selection and PHA accumulation. The acidogenic fermentation assays were carried out in discontinuous tests (batch assays) and in semi-continuous lab-scale reactors. Moreover, the selection of PHA-storing organisms was performed in a lab-scale SBR, while PHA accumulation was carried out in a batch reactor. In this section, the main characteristics of the different experimental set-ups used in this study are presented, although the specific operational conditions of each experimental work are detailed in the corresponding chapter due to the variety of operational conditions applied.

3.1.1. Fermentation batch assays

The fermentation batch tests were performed in Pyrex serum bottles (250 mL) filled with the corresponding amount of inoculum (when necessary) and substrates (see Figure 3.1). All tests were performed in triplicate, as well as their controls to assess the VFAs production of the inoculum and/or the substrates alone. Anaerobic conditions were initially achieved by flushing the headspace of the bottles with N_2 for 2 min (ca. 5L/min). Hence, they were sealed with a PTFE-butyl septum with a screwcap. Finally, the bottles were placed in a temperature-controlled incubator (UF750, Memmert GmbH) where the operating temperature was fixed depending on the conditions used in each chapter and mixed manually once a day. Once the assays started, samples were taken periodically through the septum using an 18G hypodermic needle connected to a 5 mL plastic syringe to minimise air exposure. The total sampling withdrawal always represents, as maximum, 20% of the initial volume. The duration of these assays was set between 10 and 14 days depending on the conditions of each chapter as will be later explained.

Figure 3.1. Pyrex serum bottles used in batch tests.

3.1.2. Semi-continuous lab-scale fermenters

The acidogenic fermentation or co-fermentation of organic wastes was carried out in two jacketed 5L lab-scale reactors working with different effective volumes as described in Chapters 4 and 7. Mixing was provided by a mechanical stirrer (RZR 2020, Heidolph) at 100 rpm. The operational temperature was controlled using a thermostatic bath (Termotronic, JP Selecta). The feeding and the effluent discharge were performed manually once per day, and the mixture was prepared daily before the feeding to avoid substrate degradation. In Chapter 4, the fermenter was flushed with N_2 gas when the effluent was extracted to avoid a pressure drop inside the reactor and the entrance of air (Figure 3.2A). However, the reactors' configuration was slightly modified in Chapter 7, where the flushing of nitrogen was avoided by connecting the gas outlet to a 1L Tedlar sample bag with polypropylene fitting (Tedlar® Sample Bag, SKC) and a feeding glass tube was installed to facilitate the feeding and withdrawal of the fermenter (Figure 3.2B).

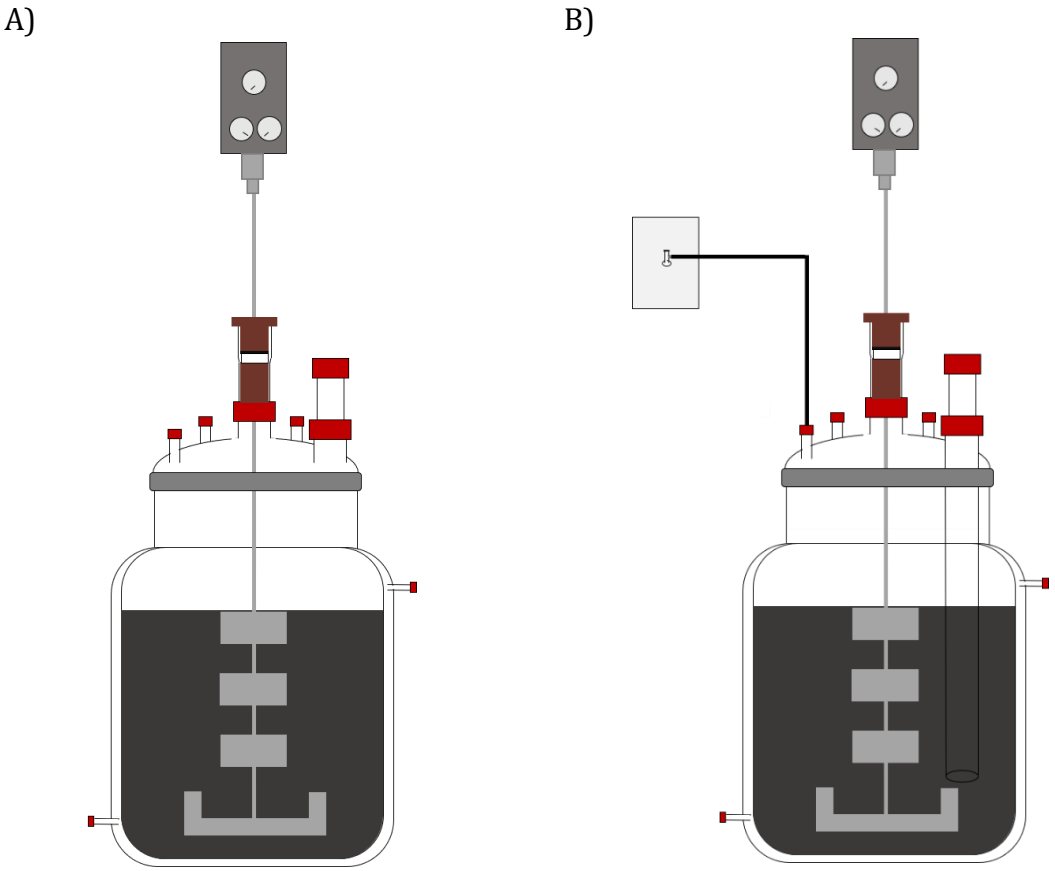

Figure 3.2. Schematic representation of the semi-continuous lab-scale reactors used in Chapter 4 (A) and Chapter 7 (B).

3.1.3. PHA selection reactor

The selection of PHA-storing biomass was accomplished in a jacketed SBR with 3L of effective volume working at 30 ºC using a thermostatic bath (Termotronic-100, JP Selecta). The reactor was equipped with a mechanical stirrer (RW 16 basic, IKA) at 80 rpm. Aeration was provided by air pumps (Mouse air pump, Epsilon) connected to porous stone diffusers located at the bottom of the reactor. A pH probe (HA405-DPA-SC-S8/225, Mettler Toledo) and a dissolved oxygen (DO) probe (CellOx 325, WTW) were used to monitor the process. In this way, the DO probe was connected to an oxygen portable meter (Oxi 3310, WTW) to transmit continuous data to the PC through a USB connection. The software MultiLab® Importer was installed to capture

data via Excel allowing the DO concentration control inside the reactor throughout the day. HRT and SRT were controlled as detailed in Chapter 8.

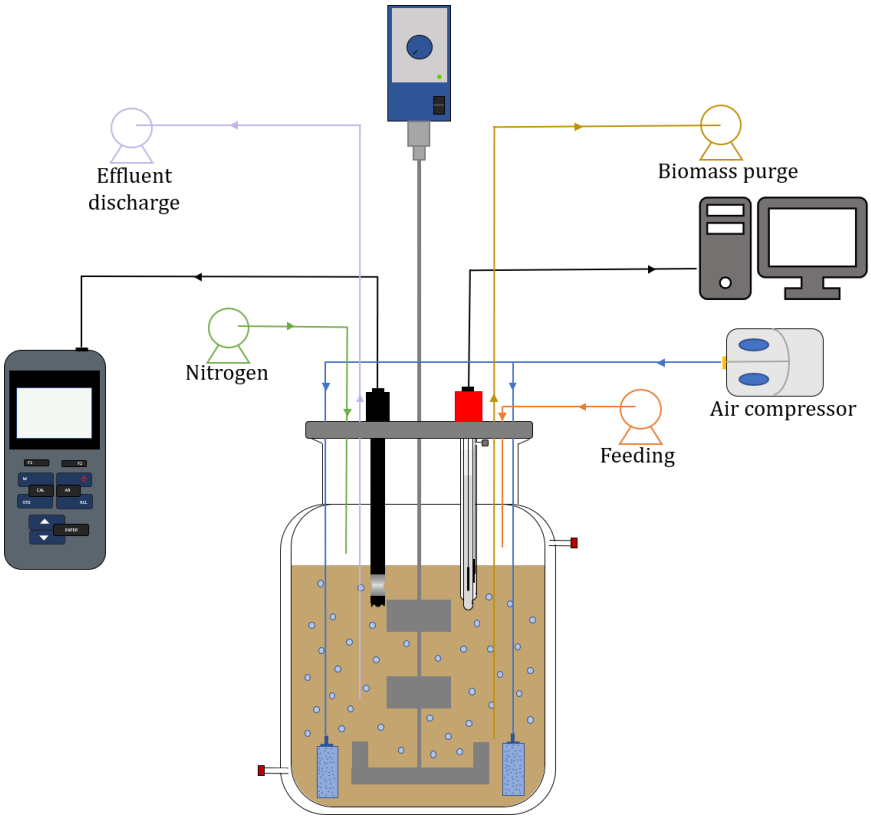

Figure 3.3. Schematic representation of the lab-scale SBR used for PHA biomass selection.

The sequence of fill and draw phases in the SBR cycles were carried out using four peristaltic pumps (Reglo Ismatec and Percom N-M, JP Selecta) connected to timers (10.047.65, Smartwares). The SBR cycles with a total length of 6h consist of: (i) carbon source feeding of VFAs in anaerobic conditions, (ii) aerobic reaction with air supply and agitation to consume the carbon source and convert it as intracellular PHA , (iii) biomass purge to obtain microorganisms with a maximum PHA accumulation capacity, (iv) aerobic ammonium feeding to allow the growth of the microorganisms, (v) biomass settling after turning off the agitation and air supply and, finally, (vi) the treated effluent discharge (Figure 3.4). The time distribution was adjusted depending on the operational phase and will be explained in Chapter 8.

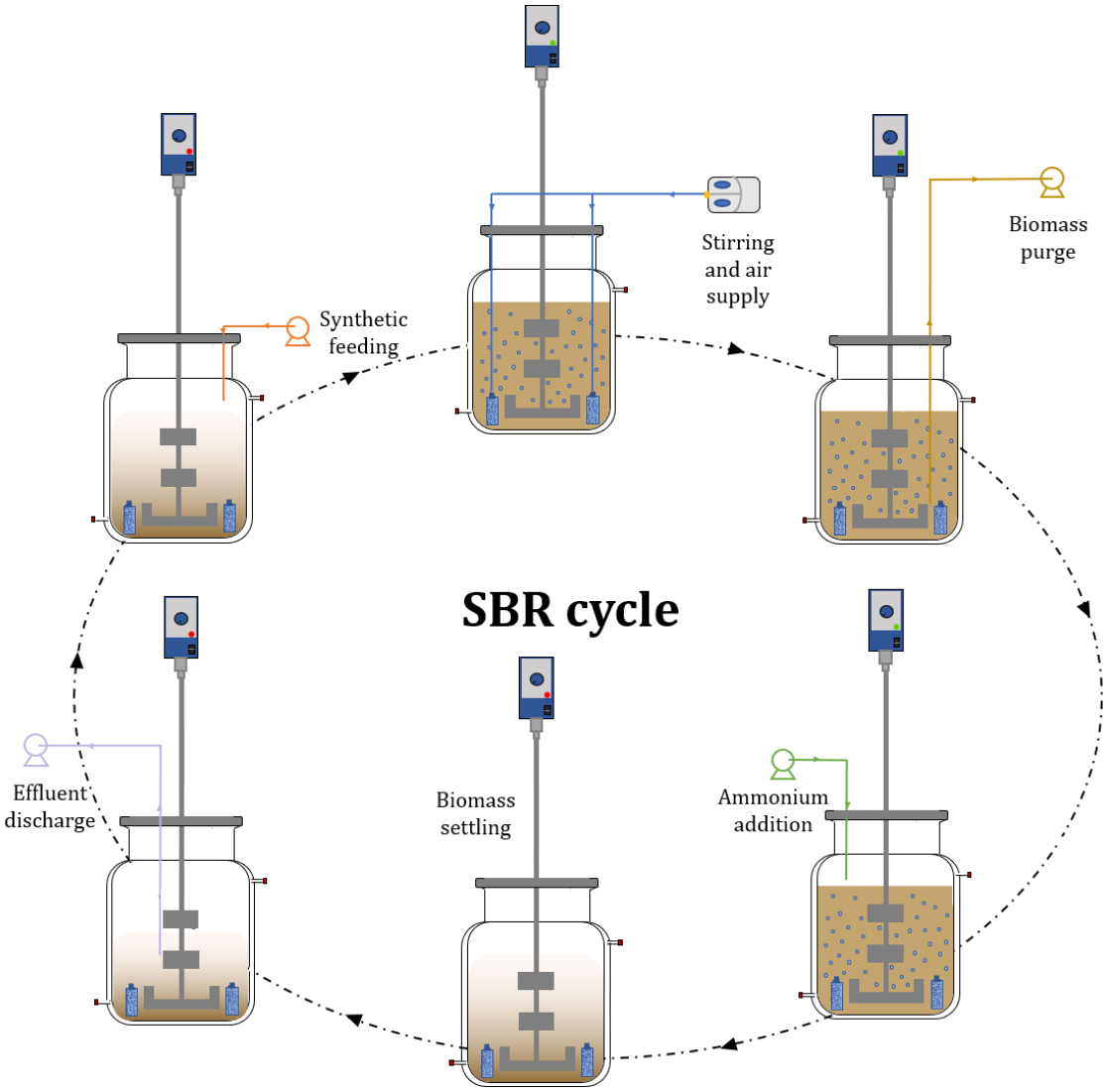

Figure 3.4. Schematic diagram of SBR performance for selection of PHA-storing biomass.

3.1.4. PHA accumulation reactor

Once PHA-storing organisms were selected in the previous SBR, the biomass purge collected was added in a jacketed glass reactor of 1L of effective volume (Figure 3.5) with the aim to maximize the PHA content of this biomass. The reactor was operated at 30 ºC by means of a water heating system (Termotronic-100, JP Selecta). Moreover, the reactor was equipped with a mechanical stirrer (RW 16 basic, IKA) and excess air was supplied through porous stone diffusers. As the selection reactor, pH and DO were monitored in the accumulation reactor using a pH probe (HA405-DPA-SC-S8/225, Mettler Toledo) and a DO probe (Cellox 325, WTW) connected to a portable meter (Oxi 3310, WTW) that allowed a continuous data acquisition through the PC. In comparison

to the selection reactor, the accumulation reactor was fed manually depending on DO concentration inside the reactor. The length was variable depending on the assay as detailed in Chapter 8.

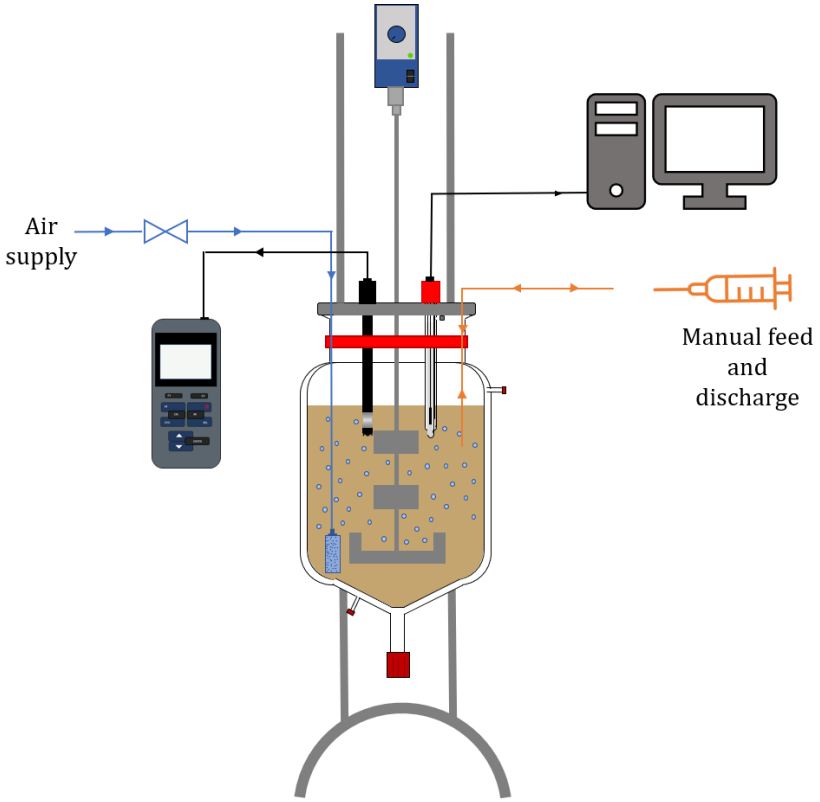

Figure 3.5. Schematic representation of batch reactor used for PHA accumulation.

3.2. Substrate and inoculum

Several organic substrates have been tested in this study: FW from university canteen, synthetic FW, WAS and synthetic feeding simulating the OFMSW for PHA production.

Real FW substrate was used in Chapter 4. FW of university canteen was collected every two weeks and immediately blended with deionised water and shredded in the laboratory. The feedstock was stored in a refrigeration chamber at 4 ºC concentrated. Deionized water was added before feeding to adjust the concentration of total solids.

To minimize the great variability of real FW synthetic FW was used in Chapters 5, 6 and 7. Moreover, synthetic FW was made to facilitate the reproducibility of the

discontinuous assays using products available in the supermarket all year-round, simulating the real FW composition. Once selected, the products were blended and diluted with deionised water to adjust the total solids content. This feedstock was prepared and stored in the refrigerator at 4 ºC a day before fermentation tests started.

WAS was also used in Chapters 5, 6 and 7 for the co-fermentation of FW. This WAS was collected from the gravity thickener after the secondary clarifier in a municipal WWTP which works with membrane bioreactor (MBR) with 300,000 population equivalent from the Barcelona metropolitan area (Spain). In Chapter 7, a second WAS from conventional anaerobic/aerobic (A/O) process with 4,000 population equivalents was used too. Once collected, this sludge was stored in the refrigerator at 4 ºC until use.

Finally, synthetic wastewater representing the fermentation liquid of OFMSW (Dosta et al., 2018) was used to produce PHA in Chapter 8. This substrate was prepared using a mixture of acetic acid, propionic acid, and butyric acid solution, and adding macronutrients and micronutrients to ensure the microorganisms' growth. The detailed information about this synthetic substrate can be found in Chapter 8.

The inoculums used are dependent on the specific chapter of this thesis. For some fermenters, WAS was used as inoculum, whereas, on some occasions, biomass withdrawn from other lab-scale reactors already in operation were used to start-up the process.

The main characteristics of these substrates and inoculums are summarised in every chapter.

3.3. Analytical methods

The analytical methods used in this work are detailed in this section. These methods were performed according to *Standards Methods for the examination of Water and Wastewater* (APHA, 2017).

3.3.1. Solids content

Total Solids (TS) and Volatile Solids (VS) content were determined using the standard methods 2540B and 2540E, respectively, from the *Standards Methods for the examination of Water and Wastewater* (APHA, 2017). To determine the TS and VS content a known volume (V) of a well-mixed sample was added in a porcelain capsule previously weighed (W_1) and it was maintained 24h at 105 ºC in the laboratory oven (Drying and sterilization oven Conterm analogue, JP Selecta) to completely evaporate the water. Right afterwards, the capsule was introduced in a desiccator until ambient temperature and was weighed (W_2) allowing obtaining the TS content calculated by means of Equation 3.1. Following this, the porcelain capsule was placed at 550 ºC for 2h in the muffle (HD-230, Hobersal). Then, the capsule was introduced in the desiccator until ambient temperature and was weighed (W_3) to calculate the VS content using Equation 3.2.

$$\mathrm{TS(g/L)} = \frac{\mathrm{W_2(g)} - \mathrm{W_1(g)}}{\mathrm{V(L)}} \tag{3.1}$$

$$\mathrm{VS(g/L)} = \frac{\mathrm{W_2(g)} - \mathrm{W_3(g)}}{\mathrm{V(L)}} \tag{3.2}$$

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were performed using the 2540D and 2540E reference methods, respectively, of the *Standard Methods for the examination of Water and Wastewater* (APHA, 2017). Firstly, a Millipore standard filter of 1.2 μm was introduced in muffle at 550 ºC for 10 minutes to ensure that the filter does not contain impurities. Then, this filter was weighed (W_4) and a known volume of well-mixed sample (V) was filtered through itself using a filtration system with a vacuum pump (D-95, Dinko) (Figure 3.6). After sample filtration, three washings of 10 mL of deionised water were carried out to remove all possible traces.

Figure 3.6. Filtration system connected to a vacuum pump.

The filter was placed at 105 ºC for 24h in the laboratory oven (Drying and sterilization oven Conterm analogue, JP Selecta), afterwards in a desiccator for 10 minutes and it was weighed (W_5). The TSS was calculated using Equation 3.3. Finally, the filter was introduced in a muffle (HD-230, Hobersal) for 15 minutes at 550 ºC. Then, it was placed in the desiccator for 10 minutes and it was weighted (W_6). Hence, the VSS content was obtained using Equation 3.4.

$$\mathrm{TSS(g/L)} = \frac{\mathrm{W_5(g)} - \mathrm{W_4(g)}}{\mathrm{V(L)}} \tag{3.3}$$

$$\mathrm{VSS(g/L)} = \frac{\mathrm{W_5(g)} - \mathrm{W_6(g)}}{\mathrm{V(L)}} \tag{3.4}$$

3.3.2. Chemical Oxygen Demand

The chemical oxygen demand (COD) indicates the quantity of matter present in the sample that is susceptible to be oxidised chemically. In this way, the COD is expressed as mg COD/L representing the oxygen-equivalents needed to oxidise the organic matter present in the sample. To analyse COD, the colorimetric reference method 5220D of Standard Methods (APHA, 2017) was used. This method is based on the complete oxidation of the organic matter contained in the sample with a strong oxidising agent (namely, potassium dichromate) under acidic conditions (achieved

using sulphuric acid in excess). The reaction of potassium dichromate with organic compounds is shown in Equation 3.5. Moreover, silver sulphate was used as a catalyst of the reaction. Mercury (II) sulphate was also added to avoid chloride interference in the COD determination (see Equation 3.6).

$$C_nH_aO_bN_c + \left(\frac{2n}{a} + \frac{a}{6} - \frac{b}{3} - \frac{c}{2}\right) Cr_2O_7^{2-} + (8d + c)H^+ \rightarrow \\ \rightarrow nCO_2 + \left(\frac{a + 8d - 3c}{2}\right) H_2O + cNH_4^+ + 2dCr^{3+} \quad (3.5)$$

$$6Cl^- + Cr_2O_7^{2-} + 14H^+ \rightarrow 3Cl_2 + 2Cr^{3+} + 7H_2O \quad (3.6)$$

Therefore, to carry out the measurement, 2.5 mL of the sample were mixed with 1.5 mL of sodium dichromate 0.04 mol/L (with 80 g/L of mercury (II) sulphate) and 3.5 mL of silver sulphate solution 10 g/L in sulphuric acid. Moreover, five patrons of 0, 50, 250, 500 and 1000 mg COD/L were prepared using potassium biphthalate with the equivalence 1.176 mgCOD/mg potassium biphthalate to perform the calibration curve. The patrons were mixed with the reagents as samples. Therefore, the samples and patrons were maintained at 150 ºC for 2h in a COD digester (ECO 25 thermoreactor, VELP Scientifica; see Figure 3.7a) to ensure complete oxidation. After the digestion, the samples were stored at ambient temperature until the next day to ensure the decantation of formed solids. Finally, the absorbance (ABS) of the samples and patrons was analysed in a spectrophotometer (UviLine 9100, SI Analytics; see Figure 3.7b) at λ=600 nm. With the obtained absorbance of the patrons, a calibration curve was obtained and used to correlate the COD concentration with the ABS of samples (see Equation 3.7). To analyse soluble chemical oxygen demand (sCOD), the samples were previously filtered through a 0.45 μm syringe filter analysis.

$$COD\ (mgCOD/L) = m\ x\ ABS + b \quad (3.7)$$

A) B)

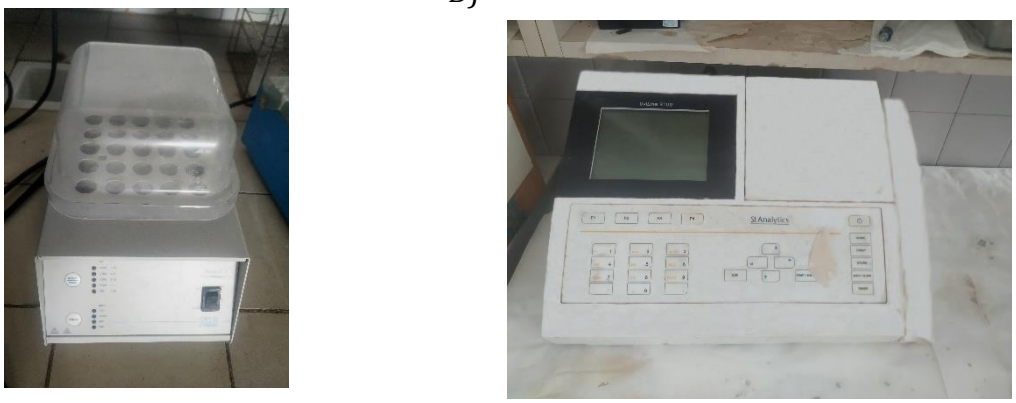

Figure 3.7. Equipments used to carry out COD analysis: COD digester(A) and spectrophotometer (B).

3.3.3. Alkalinity

The alkalinity parameter is used to determine the buffer capacity of the sample to neutralise acids. Usually, this buffer capacity is related to the bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) content. Even so, the alkalinity capacity also may include buffering substances such as borates, silicates, phosphates, ammonium, sulphides, and hydroxide (OH^-). To carry out the measurement the 2320B standard method (APHA, 2017) was followed. A titrator (pH Burette 24, CRISON; see Figure 3.8) connected to a pH probe (Basic 20 pHmeter, CRISON) was used to add automatically HCl 0.1 N in steps of 0.5 mL every 20 seconds in a known volume of sample. In this way, the pH is controlled until the desired endpoint. The volume of HCl added at each endpoint was important to determine the alkalinity. Specifically, the volume added to achieve pH 5.75 is necessary to determine the total alkalinity (Alk_T) and volume of pH 4.3 to determine partial alkalinity (Alk_p). Moreover, the acid alkalinity (Alk_a) was determined by the difference between Alk_T and Alk_P.

Figure 3.8. Automatic titration device used to measure alkalinity.

The alkalinity in terms of mg$CaCO_3$/L is calculated using the Equation 3.8.

$$\text{Alkalinity (mg } CaCO_3\text{/L)} = \frac{mL_{HCl} \text{ x } \frac{0.1 \text{ mmol HCl}}{1 \text{ ml HCl}} \text{x} \frac{1 \text{ mmol } CaCO_3}{2 \text{ mmol HCl}} \text{x} \frac{100 \text{ mg } CaCO_3}{1 \text{ mmol } CaCO_3}}{mL_{sample} \text{ x } \frac{1 \text{ L}_{sample}}{1000 \text{ mL}_{sample}}} \quad (3.8)$$

3.3.4. Total Ammonium Nitrogen

The concentration of total ammonium nitrogen (TAN, NH_4^+-N) was determined with a specific ammonia electrode (Orion 9512HPBNWP, Thermo Scientific) connected to an mV meter (Orion DualStar pH/ISE Benchtop, Thermo Scientific) (see Figure 3.9) following the standard method 4500-NH_3D (APHA, 2017).

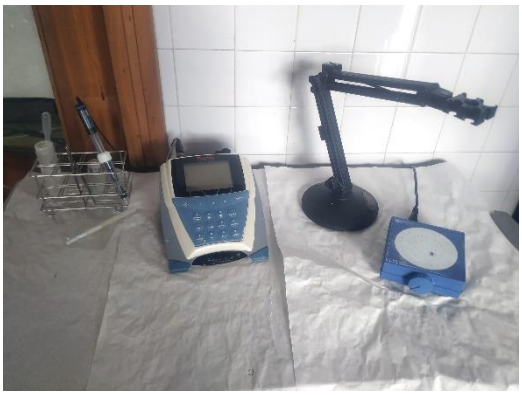

Figure 3.9. Ammonia electrode used to analyse TAN.

This method is based on the completely conversion of dissolved NH_4^+-N to free ammonia nitrogen by raising the pH above 11 adding a strong base (NaOH) (see Equation 3.9). Therefore, a subsequent NH_3(g) diffusion through the hydrophobic gas-permeable membrane of the electrode is carried out. When the base is added, the electrode is immediately submerged into the sample to detect the potential variation (ΔV) in mV. To obtain the relation between potential and concentration, five N-NH_4^+ standards of 1, 5, 25, 50 and 100 mg NH_4^+-N /L were prepared and analysed (Equation 3.10).

$$NH_4^+ (aq) + NaOH \rightarrow NH_3 (g) + H_2O + Na^+ \quad (3.9)$$

$$Ln\ (NH_4^+ - N) = a\ x\ \Delta V + b \quad (3.10)$$

3.3.5. Total Kjeldahl Nitrogen

The Total Kjeldahl Nitrogen (TKN) determines the sum of organic nitrogen and NH_4^+-N. This measurement was carried out in three steps: (i) digestion, where organic nitrogen is converted into NH_4^+-N, (ii) distillation, where NH_4^+-N (converted to free ammonia) is collected in receptor recipient, and (iii) total ammonium nitrogen measurement using a specific ammonia electrode.

The aim of the digestion is to break all nitrogen bonds of the sample and convert all organically bound nitrogen into ammonium ions (NH_4^+-N). Hence, 3g of sample were weighed and diluted with 15 mL of deionized water in a Kjeldahl tube. Three Kjeldahl

tablets (composed of 96.5% K_2SO_4, 1.5% $CuSO_4 \cdot H_2O$ and 2% Se) were added to catalyst the reaction: K_2SO_4 was used to elevate the boiling temperature of sulfuric acid, while the copper salt and selenium were used as catalysts to improve the rate and efficiency of the process. In the same tube, pumice stone was added to control the boiling process with 15 mL of H_2SO_4 96-98% to digest the sample (see the simplified equation of the process, Equation 3.11). Once the sample was prepared, it was introduced in a Kjeldahl digester (Bloc Digest 20, JP Selecta; see Figure 3.10) starting the process at 150 ºC for 15 min to evaporate the water. Then, the digestion was carried out at 250 ºC for 30 min to reduce white smoke production. Finally, the digestion was held at 400 ºC for 90 min. Once the sample was digested, it was cooled at ambient temperature to introduce 25 mL of water in each tube until distillation.

$$\text{Protein}\ (-\text{N}) + \ \text{H}_2\text{SO}_4 \rightarrow (\text{NH}_4)_2\text{SO}_4 + \ \text{CO}_2 + \ \text{H}_2\text{O} \qquad (3.11)$$

Figure 3.10. Kjeldahl digester. *Source: JP Selecta.*

The distillation process started with the conversion of ammonium ions (NH_4^+-N) of digested sample to free ammonia (NH_3) by adding a strong base (NaOH 10M) (Equation 3.12) in the distillation unit (Pro-nitro M, JP Selecta; see Figure 3.11). A water stream was then bubbled in the sample while dragging the NH_3 formed. This free ammonia was condensed and collected in an Erlenmeyer reception glass that contained 50 mL of H_2SO_4 (0.04 mol/L) to convert NH_3 into NH_4^+ (Equation 3.13).

$$(NH_4)_2SO_4 + 2NaOH \leftrightarrow 2NH_3\,(gas) + Na_2SO_4 + 2H_2O \quad (3.12)$$

$$H_2SO_4 + 2NH_3 \rightarrow SO_4^{2-} + 2NH_4^+ \quad (3.13)$$

Figure 3.11. Distiller used for the TKN analysis.

Finally, the NH_4^+-N was measured with the specific ammonia electrode explained previously in section 3.3.4.

3.3.6. Anions and cations determination

The ionic chromatograph (IC) (861 Advanced Compact IC, Metrohm) with autosampler (863 Compact Autosampler, Metrohm) was used to determine anions and cations species in a liquid sample (Figure 3.12). The ionic chromatograph was equipped with an anionic column (Metrosep A Supp 17 – 250/4.0 mm) with its guard column (Metrosep A Supp 17 Guard/4.0) and cationic column (Metrosep C 4 – 150/4.0 mm) with its guard column (Metrosep C 4 Guard/4.0). On the one hand, the anionic column of polystyrene/divinylbenzene copolymer allowed to analyse F^-, Cl^-, NO_2^-, NO_3^-, Br^-, SO_4^{2-} and PO_4^{3-} using a mobile phase composed of 0.2 $NaHCO_3$ mmol/L and 5.0 Na_2CO_3 mmol/L. On the other hand, the cationic column of silica gel with carboxyl groups was used to detect Li^+, Na^+, NH_4^+, K^+, Ca^{2+} and Mg^{2+} using a mobile phase of 1.7 mmol/L HNO_3 and 0.7 mmol/L $C_7H_5NO_4$.

Figure 3.12. Ionic chromatograph with autosampler.

To perform the measurement, it was necessary to centrifuge and filter the sample through 0.45 μm previously. Hence, the sample was injected with the autosampler into a sample loop with a known volume. The mobile phase carried the sample until the column and finally the conductivity was continuously measured at the column outlet, knowing the residence time of each ion to be determined. The only difference between cation and anion analysis was that, to analyse anions, the sample did not pass directly from the column to the detector. In this case, the sample passed through a suppressor that consisted of a rotor that contained three cartridges. While the first cartridge was used for suppression, the second cartridge was regenerated with diluted acid (H_2SO_4). The third cartridge was rinsed with water during this time ensuring a freshly regenerated suppressor cartridge available for each new sample with each rotor change. Hence, the sample passed to the detector giving a response in terms of peaks by conductivity signal, corresponding to a different species previously calibrated with the area of the peak related to its concentration. Typical chromatograms obtained in both cases are shown in Figure 3.13.

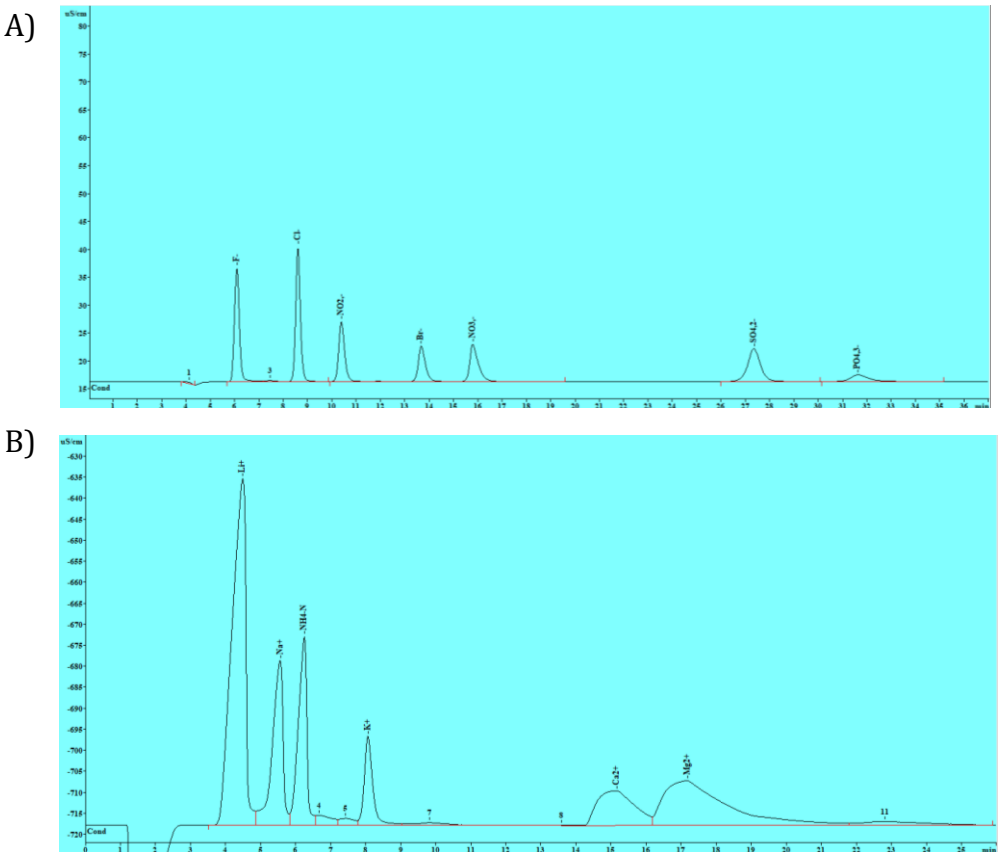

Figure 3.13. Typical chromatogram obtained with anion column (A) and cation column (B).

3.3.7. Volatile fatty acids and alcohols determination

In chapters 4 and 5, the analysis of VFAs was performed using gas chromatography (GC) (GC 2010 plus, Shimadzu), equipped with a capillary column (Nukol™, 15 m x 0.53 mm x 0.5 µm) and flame ionised detector (FID) as shown in Figure 3.14. The temperature of the capillary column started at 80 ºC and it was heated by 10 ºC/min to 110 ºC. From then, the temperature was increasing 15 ºC/min until 145 ºC and, finally, it was increased 20 ºC/min to 190 ºC. The temperature of the injector and detector was set at 280 ºC and 300 ºC, respectively. Helium was used as carrier gas, hydrogen as fuel gas and synthetic air as oxidising gas. With this configuration, the following VFAs were detected: acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic.

Figure 3.14. Gas chromatograph GC 2010 plus, Shimadzu for VFAs analysis.

In chapters 6 and 7, different configuration with another column (Agilent technologies J&W DB-FFAP, 30 m x 0.25 mm x 0.25 μm) was used to analyse alcohols (XOH) too. The temperature of the column started at 60 ºC holding the temperature 2 min. Then, the temperature was increasing 20 ºC/min until 240 ºC holding the temperature for 2 more min. In this configuration, the temperature was 220 ºC in the injector and 250 ºC in the detector. The gases used in the process are the same cited previously with the following flows: 30 mL/min, 40 mL/min and 400 mL/min for helium, hydrogen and synthetic air, respectively. In this case, the same VFAs were detected (acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic) but also ethanol, propanol and butanol were detected (see Figure 3.15 for calibration of VFAs (A) and alcohols (B)).

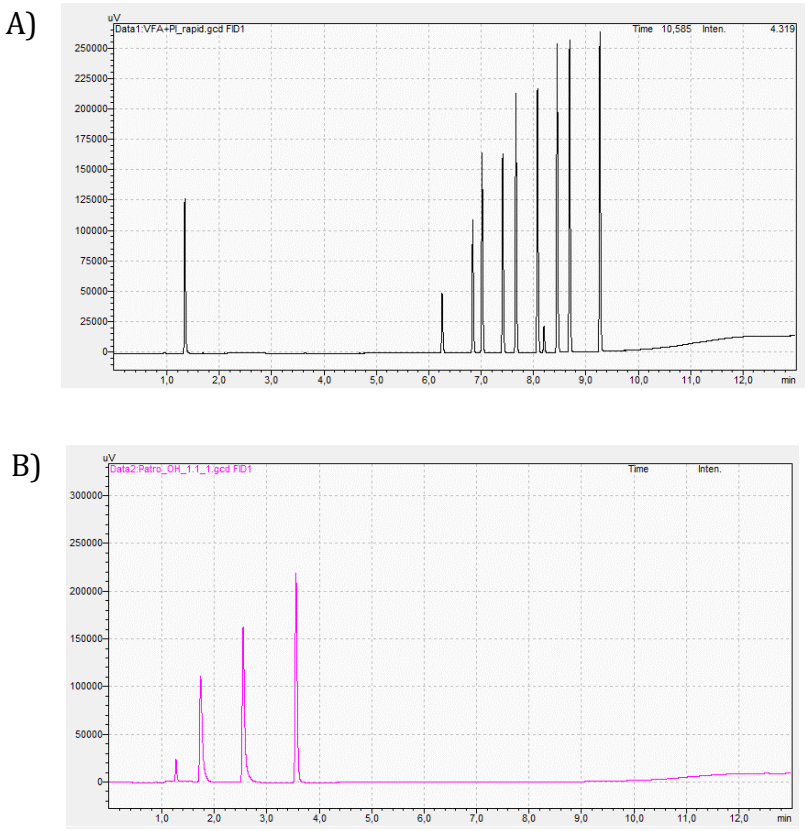

Figure 3.15. Typical chromatogram obtained for VFAs (A) and alcohols (B).

During this investigation, VFAs and alcohols mg were converted in form of COD using the following conversion factors for acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, heptanoic, ethanol, propanol and butanol: 1.07, 1.51, 1.82, 1.82, 2.04, 2.04, 2.21, 2.21, 2.34, 2.1, 2.4 and 2.6 $mgCOD/mg_{compound}$ respectively.

3.3.8. Lactic acid determination

Lactic acid was analysed in Chapter 5 using high-performance liquid chromatography (HPLC, Waters Alliance 2695, US) equipped with column (Aminex HPX-87H, 300 mm x 7.8 mm with a particle size of 9 µm) and Waters 2996 photodiode array detector (detector Waters 2996, US) with 210 nm wavelength (see Figure 3.16). The detection of acid lactic was carried out at HPLC constant temperature oven at 60 ºC. Furthermore, the solvent used was H_2SO_4 10mM with a constant flow of 0.6 mL/min and injection

volume of 100µL. As explained before, lactic acid was converted in form of COD using 1.07 $mgCOD/mg_{lacticacid}$ as conversion factor.

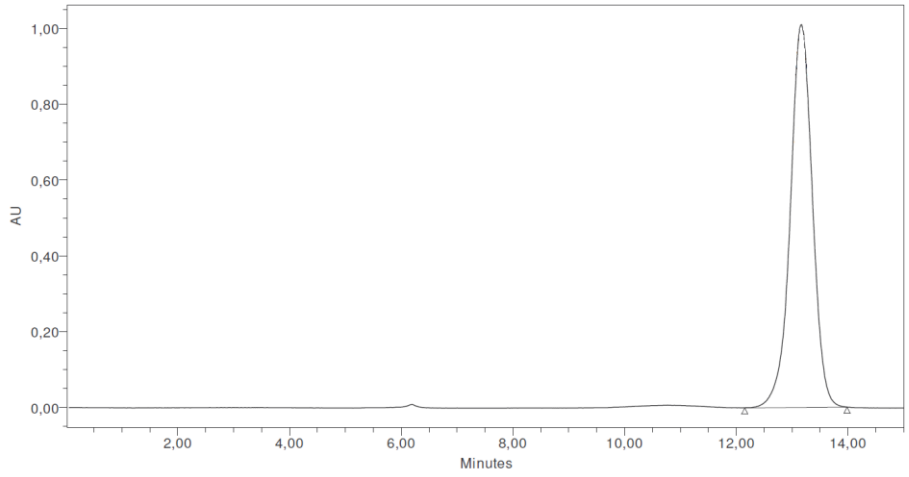

Figure 3.16. Chromatogram of lactic acid obtained with the HPLC.

3.3.9. Polyhydroxyalkanoates extraction and quantification

The analysis of PHA was carried out whit a first step of extraction followed by a PHA content quantification in a GC.

First of all, the extracted biomass of the reactor was centrifuged at 10,000 x *g* discarding the supernatant and conserving the biomass in Eppendorf tubes to be frozen at -20 ºC. These samples were later introduced in a freezer that worked at -80 ºC (Legaci refrigeration system, Revco) overnight and then, samples were freeze-dried in a lyophiliser (Telstar, LyoQuest) for 24h (see Figure 3.17).

A) B)

Figure 3.17. Lyophiliser (A) and lyophilised biomass for 24h (B).

Once the samples were lyophilised, a precise amount of biomass (error of ±0.01 mg) was weighed and introduced in a Pyrex tube with 1 mL of acidic methanol (20% sulphuric acid v/v) to break the wall cell and 1 mL of chloroform (with 1 mg/mL of benzoic acid as internal standard) to solubilise the PHA following the method proposed by Lanham et al. (2013). At the same time, a PHA standard (88% PHB and 12% PHV in molar, Sigma Aldrich) was performed in a chloroform solution with benzoic acid. In this way, a calibration curve was completed with a diverse quantity of PHA standard added in each Pyrex tube adding chloroform with benzoic acid until achieving a total volume of 1mL with a 6-point calibration curve. Furthermore, as sample preparation, 1 mL of methanol with sulfuric acid was added in each patron until obtaining a total volume of 2 mL (Figure 3.18). Once the samples and patrons were prepared, they were introduced in a digester (ECO 25 termoreactor, Velp Scientifica; see Figure 3.7a) at 100 ºC for 5 hours.

Figure 3.18. Samples preparation before introducing in a digestor for 5h.

Hence, the tubes were introduced in a recipient with ice to lower the temperature after digestion. When the samples were cooled, 0.5 mL of water was added to each tube to aid the two separate phases and the phases were mixed using a vortex (Vortex stirrer V05, lbx instruments) for 1 min (see Figure 3.19). The lower phase which contained chloroform was extracted and introduced in a GC vial with molecular sieves (VWR) to dry the sample and remove traces of water. The GC vial was closed with a metallic capsule with a silicone/PTFE septum to ensure no evaporation. This process would be carried out for each sample and patron.

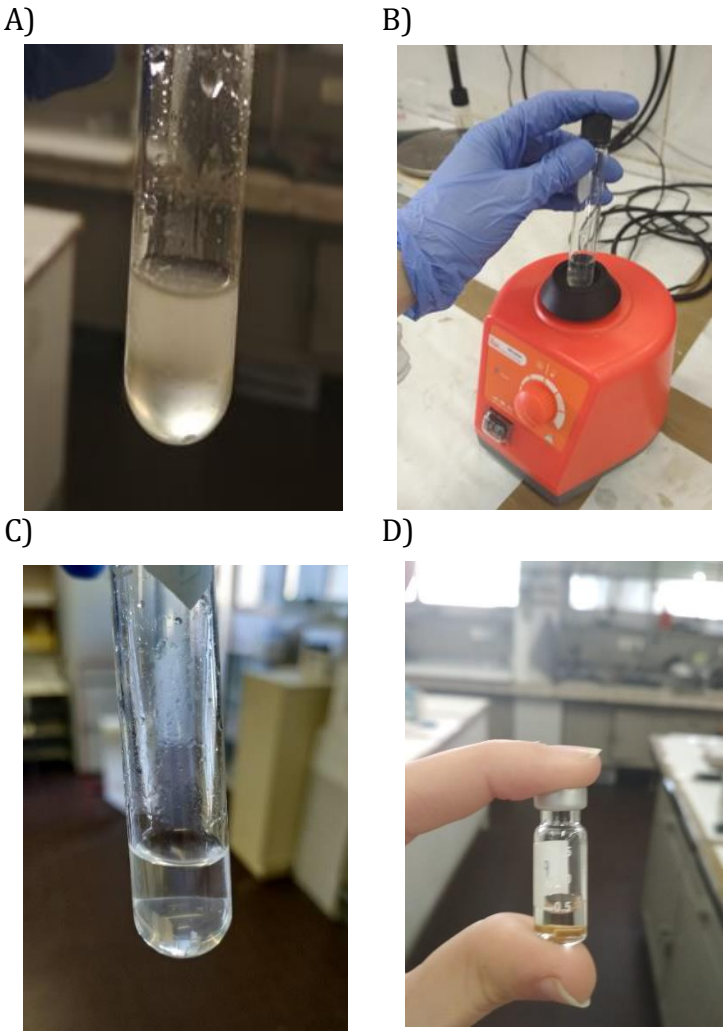

Figure 3.19. Sample after water addition (A), sample during the mixture with the vortex (B), sample with the two phases separated (C) and PHA sample in a GC vial with molecular sieves (D).

Patrons and samples were injected in GC (GC 2010, Shimadzu; see Figure 3.14) equipped with Sapiens column (60 mm x 0.25 mm x 0.25 μm) with FID. The temperature of the injector and detector were 280 ºC and 230 ºC, respectively (see Lahnam et al. (2013) for GC configuration). Helium was used as make up gas at 30.0 mL/min with hydrogen and synthetic air as fuel gas and oxidising gas at 40 mL/min and 400 mL/min, respectively. In this way, PHB, PHV and benzoic standard will be detected, by correlating the area of each peak using the calibration curve of standards. Thus, the area was transformed into concentration to obtain the percentage of PHA in the biomass using the following Equation 3.14.

$$PHB\ (\%) = \frac{PHB\ (g)}{SS\ (g)} x\ 100 \qquad PHV(\%) = \frac{PHV\ (g)}{SS\ (g)}\ x\ 100$$

$$PHA\ (\%) = PHB\ (\%) + PHV\ (\%) \tag{3.14}$$

4. Volatile fatty acids production from food waste under different working pH

ABSTRACT

This study is focused on the pH effects on the VFAs production and profile in FW fermentation. Discontinuous assays and semi-continuous fermenters were operated to test the differences between them. Batch test (pH 4.0, 6.0, 7.5, 9.0, 10.0, 11.0 and uncontrolled) obtained maximum VFAs concentration at pH between 6.0-9.0 (13.2-16.2 gCOD/L) with acetic acid as the main VFAs produced followed by caproic and butyric acid in all conditions. Semi continuous reactors working at HRT 3.5 days and 35 ºC were operated using FW from a University canteen. The acidic reactor (pH near 6) obtained a maximum VFAs concentration of 22.5 gCOD/L with acetic, caproic and butyric as the predominant acids. Alkaline condition (pH near 10) lead to a maximum VFA concentration of 14.1 gCOD/L with acetic acid as the main fermentation product (85%, in COD basis, of the total VFAs produced).

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Cheah, YK., Vidal-Antich, C., Dosta, J., Mata-Álvarez, J. (2018) Study of the effects of temperature and pH on acidogenic fermentation process from organic fraction of municipal solid waste. *6th International Conference on Sustainable Solid Waste Management*, Naxos, Greece, June 2018.

And then published in:

Cheah, YK., Vidal-Antich, C., Dosta, J., Mata-Álvarez, J. (2019) Volatile fatty acid production from mesophilic acidogenic fermentation of organic fraction of municipal solid waste and food waste under acidic and alkaline pH. *Environmental Science and Pollution Research*, 26(35), 35509-35522

4.1. Introduction

Waste generation has massively grown with consequent natural resource depletion and environmental problems in the world. This fact calls for a paradigm shift in resource and environmental management which result in the emergency of the circular economy concept where wastes are conceived as valuable resources for another purpose, closing the loops (Battista et al., 2020; Nghiem et al., 2017). In this way, biorefinery emerges as a new technology chain in which biodegradable organic waste is converted into new added-value bio-based products (Cerdán et al., 2021; Valentino et al., 2018). FW is an ideal substrate for biorefinery since it has high energetic potential by its high organic matter content (80-90% VS/TS) (Escamilla-Alvarado et al., 2017; Karthikeyan et al., 2018).

Acidogenic fermentation stands as a key unit in most waste-based biorefineries to produce value-added products by using MMCs. Specifically, acidogenic fermentation is a biological process based on hydrolysis, acidogenesis and acetogenesis phases of the AD process. During hydrolysis, the complex organic matter (i.e., proteins, carbohydrates, and fats or oils) are broken into organic monomers (sugars, amino acids and fatty acids) to be available for the acidogenic bacteria (Agler et al., 2011; Lee et al., 2014). Hydrolysis has been identified as the rate-limiting step of FW acidogenic fermentation by its particular nature and the lack of buffer capacity which could be overcome by optimising the operational parameters (Kim et al., 2003; Lim et al., 2008a). During anaerobic fermentation, not only VFAs are produced, but also other products such as alcohols, lactic acid, and hydrogen. VFAs are intermediate chemicals with several applications, at different Technological Readiness Level (TRL) including biological nutrient recovery (BNR) on WWTPs (Bahreini et al., 2021; Liu et al., 2018), bioenergy production in form of H_2 or biogas (Mu et al., 2018; Slezak et al., 2017; Zhang et al., 2014), chain elongation (De Groof et al., 2020) or biopolymers production in the form of PHAs (Fradinho et al., 2019; Valentino et al., 2018). PHA production is explained in more detail in Chapter 8 although it is important to consider that VFAs profile has a high effect on the copolymer composition which led to some different characteristics depending if is enriched on HV or HV in terms of heat resistance,

elasticity, durability, and transparency, among others (Bugnicourt et al., 2014; Chanprateep et al., 2010; Chee et al., 2010).

Regarding the VFAs profile when fermenting FW, many researchers have carried out studies in the last decades about the effect of the fermentation strategies, process configuration or operational parameters (i.e., pH, temperature, HRT and OLR) on the metabolic pathways and microbial characterisation in the fermentation acidogenic process (VFA yield and profile) (Dahiya et al., 2018; Feng et al., 2018; Gou et al., 2014; Pavan et al., 1998; Zhou et al., 2018). Specifically, the working pH during the acidogenic fermentation is a key parameter that determines the fermentation yield and VFA distribution because it affects the microbial community, metabolic pathway and enzyme activity (Tang et al., 2017). FW is characterised by its acidic pH which affects not only hydrolysis but also acidogenesis (Neyens et al., 2004; Zhou et al., 2018). Hence, it is important to find the optimal pH to promote the VFAs production avoiding the activity of methanogens, which work on the optimal pH range of 6.8-8.2 for methane production (Angelidaki & Sanders, 2004; Chaganti et al., 2011; Liu et al., 2011). Several studies have demonstrated that methanogenesis was inhibited by increasing or decreasing the pH at extreme values (Wang et al., 2014; Yuan et al., 2006). Some authors suggested the pH adjustment at acidic conditions to optimize VFAs production (Fang & Liu, 2002; Jiang et al., 2013; Wang et al., 2014). Nonetheless, other authors confirmed that alkaline pH promotes higher VFAs content than acidic pH (Garcia-Aguirre et al., 2017; Jankowska et al., 2017; Mengmeng et al., 2009). Even so, the role of the pH on FW fermentation is still inconclusive, probably due to the high variability of the substrate depending on the different sources, processing processes, eating habits or climate and seasonality (Braguglia et al., 2018; Xu et al., 2018).

This study aims to evaluate the effect of the working pH on VFAs production and its profile in acidogenic fermentation using FW from different collection periods from the university canteen. To this purpose, discontinuous assays and semi-continuous fermentations were performed under different pH values.

4.2. Materials and methods

4.2.1. Substrate and inoculum

FW used in this study was collected from a University canteen every two weeks. Once collected, the FW was immediately shredded with a kitchen blender (MMB66G5M, Bosch) to reduce the particle size (particle size reduction is a common feature of MBT plants) and a minimum amount of deionised water was added to obtain a concentrated feedstock. Hence, the FW was stored at 4 ºC until its use. If necessary, a proper quantity of water was added to dilute and control the total solid content between 4-7% until feeding the reactor. FW was collected from eight different periods to test the importance of its heterogeneity. Table 4.1 summarises the physico-chemical characterisation of each collection period (B1 to B8).

The inoculum used for the start-up of the semi-continuous acidogenic fermenter was obtained from a continuous stirred-tank reactor treating the OFMSW at pH 6 under mesophilic temperature (35 ºC) (Cheah et al., 2019). Finally, the effluent of the fermenter treating FW at pH 6 was used to inoculate FW batch tests.

4.2.2. Experimental set-up

The assays were carried out in batch mode and semi-continuous lab-scale fermenters as explained above. Details of the reactors used, and procedures are given in the next subsections.

4.2.2.1. Batch fermentation tests

Batch tests were performed to assess the influence of pH on the VFAs production and profile at short-term conditions. The bottles used were Pyrex serum bottles of 250 mL with an effective volume of 200 mL (see Section 3.1.1 for more detailed information). Each bottle was filled with inoculum:substrate ratio of 1:1 on VS basis, as Eryildiz et al. (2020) who obtained the highest VFA yield using the same ratio at pH 6.0.

The assay was performed for 10 days based on Garcia-Aguirre et al. (2017). All conditions were run by duplicate at 35 ºC. The pH value was adjusted initially using concentrated solutions of hydrochloric acid (HCl, 10M) and sodium hydroxide (NaOH, 10M). Moreover, the pH was adjusted at each sampling event to the studied value. After that, each bottle was flushed again with N_2 for 2 min (ca. 5L/min). The pH conditions studied were: 4.0, 6.0, 7.5, 9.0, 10.0, 11.0. and uncontrolled pH.

4.2.2.2. Semi-continuous fermenters

Two identical jacketed lab-scale reactors of 5L with a working volume of 4.5 L were run under mesophilic conditions (35 ºC) and HRT of 3.5 days based on Dosta et al., (2018) results (see Section 3.1.2 for more details). As mentioned in the previous section (Section 4.2.1), the purged biomass of semi-continuous fermenter working with OFMSW at pH 6.0 was used to inoculate both reactors (Cheah et al., 2019). The first fermenter was operated at acidic pH (namely 5.0-6.0) for 126 days using eight different collection periods of FW (B1 to B8). The pH was adjusted using $NaHCO_3$ (10M). At the same time, the reactor working at alkaline pH (10.0) was operated using 4 different collection periods of FW (B1 to B4) for 57 days to compare the performance at acidic and alkaline pH. In the alkaline fermenter, the alkalinity stands as a key control parameter to operate the reactor in the optimum pH conditions for VFA production (Ratanatamskul & Saleart, 2016), but the poor alkalinity of FW was insufficient to avoid a pH decrease during fermentation. Hence, $NaHCO_3$ was added to increase the buffer capacity. Nevertheless, high quantities were needed to adjust the pH at 10 and consequently, a strong alkali (NaOH, 10M) was added with $NaHCO_3$ after feeding to adjust the pH in the reactor. Specifically, different doses of $NaHCO_3$ were added: (i) 5g/L when $pH_{set\ point} - pH_{effluent}$ was ≤ 0.1, (ii) 10 g/L when $pH_{set\ point} - pH_{effluent}$ was ≤ 0.2, and (iii) 15 g/L when $pH_{set\ point} - pH_{effluent}$ was ≤ 0.5.

Both reactors were fed once per day (fed-batch culture) and effluents were characterised by analysing VFAs, sCOD, pH alkalinity, TS, VS and TAN.

Table 4.1 FW characterisation used in each collection period. Results are expressed as average ±95% confidence interval .

Parameter	Units	Period B1	Period B2	Period B3	Period B4	Period B5	Period B6	Period B7	Period B8
TS	% (w/w)	4.3 ± 0.7	5.9	4.3 ± 0.6	5.0 *	5.6 ± 1.7 *	7.3 ± 1.1 *	7.3 ± 0.3 *	6.6 ± 0.5 *
VS	% (w/w)	4.1 ± 0.7	5.6	4.1 ± 0.6	4.1 *	5.2 ± 1.7 *	6.1 ± 0.8 *	5.7 ± 0.2 *	5.5 ± 0.4 *
sCOD	gCOD/L	15.8 ± 12.7	32.4	36.7 ± 13.3	23.6 ± 6.7	40.0 ± 6.8	37.0 ± 2.9	38.7 ± 4.3	32.3 ± 14.7
VFAs	gCOD/L	1.7 ± 0.7	3.0 ± 0.7	2.2 ± 0.6	1.1 ± 0.2	1.9 ± 0.4	1.7 ± 0.1	2.1 ± 0.2	2.6 ± 0.4
TAN	mgN/L	-	153.0	81.0 ± 24.6	48.7	15.5 ± 4.5	34.1 ± 4.8	50.4 ± 1.3	26.7 ± 6.2
pH	-	4.8 ± 0.1	4.4 ± 0.4	4.2 ± 0.1	4.2 ± 0.1	5.7 ± 1.3 **	5.8 ± 0.3 **	6.6 ± 0.8 **	6.5 ± 0.8 **

* TS and VS analyzed after the addition of external alkalinity ($NaHCO_3$).

** pH measurement in periods B5 and B6 was performed after the addition of $NaHCO_3$.

4.2.3. Analytical methods

TS, VS, sCOD and TAN were analysed in accordance with the Standards Methods for the examination of Water and Wastewater (APHA, 2017) as has been detailed in Section 3.3. The pH of the fermenters was measured using a pressurised gelectrolyte electrode (HA405-DPA-SC-S8/225, Mettler Toledo). VFAs were analysed from filtered sample (through a 0.45 μm syringe filter) acidified with 85% phosphoric acid and diluted 10-fold by using a gas chromatograph (specifications were detailed in Section 3.3.7). VFAs concentration (gVFA/L) were converted in form of COD (gCOD/L) using stoichiometric conversion factors.

4.3. Results and discussion

4.3.1. Effect of pH on batch fermentation tests

Figure 4.1 shows the evolution of VFAs production with a maximum concentration of 16.3 gCOD/L and 15.4 gCOD/L at pH 7.5 and pH 9.0, respectively. Moreover, the VFAs production obtained on the 10^{th} day was also high at pH 6.0, pH 10 and uncontrolled pH (always between 5.1-5.5) achieving concentrations of 13.2, 10.7 and 10.5 gCOD/L, respectively (see Figure 4.1). However, the VFAs production at pH 4.0 and 11.0 was quite constant throughout the batch test with a maximum value of 6.6 gCOD/L for pH 4.0 and 8.0 gCOD/L for pH 11.0, since extreme pH values do not favour acidogenic bacterial survival (Strazzera et al., 2018). Wu et al. (2020) also showed that low pH (pH 3.0, 4.0 and 5.0) suppressed functional microorganisms' activities for hydrolysis and acidification resulting in low VFAs accumulation. Even so, it is well known that low pH has a negative effect on methanogenic archaea hindering the consumption of VFAs (Yu et al., 2021).

These results are in accordance with Zhang et al. (2005), who also fermented kitchen waste at pH 5.0, 7.0, 9.0 and 11.00 on batch mode at 35 ºC, reporting higher solubilization degree at pH 7.0 which in turn improved hydrolysis and acidogenesis (82% on COD content) in comparison with acidic or basic pH conditions (pH 5.0 and 11.0, respectively).

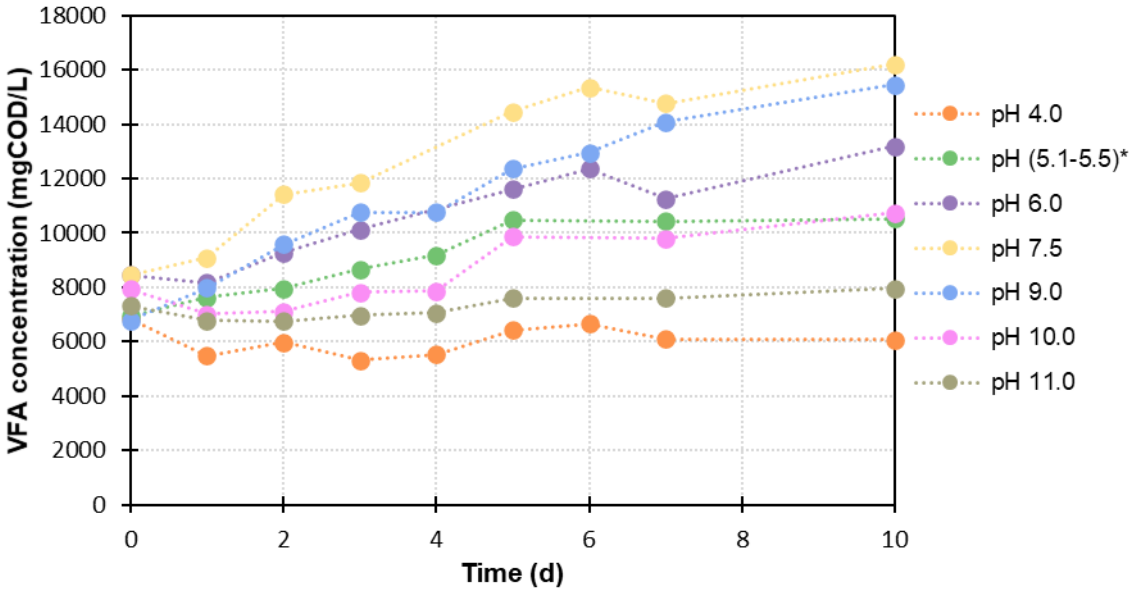

Figure 4.1. Evolution of VFAs concentration in the batch test of FW at different pH conditions.

Regarding pH profiles, Figure 4.2 shows the variations of the pH measured at each sampling event. As can be appreciated, the most significant pH fluctuations occurred at pH 6.0, pH 7.5 and pH 9.0, where the values decreased drastically from 6.0 to 4.8 (condition pH 6.0), from 7.5 to 5.3 (condition pH 7.5), and from 9.0 at 5.1 (condition 9.0) on the first day before being adjusted to the set point. These sudden pH drops could affect the VFAs production and profile produced during these days. Furthermore, at the first days of the batch test, the acidogenic bacteria were acclimated and adapted to the new conditions with consequent fluctuations and instability of the fermentation liquor (Garcia-Aguirre et al., 2017). However, from the 4th day on, the pH variations were lower within the range of ± 0.3 from their set point values. Even so, the generation of VFAs and the existence of a high concentration of soluble organics lead to a partially acidic pH (Lissens et al., 2004; Zhang et al., 2005). The importance to control the pH remains on the fact that it directly impacts the VFAs production and composition, since it has a great influence on the hydrolysis and acetogenesis phases affecting, at the same time, the metabolic pathways to obtain different VFAs (Atasoy et al., 2018; Begum et al., 2018; Zhao et al., 2018; Zhou et al., 2018).

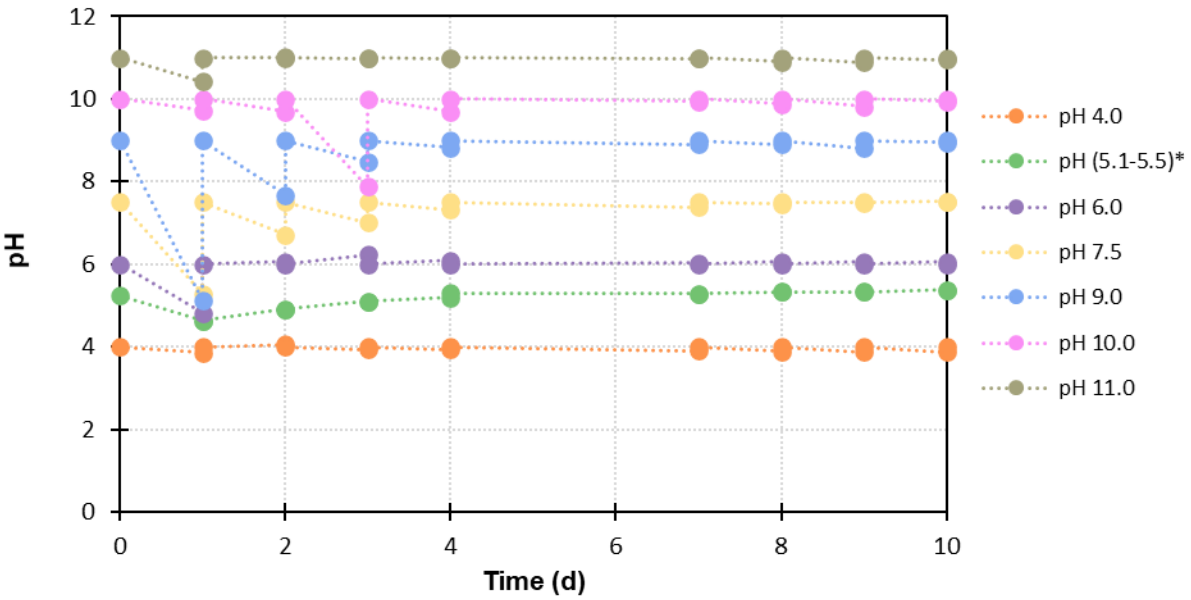

Figure 4.2. Evolution of pH control in the batch test of FW at different pH conditions.

Figure 4.3 shows the individual VFAs concentration on the 10th day and Figure 4.4 shows the proportion of each VFA on the same day. As commented before, the tests performed at highly acidic (pH 4.0), or alkaline (pH 11.0) conditions obtained a lower final VFAs concentration. The maximum VFAs concentration on the last day was obtained at pH 7.5 followed by pH 9.0 and 6.0 (i.e., 16.2, 15.5 and 13.2 gCOD/L, respectively) probably due to the higher solubilisation degree compared to extrema acidic and basic pH conditions as was studied by Zhang et al. (2005). Moreover, the use of inoculum that previously worked at pH 6.0 probably has had an influence on this maximum VFAs concentration at these pH conditions.

Acetic acid was the main fermentation product with a percentage of 33-48% throughout all conditions tested. The highest acetic acid concentration (6.6 gCOD/L) was obtained at pH 9.0, representing a 45% of the total VFAs production similar to the percentage obtained at pH 10.0 (44%). Zheng et al. (2018) also obtained acetic acid as main fermentation product at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and uncontrolled pH. Moreover, these results were consistent with those obtained by Zhang et al. (2005) who observed that acetic acid was favoured as the main acid at pH 9.0 and 11.0 when fermenting kitchen waste in comparison with pH 5.0 and 7.0.

In addition to acetic acid, butyric and caproic acids were also produced as majoritarian acids during the acidogenic fermentation tests with a variable percentage of 11-20% for butyric acid and 27-47% for caproic acid. Regarding butyric acid production, the maximum concentration was obtained at pH 7.5 (1.8 gCOD/L), pH 9.0 (1.4 gCOD/L) and pH 6.0 (1.2 gCOD/L), representing the 20, 16 and 16% of VFAs produced, respectively, on the last day. Furthermore, the caproic concentration was higher at pH 7.5 (2.4 gCOD/L), pH 6.0 (2.3 gCOD/L) and uncontrolled pH (1.9 gCOD/L) representing 32%, 38% and 40%, respectively, of the distribution. The dominance of butyric acid in the VFA profile can be related to pH because it seems to be favoured at pH values lower than 5.5 as occurs in the batch test, while acetic acid is usually produced as majoritarian acid at neutral and alkaline pH (Fang et al., 2020; Wang et al., 2014). In this way, the majoritarian acids obtained in most studies of FW fermentation were acetic and butyric (Dahiya et al., 2015; Garcia-Aguirre et al., 2019; Li et al., 2018).

Hence, the working pH affected not only the VFAs production but also the VFA profile. Nevertheless, it is important to consider that these batch experiments were carried out at short-term conditions using the inoculum of a semi-continuous fermenter working at pH 6.0, which could influence the VFAs production performance. In this way, the results need to be confirmed under continuous operation to check the ability of microorganisms to adapt and evolve to the new operation conditions.

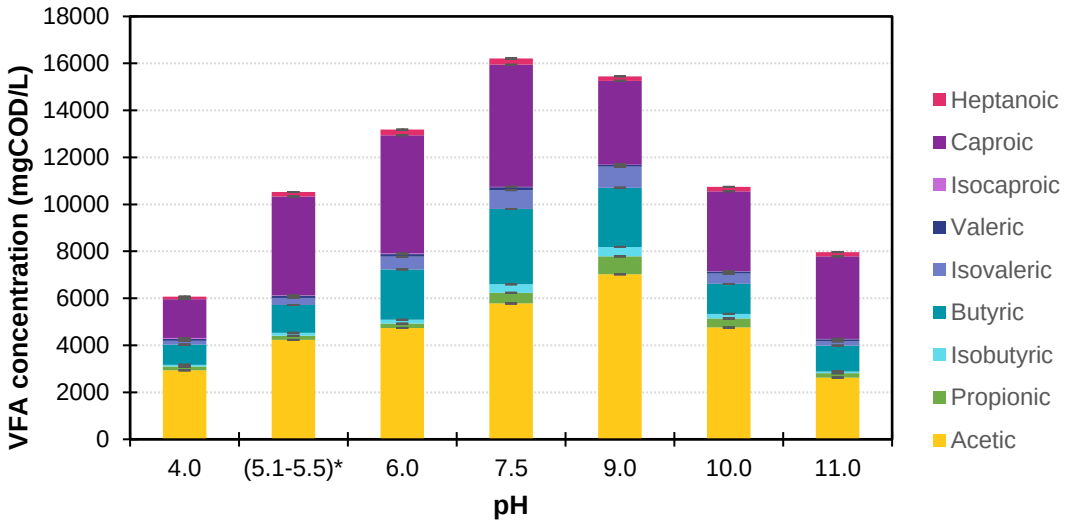

Figure 4.3. Individual VFAs production at 10th day in the batch test of FW at different pH conditions.

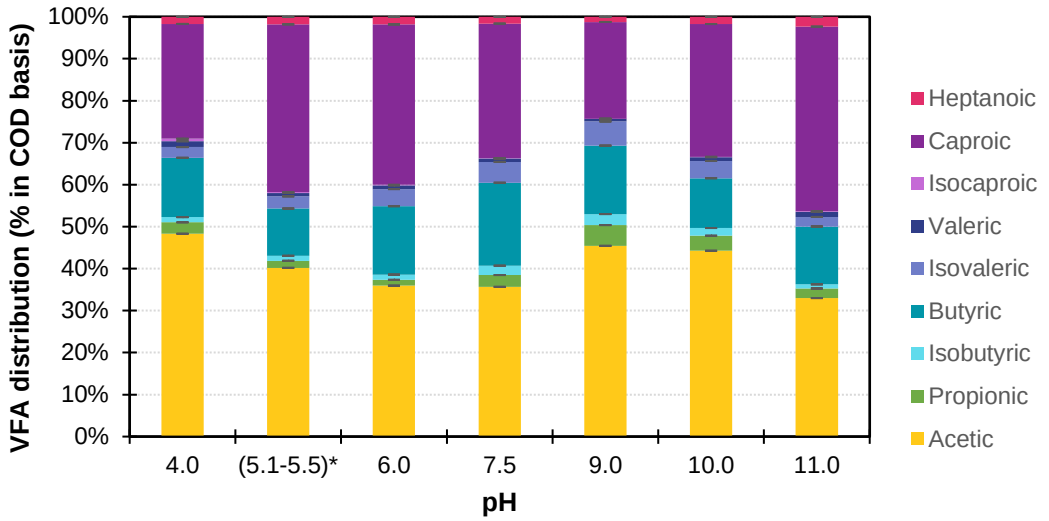

Figure 4.4. Individual VFAs percentage (COD basis) at 10th day in the batch test of FW at different pH conditions.

4.3.2. Effect of pH on semi-continuous acidogenic fermenters

The study of the effect of the pH was carried out in two reactors working at pH 6.0 and 10.0 using FW of different collection periods as has been explained in Section 4.2.1 to test the effects of acidic and alkaline pH. TS content between B1 and B3 was quite similar with a 4.3 % TS and a VS/TS ratio of 95%. From B4, the TS content was slightly higher, between 5.0 and 7.3% (w/w) due to the TS content calculation after external alkalinity ($NaHCO_3$) addition decreasing the ratio VS/TS until approximately 80%. The VFAs of the influent (B1-B8) ranged from 1.1 to 3.0 gCOD/L with acidic pH (4.2-4.8, before $NaHCO_3$ addition) and TAN concentration lower than 160 mgN/L.

Tables 4.2 and 4.3 show the average composition of the effluent of both fermenters working at pH 6.0 and 10.0, respectively. Moreover, Figures 4.5 and 4.6 depict the VFAs evolution and VFAs composition throughout the experimentation of reactor working at pH values around 6 and 10 with an operating period of 126 days and 57 days, respectively. As expected, some fluctuations were observed during the start-up due to the initial inoculum used, in which there was remaining unfermented or insoluble organic matter from OFMSW. The addition of FW on the feeding might have resulted in an unstable fermentation liquor with high organic matter from OFMSW that might

aggravate undesirable impacts to the system (Mata-Alvarez et al., 2014). Nevertheless, the start-up of both reactors worked similarly with a maximum VFAs production of 10.7 gCOD/L for acidic reactor and 10.2 gCOD/L for the basic reactor with acetic as the main acid (44-54%) followed by butyric (15-21%) and propionic acid (10-13%). No big differences were observed between both reactors, probably because the initial microbial cultures of the reactor working with OFMSW were still in adaptation to the new substrate.

As Figures 4.5 and 4.6 show, FW of different collection periods also had a different initial composition, which resulted in a shift in the VFAs distribution. For example, FW of the second collection period (B2) contained a higher TAN concentration at the influent (150 mgN/L) due to a significantly higher quantity of meat and fish residues, a source of proteins, which led to an unusual peak of TAN in the effluent (1075 and 876 mgN/L on the acidic and the alkaline reactor, respectively). Furthermore, the second period (B2) was characterised by the highest VFAs production in both reactors with a maximum VFAs concentration of 22.5 gCOD/L and 14.1 gCOD/L for the acidic and alkaline reactor, respectively. The highest production of the acidic reactor was in accordance with Yin et al. (2014) who obtained a maximum production of 23.1 gVFA/L fermenting FW at pH 6.0 at 30 ± 2 ºC. Even so, Khatami et al. (2021) obtained a maximum VFAs concentration of 12.9, 12.3 and 7.0 gCOD/L on the 15th day of the batch test at pH 5.0 using three different inoculums demonstrating that not only does the pH or feed substrate have an important effect, but also the use of one type or another of inoculum.

Table 4.2 Characteristics of the FW semi-continuous fermenter effluent at acidic pH (6.0) under mesophilic conditions. Results are expressed as average ±95% confidence interval.

Parameter	Units	Period B1	Period B2	Period B3	Period B4	Period B5	Period B6	Period B7	Period B8
Alkalinity	$gCaCO_3/L$	-	-	-	0.78 ± 0.78	1.97 ± 0.30	3.22 ± 0.67	-	-
TAN	mgN/L	473	1075	652 ± 288	440	618 ± 272	517 ± 42	670 ± 40	390 ± 210
pH	-	4.95 ± 0.28	6.03 ± 0.56	5.84 ± 0.53	5.89 ± 0.60	5.71 ± 0.33	5.83 ± 0.27	5.91 ± 0.16	5.95 ± 0.20
sCOD	gCOD/L	56.42 ± 35.39	38.08	43.58 ± 11.51	36.17 ± 0.17	44.74 ± 6.87	48.09 ± 3.68	49.92 ± 2.51	48.53 ± 1.62
$COD_{VFA}/sCOD$	%	12.98 ± 9.70	59.12	23.55 ± 5.24	14.60 ± 1.94	20.82 ± 4.10	22.28 ± 3.08	26.83 ± 2.40	29.50 ± 1.81
VFAs	gCOD/L	6.82 ± 1.85	20.12 ± 6.84	7.60 ± 2.65	6.81 ± 1.80	8.56 ± 1.24	12.29 ± 1.26	14.12 ± 0.75	14.72 ± 1.43
$(C_2+C_4)/(C_3+C_5)$	-	4.27 ± 3.25	4.53 ± 3.04	6.86 ± 3.34	12.87 ± 4.60	10.14 ± 1.42	12.69 ± 1.67	12.81 ± 1.25	12.77 ± 2.67
Acetic acid	%*	53.92 ± 31.70	21.24 ± 5.00	32.72 ± 8.30	42.38 ± 12.93	33.89 ± 7.68	42.04 ± 5.33	44.41 ± 4.74	42.72 ± 5.13
Propionic acid	%*	10.04 ± 5.42	8.00 ± 3.22	2.78 ± 1.11	1.27 ± 1.23	1.67 ± 0.11	1.25 ± 0.13	1.30 ± 0.12	1.02 ± 0.52
Butyric acid	%*	14.36 ± 2.24	35.92 ± 3.93	13.38 ± 2.82	13.38 ± 2.82	16.04 ± 2.30	14.14 ± 2.48	16.46 ± 1.46	13.59 ± 1.30
Valeric acid	%*	10.21 ± 1.25	12.26 ± 0.62	3.79 ± 0.71	3.79 ± 0.71	3.25 ± 0.16	3.23 ± 0.35	3.48 ± 0.24	3.56 ± 0.38
Caproic acid	%*	7.53 ± 0.07	18.79 ± 1.09	39.37 ± 7.48	37.10 ± 6.10	43.35 ± 7.52	38.00 ± 2.95	33.07 ± 1.06	37.66 ± 3.08
Heptanoic	%*	3.93 ± 2.79	3.78 ± 0.75	5.12 ± 5.25	2.08 ± 0.76	1.80 ± 0.23	1.33 ± 0.05	1.28 ± 0.06	1.46 ± 0.15

* VFAs percentages reported in COD basis.

Table 4.3 Characteristics of the FW semi-continuous fermenter effluent at basic pH (10.0) under mesophilic conditions. Results are expressed as average ± 95% confidence interval.

Parameters	Units	Period B1	Period B2	Period B3	Period B4
Alkalinity	$gCaCO_3/L$	-	-	-	5.81 ± 0.26
TAN	mgN/L	-	875.0	445.3 ± 83.8	394.0
pH	-	8.64 ± 0.97	7.87 ± 0.75	9.54 ± 0.46	9.22 ± 0.32
sCOD	gCOD/L	61.42 ± 42.47	44.87	49.51 ± 2.52	41.97 ± 4.95
COD_{VFA}/sCOD	%	14.40 ± 11.32	31.42	20.50 ± 4.84	19.26 ± 1.98
VFAs	gCOD/L	7.53 ± 1.96	12.06 ± 2.58	7.23 ± 1.94	7.19 ± 3.76
$(C_2+C_4)/(C_3+C_5)$	-	3.11 ± 1.51	4.67 ± 2.57	9.31 ± 3.59	8.88 ± 6.23
Acetic acid	%	43.97 ± 18.05	71.34 ± 9.76	72.11 ± 30.47	85.09 ± 20.88
Propionic acid	%	12.83 ± 5.09	3.41 ± 0.78	3.10 ± 1.28	2.54 ± 0.31
Butyric acid	%	20.66 ± 3.94	12.19 ± 1.66	11.25 ± 3.05	5.97 ± 0.05
Valeric acid	%	10.44 ± 0.70	6.14 ± 0.55	7.76 ± 0.04	4.70 ± 0.32
Caproic acid	%	7.78 ± 1.61	4.94 ± 0.05	3.84 ± 0.01	0.84 ± 0.10
Heptanoic acid	%	4.32 ± 3.67	1.98 ± 0.07	1.95 ± 1.72	0.87 ± 0.81

*VFAs percentages reported in COD basis.

As shown in Table 4.3, it is important to highlight that the pH of the alkaline reactor effluent on period B2 was 7.87 ± 0.75 due to the difficulties to control the pH value near 10. In this period (B2), the VFA distribution between the fermenters was very different with an average distribution of 36% butyric, 21% acetic and 19% caproic acid on the acidic reactor and 71% of acetic, 13% of butyric and 6% of valeric acid on the alkaline reactor (see Tables 4.2, 4.3 and Figure 4.8). In the alkaline reactor, the percentages of propionic acid (3%) and valeric acid (6%) decreased from the start-up period affecting the ratio between odd and even carbons $(C_2+C_4)/(C_3+C_5)$ which is an important parameter to consider, especially for PHA production, since it has been reported that acetic and butyric acids are mainly involved in PHB production and propionic and valeric acid in PHV production (Bengtsson et al., 2010; Patel et al., 2009). Therefore, if the $(C_2+C_4)/(C_3+C_5)$ ratio increases, major PHB presence is expected on the copolymer PHBV obtained from this fermentation liquid, which changes the main properties of the bioplastic.

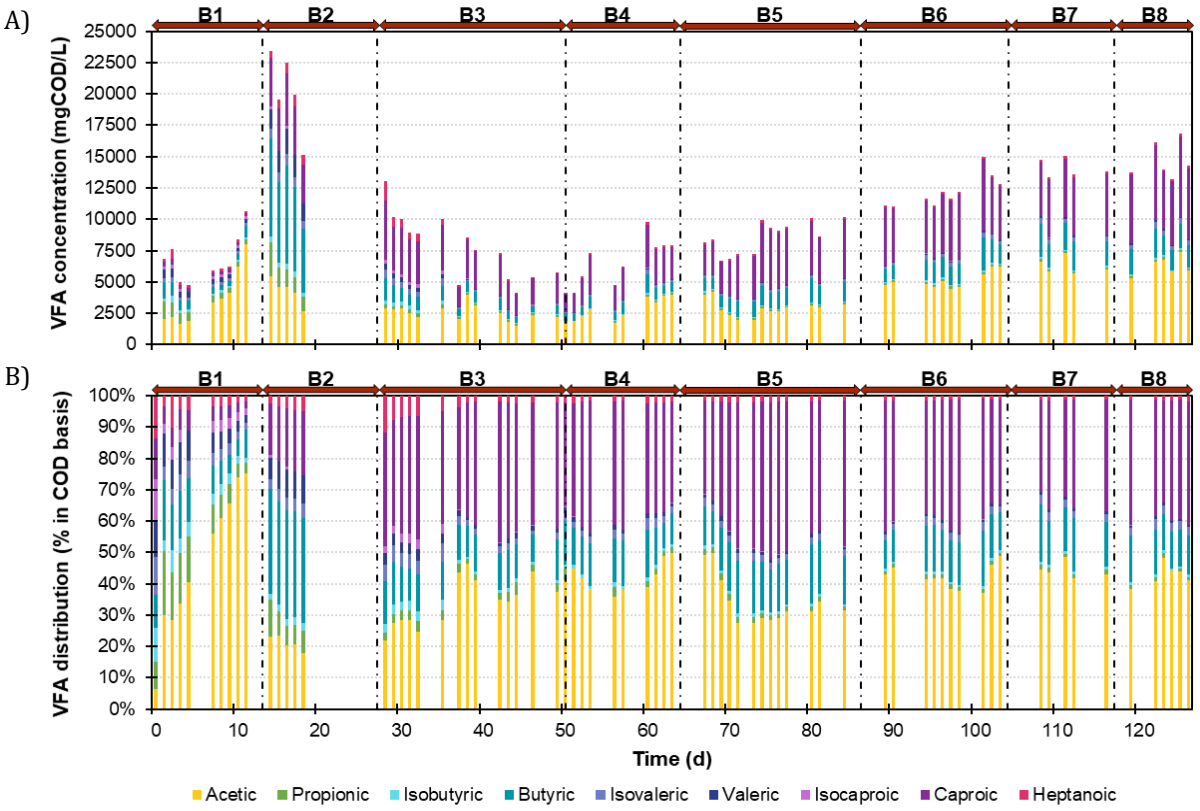

Figure 4.5. Evolution of VFA production (A) and VFA distribution in COD basis (B) of acidogenic fermentation at pH 6.

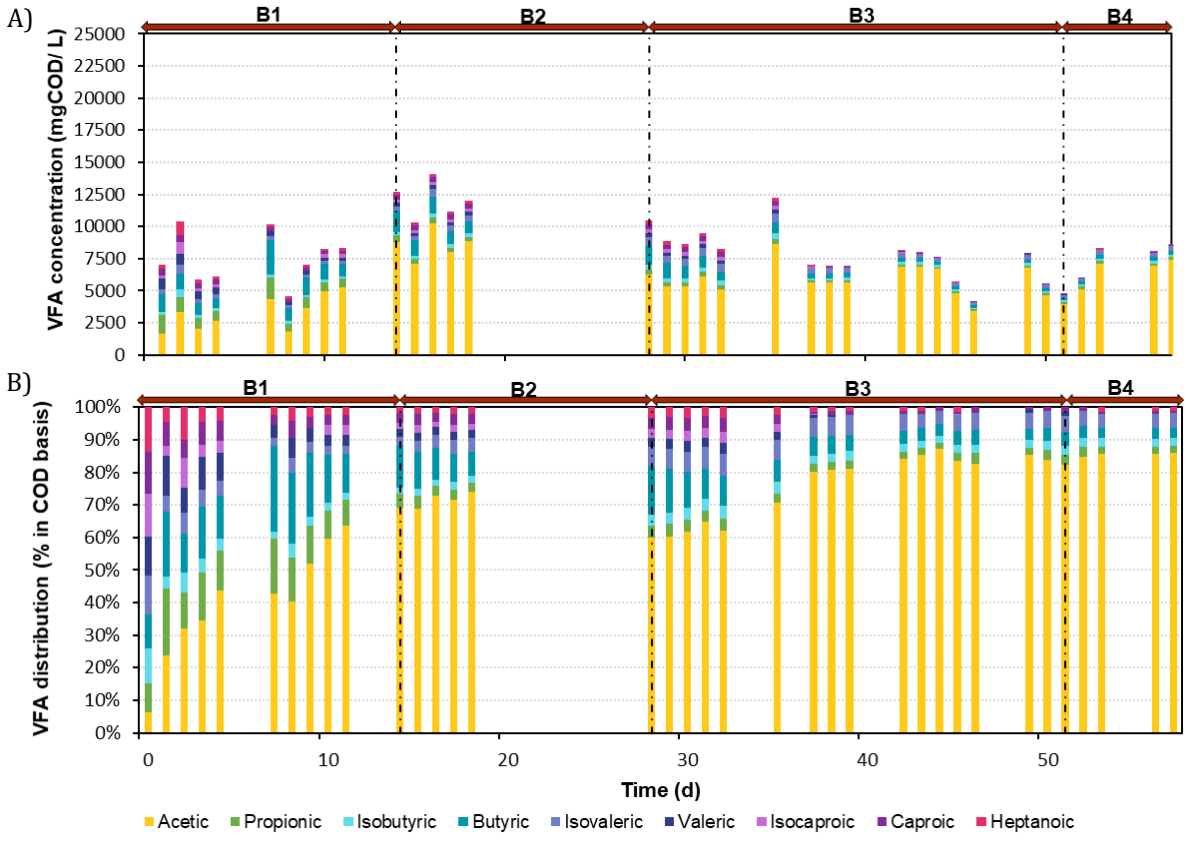

Figure 4.6. Evolution of VFA production (A) and VFA distribution in COD basis (B) of acidogenic fermentation at pH 10.

At period B3 and B4, the VFAs concentration average on the effluent was similar on the acidic reactor (7.60 ± 2.65 gCOD/L and 6.81 ± 1.80 gCOD/L, respectively) and the alkaline reactor (7.23 ± 1.94 gCOD/L and 7.19 ± 3.76 gCOD/L, respectively), but the VFA profile was different (see Figure 4.7). The alkaline reactor (pH 10) was characterised by a constant increase of acetic acid percentage up to 85% of the total VFAs on the last day with a consequent decrease in butyric, valeric and caproic contents (6%, 5% and 1%, respectively; see Figure 4.6). This fact might be caused by the preference of phosphoroclastic degradation pathway as was reported by some authors which obtained values of approximately 75% of acetic acid fermenting FW and OFMSW at pH 10 (Dahiya et al., 2015; Garcia-Aguirre et al., 2017; Khatami et al., 2021). This high acetic acid content could be interesting for biological heterotrophic denitrification as has been studied by Elefsiniotis & Wareham (2007) who demonstrated that denitrifies preferred acetic acid followed by butyric and propionic acid. Furthermore, it is important to note that acetic acid percentage at pH 10 on the previous batch tests was lower (44%) than that obtained on the continuous assay (up to 85%). This fact suggests the microorganisms were adapted to alkaline conditions throughout the experiment, a fact that did not occur on batch test assay. Hence, the batch tests assays were important to test various conditions at the same time, but the continuous experiments offer more information considering the microorganisms adaptation and evolution throughout the experiment.

Regarding alkaline conditions during acidogenic fermentation, some authors remark that it promotes the accessibility of the soluble compounds and improve the hydrolysis of the carbohydrates and proteins providing readily fermentation substrate for the VFAs production (Dahiya et al., 2015; Khatami et al., 2021; Park et al., 2014). In this way, Zheng et al. (2013) described an increase in bacteria related to hydrolysis and acidification of the sludge at pH 10.0 compared to uncontrolled pH. Besides, at pH 10 a decrease in methanogenic archaea was found leading to a higher VFAs production at this condition because it is not converted into biogas.

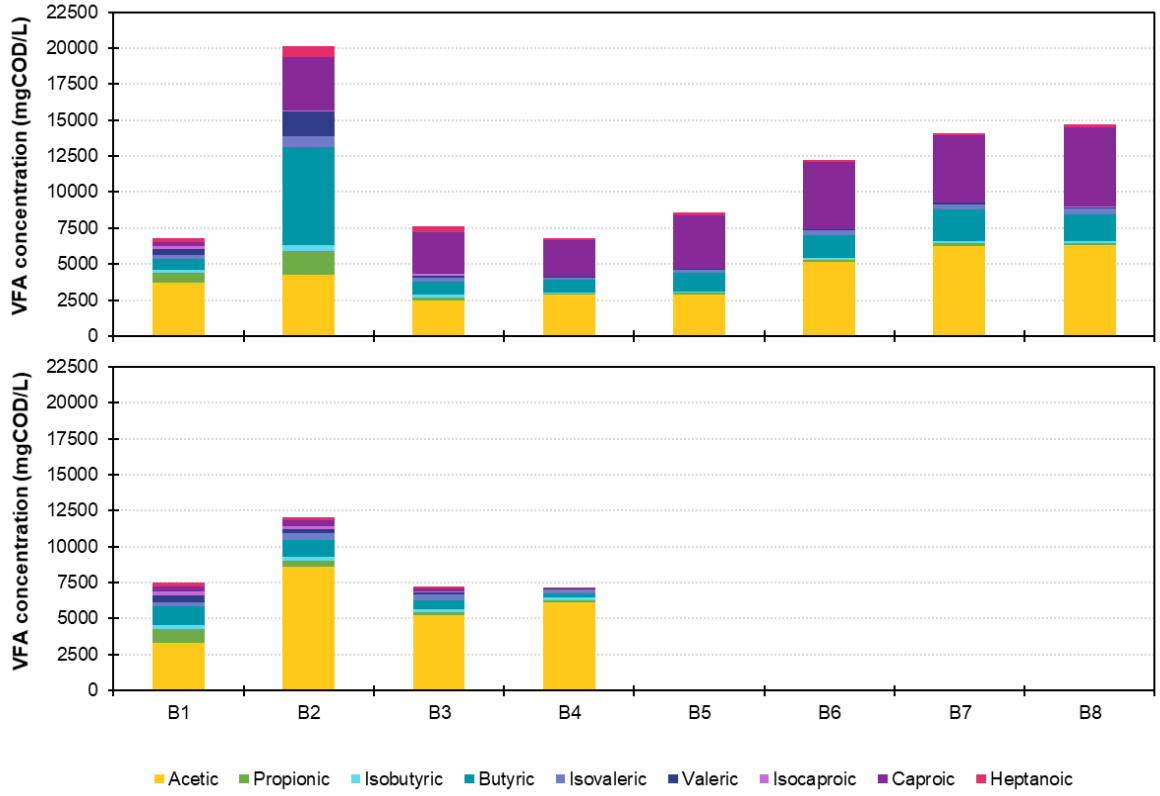

Figure 4.7. Average VFA production on the effluent of acidic reactor (top) and alkaline reactor (bottom).

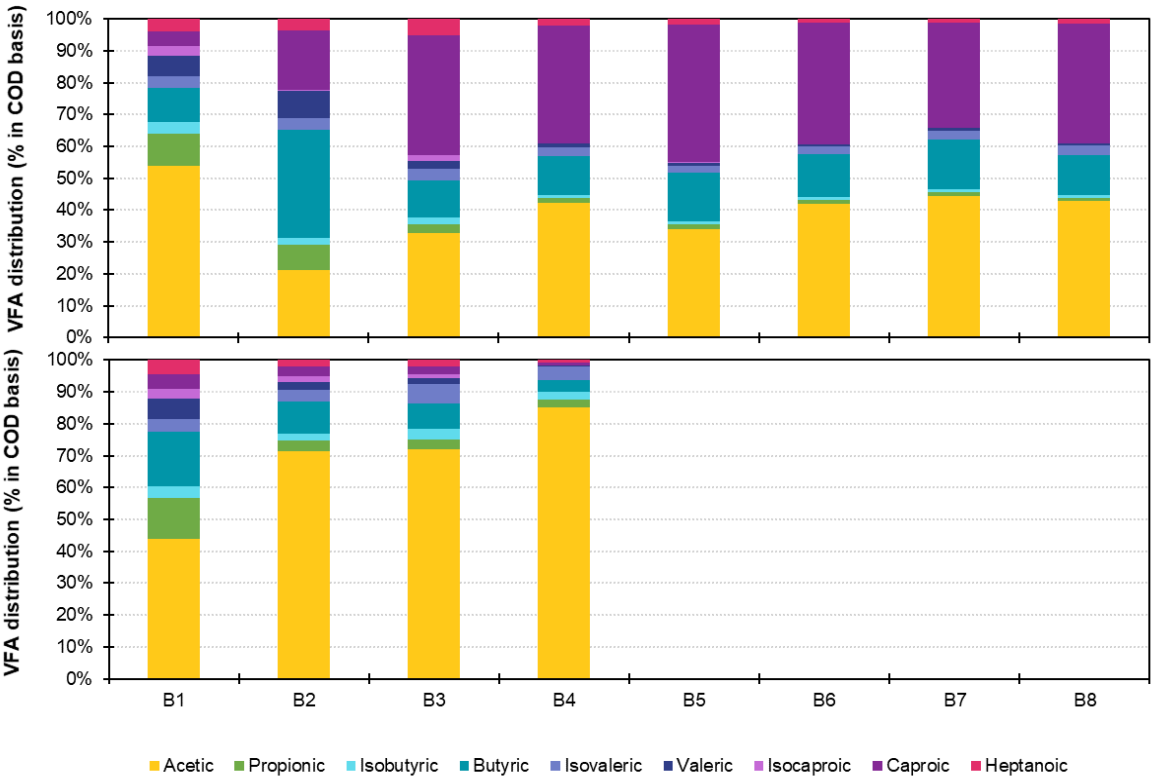

Figure 4.8. Average VFA distribution in COD basis on the effluent of acidic reactor (top) and alkaline reactor (bottom).

After the 57th day, the alkaline fermenter (pH 10) was stopped because it was clearly observed that alkaline pH favours acetic acid production and stable VFAs concentration was obtained on B3 and B4 periods, although FW composition was quite different (see Table 4.1). Hence, only the fermenter working at pH 6.0 was still operating at that date to evaluate the relation between the VFAs evolution and the different collection period. From period B5, the VFAs concentration started to increase from 8.1 to 16.1 gCOD/L on period B8 probably related to an increase of VS during B5-B8 compared to B1-B4. This fact demonstrated that an increase on VS content, lead to higher VFAs production as has been studied by several authors (De Groof et al., 2020; Jiang et al., 2013; Lim et al., 2008a; Llamas et al., 2022).

Regarding VFAs distribution, acetic acid (34-44%) and caproic acid (33-43%) were the dominant products, followed by butyric acid (14-16%) throughout periods B5 and B8 remaining quite constant with a $(C_2+C_4)/(C_3+C_5)$ ratio between 10-13. These results were consistent with the distribution obtained by Venkateswar Reddy & Venkata Mohan (2012) who ferment FW at pH 6.0 obtaining acetic acid and butyric acid with a low amount of propionic acid and valeric acid, contributing to the PHB accumulation. Moreover, the concentrations of propionic (1-2%) and valeric (3-4%) acids during B5-B8 periods were very insignificant. This fact could be attributed to the lack of nitrogen-rich substrates on the FW which enhance propionic and valeric generation during protein degradation (Ma et al., 2017). Most authors studied the VFA profile of FW acidogenic fermentation due to the importance of VFAs composition. Acetic and butyric acids were the main VFAs in most FW fermentation studies at pH 6.0 due to the high carbohydrate content on the FW. Particularly, FW studied in this experiment was mainly composed of carbohydrates (i.e., pasta, and rice). As it is well known, the fermentation of carbohydrates produces acetic and butyric acid (Alibardi & Cossu, 2016; Strazzera et al., 2018; Wang et al., 2014). Moreover, the butyric acid production is favoured at pH 5.0-6.0 (Dahiya et al., 2015; Ren et al., 2007) as shown in the acidic reactor compared to the alkaline reactor.

Acetic and butyric acids presence on FW fermentation have been reported, but the presence of caproic acid on FW fermentation is less studied and analysed. Even so, Khatami et al. (2021) found a decrease in the acetic acid concentration of acidic reactor (pH 5.0) which increase caproic acid concentration. Furthermore, Capson-Tojo et al.,

(2018) also recorded acetic and butyric acids as majoritarian species followed by caproic acid with FW fermentation and carboard and FW co-fermentation suggesting that caproic acid is synthetized by elongation of acetate or ethanol as an electron donor. Lu et al. (2020) also achieved a caproic content of about 20% at the end of the fermentation process attributed to the chain elongation between acetate, butyrate and ethanol (Agler et al., 2011). Hence, caproic acid is less common in FW fermentation literature but some authors confirm its presence due to the chain elongation process.

The concentrations of propionic and valeric acids were minority in periods between B3 and B8, and low valeric acid production could reflect that the treated FW had a small amount of protein content (Shen et al., 2017b).

4.4. Conclusions

Batch fermentation tests using FW yielded a maximum VFAs concentration in the pH range of 6.0-9.0 (13.2-16.2 gCOD/L), with acetic acid being the main VFAs produced (33-48%), followed by butyric acid (16-20%) and caproic acid (23-38%). The extreme pH conditions (pH 4.0 and 11.0) led to lower VFAs concentrations (6.1 and 8.0 gCOD/L, respectively), with acetic acid as the main component. Batch tests were performed using inoculum from a semi-continuous OFMSW fermenter working at pH 6.0, and therefore short-term results could be affected by this inoculum since the microorganisms were not completely adapted to the new working conditions.

Semi-continuous mesophilic fermenters took around 30 days to start-up with some fluctuations. From then, the acidic reactor and the alkaline reactor obtained similar VFAs concentrations (period B3 and B4) but highly differentiated VFA profiles. The alkaline reactor led to acetic acid production until achieving a percentage up to 85%. On the contrary, the acidic reactor was characterised by acetic (34-44%) and caproic acids (33-43%), the latter due to the chain elongation process, as the main VFAs obtained. Therefore, these results indicate that pH adjustment could be an excellent strategy to adjust the VFAs profile depending on the end-use of the desired products.

5. Assessing the potential of waste activated sludge and food waste co-fermentation for carboxylic acids production

ABSTRACT

The co-fermentation of WAS and FW was studied in batch mode to produce carboxylic acids using different mixtures on VS basis. Furthermore, the effect of the alkalinity addition and WAS auto-hydrolysis pre-treatment was studied. All experiments were carried out at 35 ºC, without pH adjustment and without external inoculum. Results showed that co-fermentation yields were always higher than mono-fermentation yields increasing as the proportion of FW in the mixture increased with a maximum of 480 mgCOD/gVS for the WAS/FW_50/50 mixture. The proportion of WAS in the mixture is also important to keep the pH above 5.0. Moreover, the WAS mono-fermentation showed propionic acid as prevailing without enhance large improvements with auto-hydrolysis pre-treatments. On the other hand, butyric acid was enriched as the proportion of FW increased in the mixture with concomitant pH decreasing.

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5.1. Introduction

Shortage of natural resources and increasing environmental awareness are driving a change in production systems from end-of-pipe waste treatment towards integrated resource recovery schemes (Puyol et al., 2017). This new paradigm requires the transformation of WWTPs for municipal sewage treatment and MBT plants for biowaste treatment into biorefineries. In biorefineries, waste streams are conceived as a source of energy, chemicals, nutrients and water rather than as a source of pollution (Pikaar et al., 2020; Vinardell et al., 2020).

Fermentation is a key unit in most waste-based biorefineries due to its capacity to break down organic matter into easily assimilable compounds such as VFAs (i.e., acetic, propionic, butyric and valeric acid), lactic acid and alcohols (Capson-Tojo et al., 2018; Dahiya et al., 2018). These fermentation products can be directly used to provide the carbon source needed to sustain other microbially-mediated units in the biorefinery, such as biopolymers production (e.g., PHB and PHV), biological nutrient recovery (i.e., N, P and S) and chain elongation (Basset et al., 2016; Duber et al., 2020; Frison et al., 2013; Serrano et al., 2020).

FW and sewage sludge are among the most attractive organic waste streams for waste-based biorefineries since constant and large amounts are produced in municipalities. FW generation is estimated at 20% of the total food production in the EU, representing about 90 million tons per year (Braguglia et al., 2018; Poças Ribeiro et al., 2019). AD and composting are the most common treatment options for FW in the EU; however, fermentation products have a higher potential market value and a wider range of applications than biogas and compost (Dahiya et al., 2018; Fernández-Domínguez et al., 2020). FW stands as an ideal substrate for acidogenic fermentation, with reported VFA yields ranging between 50 and 400 mgCOD/gVS depending on the fermentation conditions (Jiang et al., 2013; Li et al., 2018; Lim et al., 2008a). Nonetheless, FW fermentation is constrained by (i) its particulate nature, which makes hydrolysis the rate-limiting step and (ii) the lack of buffer capacity, which requires alkali dosage to prevent hydrolytic-fermentative bacteria pH inhibition (Cheah et al., 2019; Kim et al., 2003). Pre-treatments can be carried out to overcome hydrolysis rate limitations and increase fermentation yields. However, pre-treatments require an environmental and

economic evaluation since fermentation yield improvements may not be enough to justify the pre-treatment capital and operation costs (Bolzonella et al., 2018; Strazzera et al., 2018). Nevertheless, except for alkaline pre-treatments, pre-treatments do not solve the constrains related to FW lack of alkalinity.

Co-fermentation, the combined fermentation of two or more waste, stands as another approach to improve FW fermentation yields. The increased FW fermentation performance achieved under co-fermentation conditions has been associated with: (i) a higher organic matter content, (ii) an improved buffer capacity, (iii) the balance of macronutrients, micronutrients and moisture, (iv) the dilution of inhibitory and toxic compounds, and (v) a diversification of the hydrolytic-fermentative bacteria (Fang et al., 2020; Feng et al., 2011; Peces et al., 2020; Wu et al., 2016).

Among the different wastes, WAS from WWTP is the most researched co-substrate for FW fermentation (Fang et al., 2020). This is likely due to its availability and buffer capacity, which allows maintaining the pH above inhibitory levels (pH > 5.0) without external chemicals addition. WAS/FW co-fermentation has been successfully carried out in several studies. Feng et al. (2011) who co-fermented FW and WAS at different pH, showed that co-fermentation mixtures produce more VFA than mono-fermentation controls (only FW or WAS). Feng et al. (2011) achieved higher VFA yields at pH between 7 and 9, with the highest VFA yield at pH 8 (ca. 0.57 gCOD/gVSS). Garcia-Aguirre et al. (2019), who co-fermented WAS and FW in a pilot-scale reactor (41/59 ratio on VS basis, SRT 5 d, 55 °C), reported that pH 9 favoured the accumulation of acetic acid and pH 6 favoured the accumulatio of butyric acid. Valentino et al. (2019) and Moretto et al. (2020) co-fermented WAS and FW (28/72 ratio on VS basis) to generate a VFA-rich effluent for PHA production. In both publications, the pH self-regulated at around 5.0. Valentino et al. (2019) compared the co-fermentation performance between mesophilic (37 °C) and thermophilic (55 °C) conditions (SRT 6 d, OLR 7.0 kgVS/(m^3·d)). The VFA yield was 0.41 and 0.44 gCOD/gVS, respectively, and butyric acid was the main VFA in both conditions (42 and 51%, respectively). Valentino et al. (2019) recommended co-fermentation at mesophilic conditions due to the higher process stability. In the subsequent study, Moretto et al. (2020) included a 72 °C pre-treatment (SRT of 2 d) before the mesophilic co-fermenter (37 °C, SRT 5 d, OLR 12–15 kgVS/(m^3·d)), which increased the VFA yield from 0.37 to 0.65 $gCOD_{VFA}$/gVS.

The improvement of co-fermentation performance observed in Moretto et al. (2020) may be related to the higher hydrolysis rate at 72 °C (Carrère et al., 2010). Additionally, short-term low temperature pre-treatments (55–70 °C) have been reported to release enzymes trapped in the WAS EPS matrix (Arias et al., 2018; Carvajal et al., 2013; Ferrer et al., 2008). This pre-treatment, known as auto-hydrolysis, could have a double beneficial effect on WAS/FW co-fermentation. On the one hand, the released enzymes could facilitate FW hydrolysis once both wastes are mixed. On the other hand, temperature could facilitate the disruption of the microbial cell wall and floc structure of WAS making it more bioavailable for hydrolytic fermentative bacteria (Carrère et al., 2010; Carvajal et al., 2013; Ruiz-Hernando et al., 2014).

Despite these research efforts, co-fermentation literature is still inconclusive in some issues and further research is required to clarify them. For instance, the existing literature does not clearly elucidate (i) the impact that the co-fermentation mixture composition has on VFA yield and VFA profile (i.e. acids distribution in the VFA mix), (ii) the relative importance of WAS alkalinity on the pH of the fermentation liquor and the improved fermentation performance, (iii) the impact of pre-treatments on co-fermentation performance, nor (iv) how co-fermentation behaves when WAS, instead of FW, is the main substrate in co-fermentation mixture.

The aim of this study was to investigate the performance of WAS/FW co-fermentation (using WAS as main substrate) under different experimental conditions to understand the benefits and constraints of this approach. This goal was achieved by studying: (i) the impact of WAS/FW mixture ratio on fermentation yield and product profile, (ii) the impact of pH on co-fermentation performance, (iii) the feasibility of WAS autohydrolysis pre-treatment to improve fermentation yields. Furthermore, testing the same mixtures through three independent fermentation assays allowed assessing co-fermentation reproducibility.

5.2. Materials and methods

5.2.1. Substrates' origin

Thickened WAS was collected from a municipal WWTP (ca. 400,000 population equivalent) in Barcelona metropolitan area (Spain). At the WWTP, WAS is thickened by a gravity thickener after the secondary clarifier. Once collected, WAS was stored in a refrigerated chamber at 4 °C until use (the maximum storage time was 5 days). Synthetic FW was used in this study to facilitate fermentation experiments reproducibility due to the highly heterogeneous nature of FW. Synthetic FW composition was formulated by averaging reported real FW composition (Braguglia et al., 2018; Capson-Tojo et al., 2018; Hassan et al., 2019). Specifically, the synthetic FW contained (wet_{weight} basis): vegetables (30%), fruits (30%), carbohydrates (20%), meat (10%), and fish and seafood (10%). To further ensure FW reproducibility, the ingredients were products found in the supermarket all the year-round, i.e., potato and onion for vegetables, apple and banana for fruits, boiled pasta for carbohydrates, canned ham for meat, and surimi sea sticks for fish and seafood. The FW used was shredded with a benchtop blender for 3–4min (particle size reduction is a common feature of MBT plants) and diluted with deionised water to adjust de TS concentration to around 15% (Abreu et al., 2019; Moretto et al., 2020; Xiong et al., 2019). Synthetic FW was prepared 24 h before starting the fermentation tests and stored in the refrigerator at 4 °C until use. Table 5.1 shows the physicochemical characterisation of WAS and FW in each experiment.

Table 5.1. FW and WAS characterisation for each experiment. Results are expressed as average ± 95% confidence interval (n = 3).

		Experiment 1		Experiment 2		Experiment 3	
Parameter	**Units**	**WAS**	**FW**	**WAS**	**FW**	**WAS**	**FW**
TS	gTS/L	48.5 ± 1.4	172.7 ± 0.4	45.6 ± 0.1	174.3 ± 0.5	57.6 ± 0.2	151.3 ± 0.3
VS	gVS/L	34.2 ± 1.3	164.7± 0.4	31.7 ± 0.1	166.6 ± 0.5	42.9 ± 0.2	146.0 ± 0.1
pH	-	7.3 ± 0.1	6.0 ± 0.1	6.5 ± 0.1	6.0 ± 0.1	6.8 ± 0.1	5.4 ± 0.1
VFAs	mgCOD/L	815.5	2424.9	2581.9	1827.6	2089.0	2497.7
Acetic (HAc)	%*	16.0	15.7	39.2	43.5	35.5	32.6
Propionic (HPr)	%*	15.5	5.7	19.4	7.3	19.1	3.2
Butyric (HBu)	%*	11.8	15.6	14.9	16.7	12.3	20.9
Valeric (HVa)	%*	34.0	29.3	13.9	15.9	21.8	30.4
Caproic (HCa)	%*	7.4	6.0	9.1	13.6	10.2	11.6
Heptanoic (HHep)	%*	15.3	27.7	3.4	2.9	1.1	1.2

*VFAs percentages are reported in COD basis

5.2.2. Fermentation batch assays set-up

Fermentation batch assays were performed in 250 mL serum bottles under anaerobic conditions at mesophilic conditions (35 °C). Mesophilic conditions were selected since higher fermentation yields have been reported compared to psychrophilic and thermophilic conditions (Fernández-Domínguez et al., 2020; Jiang et al., 2013; Komemoto et al., 2009). Both mono-fermentation (FW and WAS controls) and WAS/FW co-fermentation assays contained a total of 150 g (on wet basis). No inoculum was added, hence the fermentation process relied on the native fermentative bacteria. The pH of the fermentation liquor was not adjusted at the beginning nor during the experiment. Therefore, the pH control relied on the WAS buffer capacity to avoid the use of chemical reagents. All tests were carried out in triplicate. Anaerobic conditions were achieved by flushing the headspace of the bottles with N_2 gas for 2 min (ca. 5 L/min) before they were sealed with a PTFE-butyl septum retained with a screwcap. Finally, test bottles were placed in a temperature-controlled incubator set at 35 ± 1 °C. Each fermentation batch was run for 14 days since VFAs production ceased after about 10 days in all assays. Fermentation performance was monitored from 8 sampling events where VFAs, lactic acid, sCOD, and pH were measured. In each sampling event, the pressure accumulated in the headspace of the bottle was vented to atmospheric pressure and subsequently, 4 mL of liquid sample were withdrawn (the total

withdrawn samples represented about 20% of the initial volume). Samples were taken through the septum with an 18G hypodermic needle and 5mL plastic syringe to minimise air exposure.

Three fermentation experiments were performed in this study, which used the same three mixtures of WAS and FW (on VS basis): (i) 50%WAS +50% FW(WAS/FW_50/50), (ii) 70%WAS+30% FW(WAS/FW_70/30), and (iii) 90% WAS + 10% FW (WAS/FW_90/10). A key difference with most previous publications is that in this study WAS, instead of FW, is the main substrate. Additionally, WAS and FW mono-fermentation controls were included in each experiment. Table 5.2 provides detailed information about bottles composition in each experiment. The specific details and goals of each experiment are given in the following sub-sections.

Table 5.2. Composition of the three co-fermentation assays.

	Experiment 1		**Experiment 2**			**Experiment 3**		
Condition	FW (g)	WAS (g)	FW (g)	FW* [1] (g)	WAS (g)	FW (g)	WASp [2] (g)	WAS (g)
FW	150.0	-	150.0	-	-	150.0	-	-
FW*	-	-	-	150.0	-	-	-	-
WAS	-	150.0	-	-	-	-	150.0	-
WASp	-	-	-	-	150.0	-	-	150.0
WAS/FW_50/50	25.8	124.2	24.0	-	126.0	34.1	-	115.9
WAS/FW_70/30	12.3	137.7	8.9	-	141.1	17.0	-	133.0
WAS/FW_90/10	3.4	146.6	3.1	-	146.9	4.7	-	145.3
WAS/FW*_50/50	-	-	-	24.0	126.0	-	-	-
WAS/FW*_70/30	-	-	-	8.9	141.1	-	-	-
WAS/FW*_90/10	-	-	-	3.1	146.9	-	-	-
WASp/FW_50/50	-	-	-	-	-	34.1	115.9	-
WASp/FW_70/30	-	-	-	-	-	17.0	133.0	-
WASp/FW_90/10	-	-	-	-	-	4.7	145.3	-

[1] FW* identifies FW samples with extra alkalinity (addition of 30 g $NaHCO_3$/kg)
[2] WASp identifies WAS samples after WAS auto-hydrolysis pre-treatment (55 ºC for 2.5 h)

Experiment 1: impact of WAS/FW mixture on co-fermentation performance

The first experiment aimed to understand the impact of the mixture composition on co-fermentation performance, i.e., pH evolution, VFA yield, VFA profile and lactic acid. This experiment included three WAS/FW mixtures (i.e., WAS/FW_50/50, WAS/FW_70/30 and WAS/FW_90/10) and mono-fermentation controls for each substrate (i.e. FW only and WAS only).

Experiment 2: impact of FW buffer capacity on co-fermentation performance

The second experiment was carried out (i) to assess the reproductivity of experiment 1 results and (ii) to improve the understanding of the fermentation liquor buffer capacity (alkalinity) on co-fermentation performance. This is important because (i) the alkalinity provided by WAS varies seasonally and from plant to plant (Astals et al., 2013; Toutian et al., 2020), and (ii) the importance of pH on fermentation performance (Fang et al., 2020; Jie et al., 2014). The synthetic FW was split into two lots, one without additional alkalinity and the other with additional alkalinity (30 $gNaHCO_3/kg_{ww}$). The FW with extra buffer capacity alkalinity is symbolised in the manuscript as FW*. All co-fermentation mixtures and FW control were carried out with and without additional alkalinity (see Table 5.2). The WAS control was the only test condition that was not supplemented with extra alkalinity. The tests carried out in this second experiment were: WAS/FW_50/50, WAS/FW_70/30, WAS/FW_90/10, WAS/FW*_50/50, WAS/FW*_70/30, WAS/FW*_90/10, FW control, FW* control, and WAS control.

Experiment 3: impact of WAS auto-hydrolysis on co-fermentation performance

The third experiment was carried out to assess the potential of WAS auto-hydrolysis pre-treatment (abbreviated as WASp) on co-fermentation performance. Auto-hydrolysis was carried out by placing several tightly capped 1-L bottles in a 55 ± 1 °C temperature-controlled incubator for 2 h 30 min (each bottle contained 800 mL of WAS). 55 °C were chosen according to previous publications (Arias et al., 2018; Carvajal et al., 2013). The auto-hydrolysis time was selected based on preliminary experiments as the time when the sCOD production rate was higher. Anaerobic conditions were achieved by flushing the headspace with N_2 (2 min at 5 L/min) at the beginning of the experiment. Auto-hydrolysis performance was monitored by analysing the sCOD and VFAs concentrations over time. As for the

fermentation tests, samples were taken through the septum with an 18G hypodermic needle and a 5 mL plastic syringe. Once the auto-hydrolysis pretreatment finished, the pre-treated WAS (WASp) was immediately used in the fermentation batch assays. The three co-fermentation mixtures under study were performed with and without pre-treated WAS. The mixtures tested were: WAS/ FW_50/50, WAS/FW_70/30, WAS/FW_90/10, WASp/FW_50/50, WASp/FW_70/30 and WASp/FW_90/10. Additionally, three mono-fermentation controls were included: WAS, WASp and FW.

5.2.3. Analytical procedures

TS, VS, COD and sCOD were analysed following the Standards Methods for the examination of Water and Wastewater (APHA, 2017) as has been explained in Section 3.3. Moreover, the pH was measured using semi-micro pH electrode (PHEL-GB3-001) connected to benchtop multi-meter (Crison, MultiMeter MM 41). VFAs were analysed using a Shimadzu GC-2010 plus gas chromatograph equipped with a Nukol™ capillary column and flame ionised detector (see Section 3.3.7 for GC configuration and procedure). Lactic acid was analysed using a high-performance liquid chromatograph (HPLC, Waters Alliance 2695, US) as has been explained detailed in Section 3.3.8. Individual VFA and lactic acid concentrations were converted to COD equivalents using the theoretical value based on their elemental composition.

5.3. Results and discussion

5.3.1. Impact of WAS/FW mixture on co-fermentation performance

Figure 5.1. shows the evolution of fermentation yield over time for co-fermentation mixtures and mono-fermentation controls (i.e. WAS and FW) of the first experiment. In Figure 5.1, it can be observed that the yield of the co-fermentation mixtures was much higher than the yield of the WAS and FW controls (95 and 80 mgCOD/gVS, respectively). It can also be observed that the pH decreased from neutral (WAS control) to below 4 (FW control) as the FW content in the mixture increased.

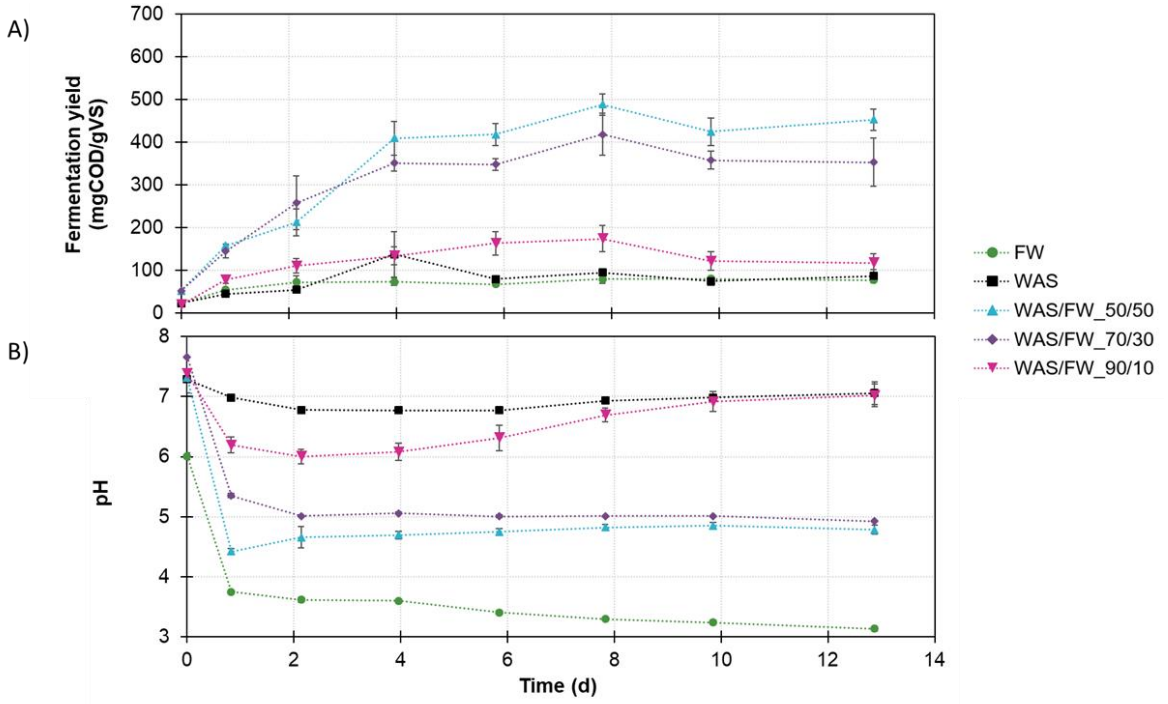

Figure 5.1. Evolution of the fermentation yield (A) and pH (B) in Experiment 1. Error bars indicate the standard deviation.

The highest fermentation yield was achieved by the mixture WAS/FW_50/50 (489 mgCOD/gVS), which was the mixture with the highest amount of FW. The yield of the co-fermentation mixtures decreased as the amount of FW in the mixture decreased. Specifically, the maximum fermentation yield of WAS/FW_50/50, WAS/FW_70/30, and WAS/FW_90/10 was 489, 419 and 175 mgCOD/gVS, respectively. The fact that the fermentation yield increased with the increase of FW proportion suggests that the improvement on the fermentation yield can be primarily related to a higher extent of FW fermentation under co-fermentation conditions. Assuming that the fermentation yield from WAS did not change under co-fermentation conditions, the FW yield under co-fermentation conditions was estimated at 900 mgCOD/gVS for the three co-fermentation mixtures at the peak concentration.

The low fermentation yield of the FW control can be attributed to pH inhibition since the pH of the fermentation liquor dropped from 6.0 to 3.7 in the first day (Figure 5.1B). Several publications have consistently reported that pH below 5.0 is inhibitory for the

hydrolytic-fermentative bacteria (Feng et al., 2011; Wang et al., 2014). Indeed, FW low buffer capacity and the associated pH drop have been previously reported to limit the extent of FW fermentation (Feng et al., 2018; Luo, et al., 2020a; Xiong et al., 2019). Therefore, WAS improved FW fermentation by providing the buffer capacity needed to keep the pH above severe inhibitory values (Cabbai et al., 2016; Zhang et al., 2017a), but also could have diversified the starting microbial community (Wu et al., 2016).

The pH of the co-fermentation mixtures with a higher proportion of FW (i.e., WAS/FW_50/50 and WAS/FW_70/30) dropped from 7.3 to around 5.0 in the first two days and remained at this level until the end of the experiment (Figure 5.1B). pH around 5.0 is within reported inhibitory levels (Xing et al., 2020). However, the steady accumulation of VFAs until day 8 indicates that the fermentation process was not severely inhibited under these conditions. The pH of the WAS/FW_90/10 varied between 6.0 and 7.0. The neutral pH of the WAS/FW_90/10 mixture could have facilitated the net degradation of acetic acid after reaching the maximum concentration at day 6 which is shown in Figure 5.2. that illustrates the evolution of the concentration of each condition over time. Between day 6 and 13, the acetic acid concentration dropped from 1500 mgCOD/L to about 200 mgCOD/L and the pH increased from 6.3 to 7.0. The consumption of acetic acid is a recurrent phenomenon in fermentation batch assays (Pang et al., 2020; Peces et al., 2020), and in this experiment, it may be related to the presence of sulphate reducing bacteria or methanogenic archaea in WAS (Chen et al., 2013; Xiong et al., 2019). These experimental results indicate that mixtures with a high proportion of WAS may not be favourable for WAS/FW co-fermentation, particularly when targeting acetic acid accumulation, due to the constant immigration of acetic acid consumers into the system. However, this hypothesis needs to be validated with continuous experiments due to the hydraulic selective pressure of continuous systems on the microbial community.

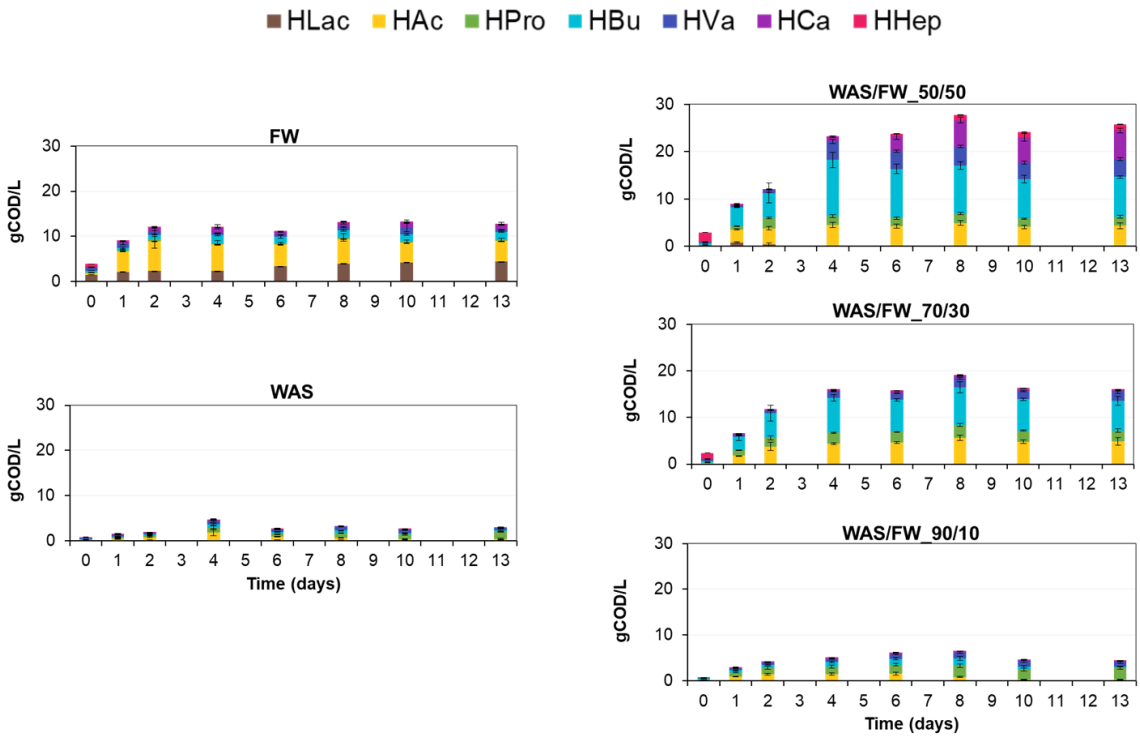

Figure 5.2. Evolution of the individual VFAs and lactate for each condition in Experiment 1. Error bars indicate the standard deviation.

Figure 5.3 shows the fermentation profile of the five conditions in experiment 1 on day 8, since this was the day when the co-fermentation mixtures reached the maximum fermentation yield. WAS mono-fermentation profile was primarily composed of acetic (15%), propionic (34%), butyric (17%) and valeric acid (26%), which is consistent with the results reported by Pang et al. (2020a) and (2020b). In contrast, the FW mono-fermentation profile was dominated by acetic (42%) and lactic acid (30%). This FW profile is similar to the profile reported by Komemoto et al. (2009). Substrate composition and pH are two well-known controlling factors of the fermentation product profile (Hoelzle et al., 2014; Lin & Li, 2018). The high presence of lactic acid in FW mono-fermentation could be related to its starch content (Li et al., 2015; Ma et al., 2017), the low pH of the fermentation liquor (Itoh et al., 2012), and the presence of lactic acid producing bacteria in FW (Wang et al., 2015; Wu et al., 2015; Zhang et al., 2016). According to Tang et al. (2016), lactic acid production from FW is favoured at pH values between 5.0 and 6.0, which is subsequently converted to propionic acid. However, in the FW control propionic acid production from lactic acid may have been

inhibited due to low pH, promoting the accumulation of lactic acid in the fermentation liquor (Li et al., 2014; Zhang et al., 2005).

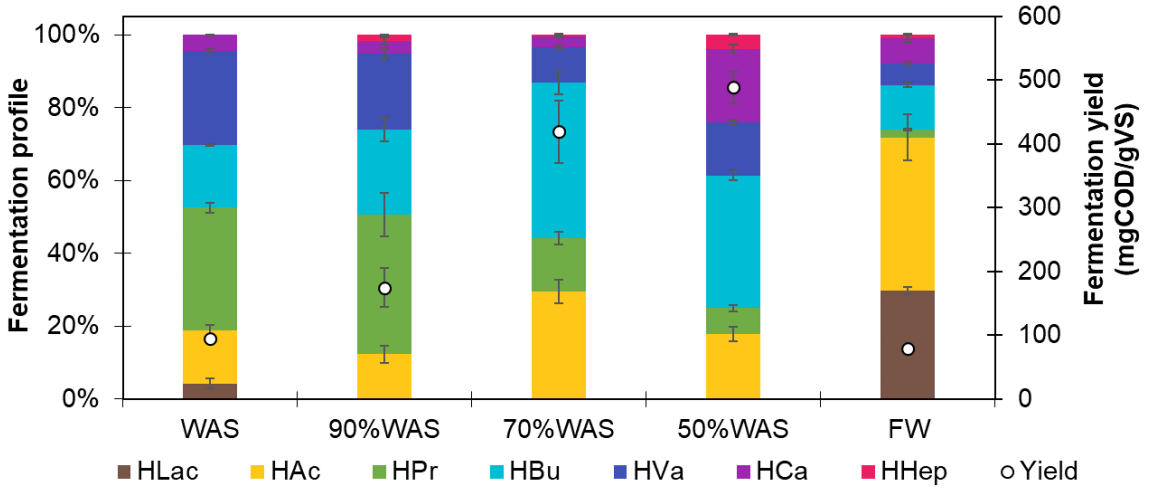

Figure 5.3. Fermentation profile in COD basis at the maximum fermentation yield (day 8) in Experiment 1. Error bars indicate the standard deviation.

The impact of substrate composition can be assessed by comparing the fermentation profile of the WAS/FW_50/50 and WAS/FW_70/30 mixtures since both tests displayed similar pH values and yield over time. The WAS/FW_50/50 mixture was dominated by butyric acid (36%) followed by caproic (20%), acetic (18%), valeric (15%) and propionic acid (7%). On the other hand, the WAS/FW_70/30 mixture was still dominated by butyric acid (43%), but the contribution of acetic (30%) and propionic acid (15%) increased to the detriment of valeric (10%) and caproic acid (3%). The dominance of butyric acid in the fermentation profile despite the different substrate composition can be related to pH, since butyric acid accumulation appears to be favoured at pH values between 5.5 and 4.0 (Fang et al., 2020; Wang et al., 2014). Besides butyric acid, the different fermentation profile between WAS/FW_50/50 and WAS/FW_70/30 could be related to the substrate composition. Finally, the fermentation profile of the WAS/FW_90/10 mixture (i.e., acetic (12%), propionic (38%) and butyric acid (23%)) was closer to the WAS mono-fermentation profile than to the other mixtures, which can be consistently related to both substrate composition and pH.

5.3.2. Impact of FW buffer capacity on co-fermentation performance

The second experiment aimed to check the reproducibility of experiment 1 and assess the impact of a higher FW alkalinity (FW* indicates test carried out with extra alkalinity). As in experiment 1, the fermentation yield of the co-fermentation mixtures was much higher than the obtained in the WAS and FW mono-fermentation controls (96 and 72 mgCOD/gVS, respectively) (see Figure 5.4A). Importantly, fermentation yield, fermentation profile and pH of both controls were similar to the obtained in experiment 1, which facilitates co-fermentation results comparison. The fermentation yield of the three co-fermentation mixtures without extra alkalinity (i.e., WAS/FW_50/50, WAS/FW_70/30 and WAS/FW_90/10) increased as the amount of FW in the mixture increased, further supporting that the yield improvement was primarily due to the higher FW fermentation under co-fermentation conditions. The maximum fermentation yield for the WAS/FW_50/50 and WAS/FW_70/30 mixtures was similar to the obtained in experiment 1, (i.e., 463 mgCOD/gVS for WAS/FW_50/50, and 397 mgCOD/gVS for WAS/FW_70/30). However, in experiment 2, the FW/WAS_90/10 mixtures showed a higher yield (327 mgCOD/gVS) than in experiment 1 (175mgCOD/gVS). The higher yield in experiment 2 could be related to the lower pH in the first days of the experiments, which may have increased fermentative bacteria activity and/or inhibited VFA consumers (e.g. methanogenic archaea) (Jiang et al., 2013; Lim et al., 2008a; Wang et al., 2014; Yuan et al., 2019; Zhou et al., 2013). The latter appears more likely since from day 6 the concentration of VFAs in the fermentation liquor (mainly acetic acid) decreased from about 3400 mgCOD/L to 600 mgCOD/L, which was concomitant to a pH increase from 5.9 to 7.2 (see Figure 5.4B and Figure 5.5). These results reinforce the idea that fermenters operational conditions should prevent the enrichment of acetic acid degraders (e.g., methanogenic archaea and sulphate reducing bacteria), which is particularly relevant when one of the co-substrates provides a constant inflow of microorganisms (e.g., WAS and animal manure).

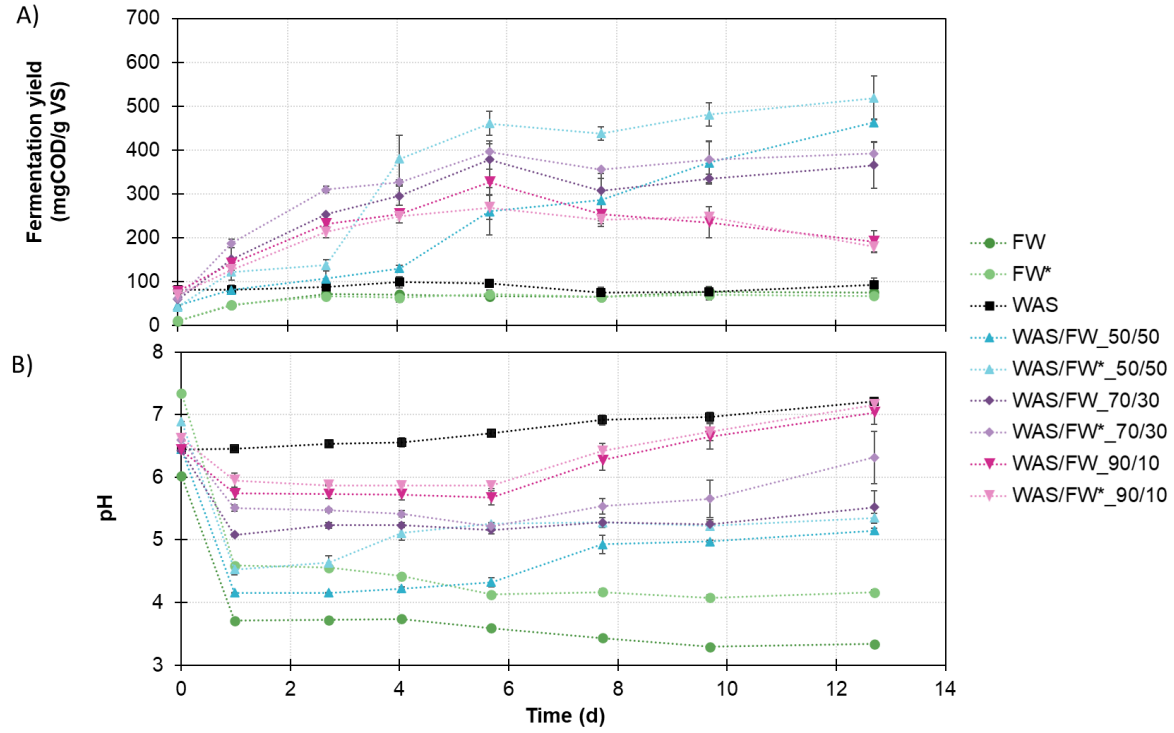

Figure 5.4. Evolution of the fermentation yield (A) and pH (B) for Experiment 2.

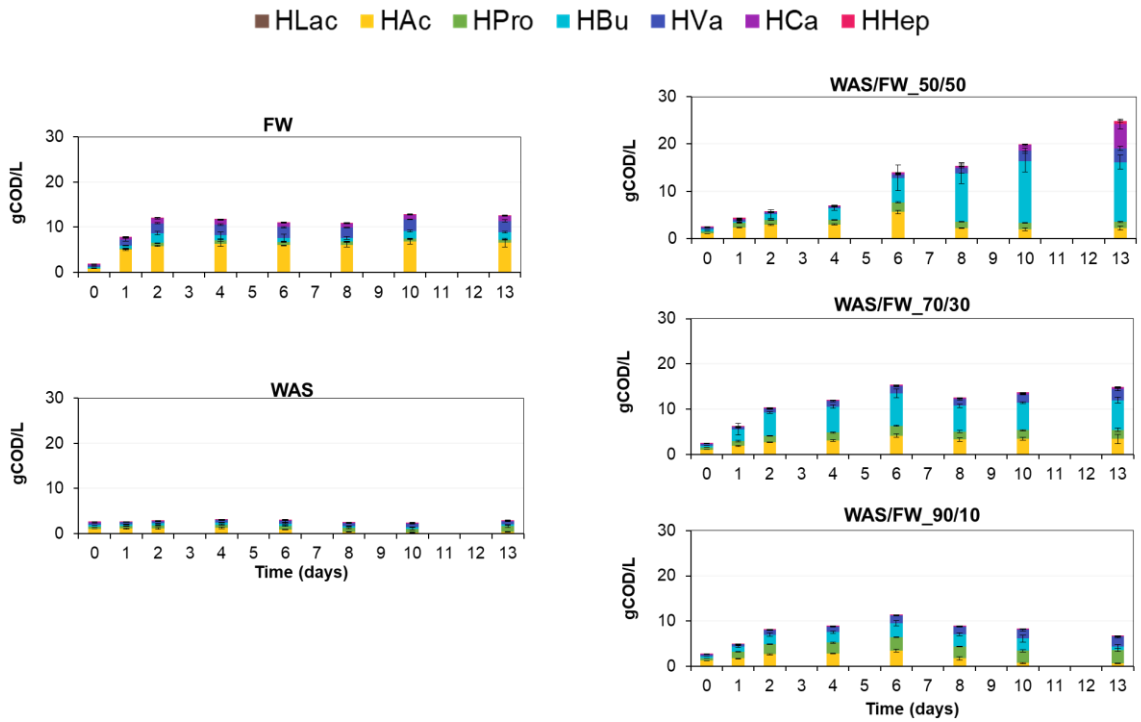

Figure 5.5. Evolution of the individual VFAs and lactate for each condition without alkalinity addition in Experiment 2. Error bars indicate the standard deviation.

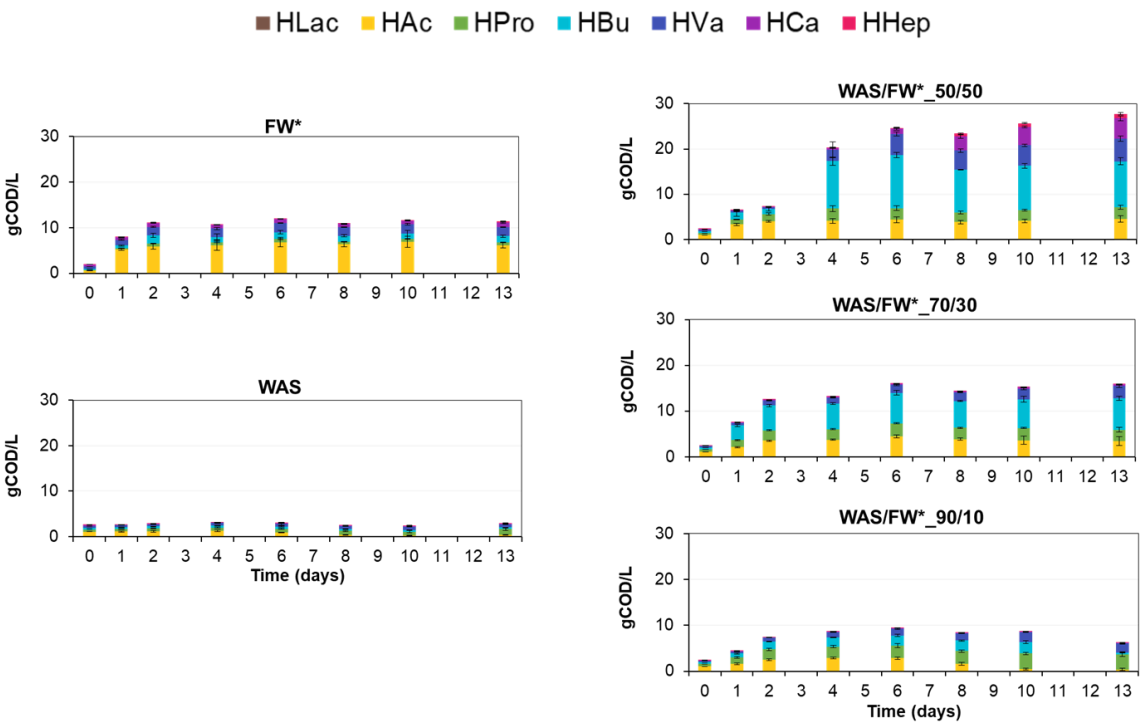

Figure 5.6. Evolution of the individual VFAs and lactate for each condition with alkalinity addition (FW*) in Experiment 2. Error bars indicate the standard deviation.

The addition of alkalinity to FW (30 g$NaHCO_3$/kg) was not enough to have a notable effect on the fermentation yield nor the fermentation profile of the WAS/FW_70/30 and the WAS/FW_90/10 mixtures (see Figure 5.4A, 5.5 and 5.6), which can be attributed to the relatively low proportion of FW in the mixture (see Table 5.2). The maximum fermentation yield of the WAS/FW_70/30 and WAS/FW*_70/30 mixtures were 379 and 397 mgCOD/gVS and for the WAS/FW_90/10 and WAS/FW*_90/10 mixtures were 327 and 269mgCOD/gVS. However, the fermentation yield (and its evolution over time) was remarkably different between WAS/FW_50/50 and WAS/FW*_50/50 (Figure 5.4). A boost in VFA and lactic acid production occurred in WAS/FW*_50/50 at day 3, which was synchronic with a pH increase from 4.6 to 5.1. The better performance of WAS/FW*_50/50 can be related to the higher pH of the fermentation liquor as a result of its higher buffer capacity. These results indicate that the amount of FW in the co-fermentation mixture should be limited to keep the pH above 5.0. Higher proportions of FW are possible but at the expense of constantly dosing external alkali chemicals, which should be considered in the techno-economic

analysis. Note that the pH is not only affected by the mixture composition but also by the operational conditions of the fermenter.

The fermentation profile at the maximum fermentation yield (day 6) of the mono-fermentation and co-fermentation test carried out without extra alkalinity in experiment 2 (Figure 5.7) were similar to the obtained in experiment 1 (Figure 5.3). The only remarkable difference was the profile of WAS/FW_50/50 since in experiment 1 the fermentation profile was dominated by butyric (36%), valeric (20%) and acetic acid (18%), while in experiment 2 the fermentation profile was dominated by acetic (40%), butyric (26%) and propionic acid (15%). The difference on fermentation profile can be attributed to the pH difference between both experiments (pH of 4.8 vs. 4.3 in experiment 1 and 2, respectively) since lower pH tends to favour the accumulation of acetic and lactic acid over butyric acid (Luo et al., 2020b). This observation is further supported by comparing the fermentation profile of the WAS/FW_50/50 and WAS/FW*_50/50 (pH of 4.3 vs. 5.3 at day 6, respectively). The fermentation profile of WAS/FW_50/50 was dominated by acetic (40%), butyric (26%) and propionic acid (15%), while WAS/FW*_50/50 was dominated by butyric (47%) followed by valeric (19%) and acetic acid (18%). The fermentation profile of the other two co-fermentation mixtures was not affected by the extra FW alkalinity (WAS/FW_70/30 vs. WAS/FW*_70/30 and WAS/FW_90/10 vs. WAS/FW*_90/10) as expected due to the similar pH and fermentation yield over time. Experiment 2 results indicate that butyric acid was enriched as the proportion of FW in the mixture increased and the concomitant pH decreased, while propionic acid prevailed at higher WAS proportions and concomitant neutral pH.

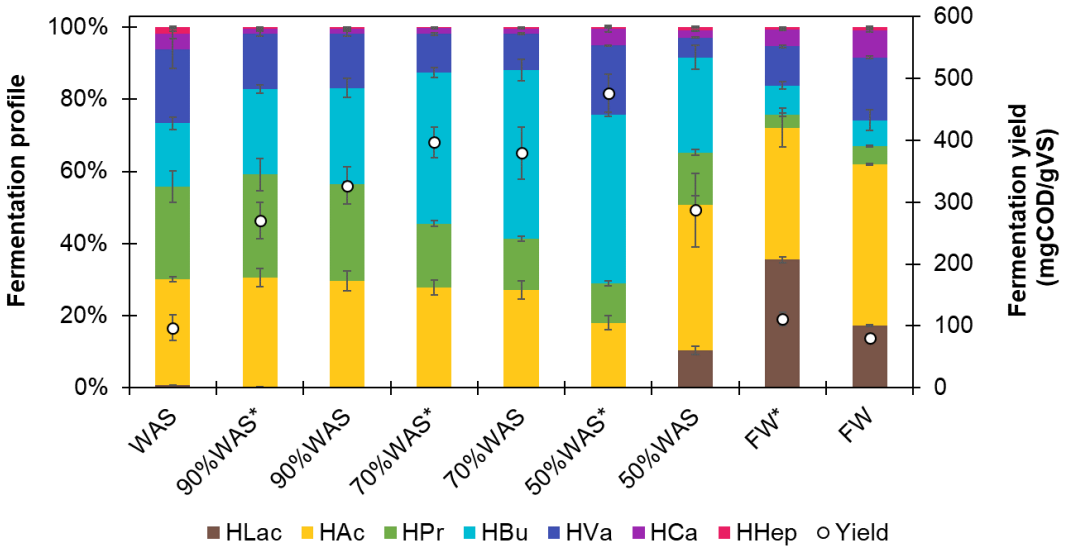

Figure 5.7. Fermentation profile in COD basis at the maximum fermentation yield (day 6) in Experiment 2. Error bars indicate the standard deviation.

5.3.3. Impact of WAS auto-hydrolysis on co-fermentation performance

5.3.3.1. WAS auto-hydrolysis pre-treatment

WAS auto-hydrolysis pre-treatment at 55 °C was monitored by measuring the sCOD and VFA concentration over time. The auto-hydrolysis pre-treatment was carried out with four different WAS batches, three of them were preliminary experiments to determine the impact of pretreatment time on WAS solubilisation and co-fermentation performance. All the auto-hydrolysis pre-treatments showed the same pattern, a steady increase of sCOD concentration and constant VFA concentration. Figure 5.8 illustrates the auto-hydrolysis results of the WAS batch used in the co-fermentation experiments. During the auto-hydrolysis pre-treatment (2.5 h), WAS sCOD concentration increased from 3.0 gCOD/L to 8.6 gCOD/L while the VFA concentration remained constant at 0.6 gCOD/L. These results showed that auto-hydrolysis pre-treatment promotes WAS solubilisation but not WAS fermentation, which is in agreement with the results reported by Arias et al. (2018) and Carvajal et al. (2013).

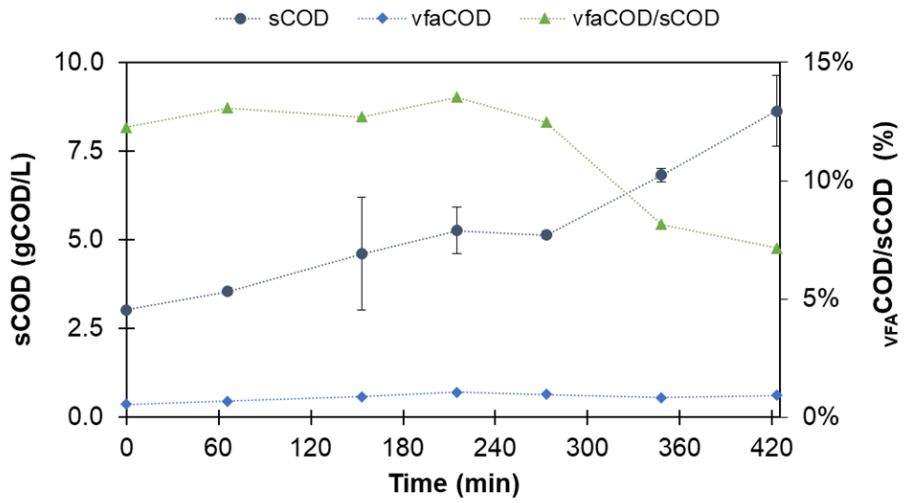

Figure 5.8. Auto-hydrolysis pre-treatment assay in experiment 3. Error bars indicate the standard deviation.

Preliminary experiments also showed that the best WAS/FW co-fermentation performance was achieved after 2.5 h of pre-treatment, which corresponds to the time when the sCOD production rate was higher (Figure 5.8). This pre-treatment time is shorter than the reported by Arias et al. (2018) and Carvajal et al. (2013); however, their research was devoted to anaerobic digestion while this research targets acidogenic fermentation.

5.3.3.2. Effect of WAS auto-hydrolysis pre-treatment on co-fermentation

The comparison of the WAS and WASp mono-fermentation controls showed that auto-hydrolysis pre-treatment led to a ~ 25% improvement of the WAS fermentation yield during the first 4 days (135 mgCOD/gVS vs. 167 mgCOD/gVS at day 4) (see Figure 5.9, Figure 5.10 and Figure 5.11). However, from day 5 onwards the fermentation yield of both samples was similar, which indicates that the auto-hydrolysis pre-treatment speeds up fermentation but does not increase WAS biodegradability (i.e., the amount of organic matter available for fermentation). This is likely due to the short duration of the auto-hydrolysis pre-treatment (2.5 h). It is worth mentioning that the WAS maximum yield in experiment 3 (194 mgCOD/gVS) was higher than the achieved in experiment 1 and 2 (95 mgCOD/gVS and 96 mgCOD/gVS, respectively). The WAS batch used in experiment 3 was collected in July (summer), therefore, the higher yield can be explained due to the higher biodegradability of WAS in summer months. Regarding the

fermentation profile, the WAS mono-fermentation at the maximum fermentation yield was composed of acetic (37%), propionic (23%), butyric (18%) and valeric acid (18%).

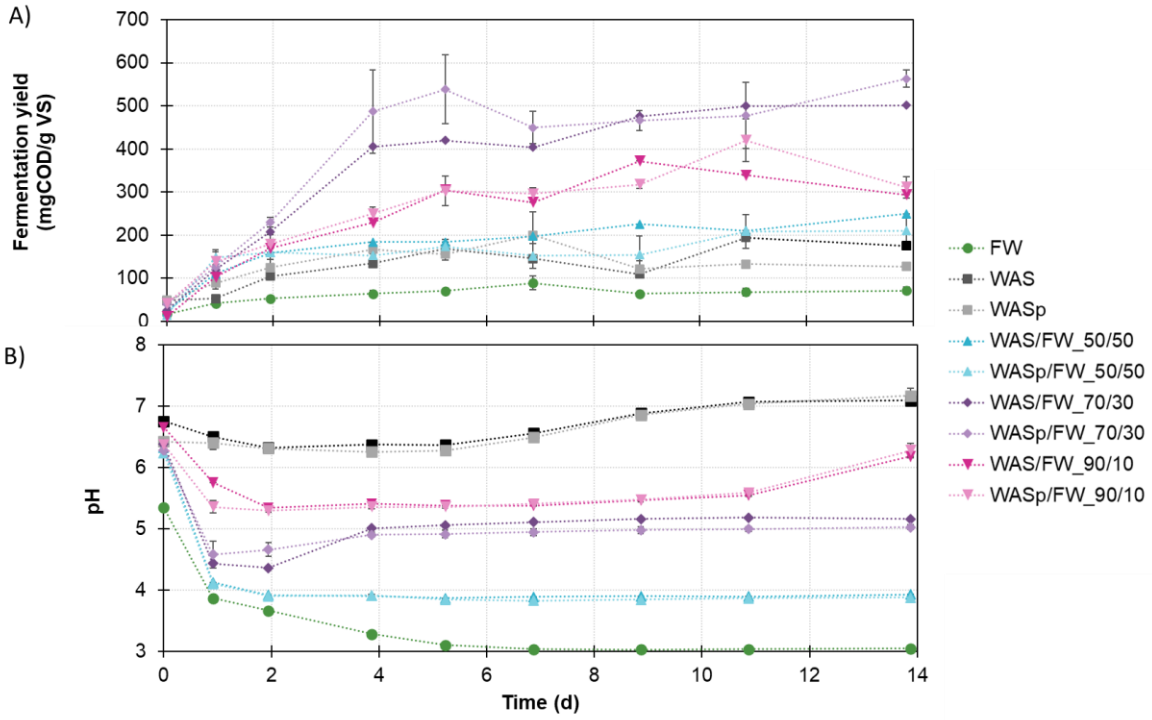

Figure 5.9. Evolution of the fermentation yield (top) and pH (bottom) in experiment 3. Error bars indicate the standard deviation.

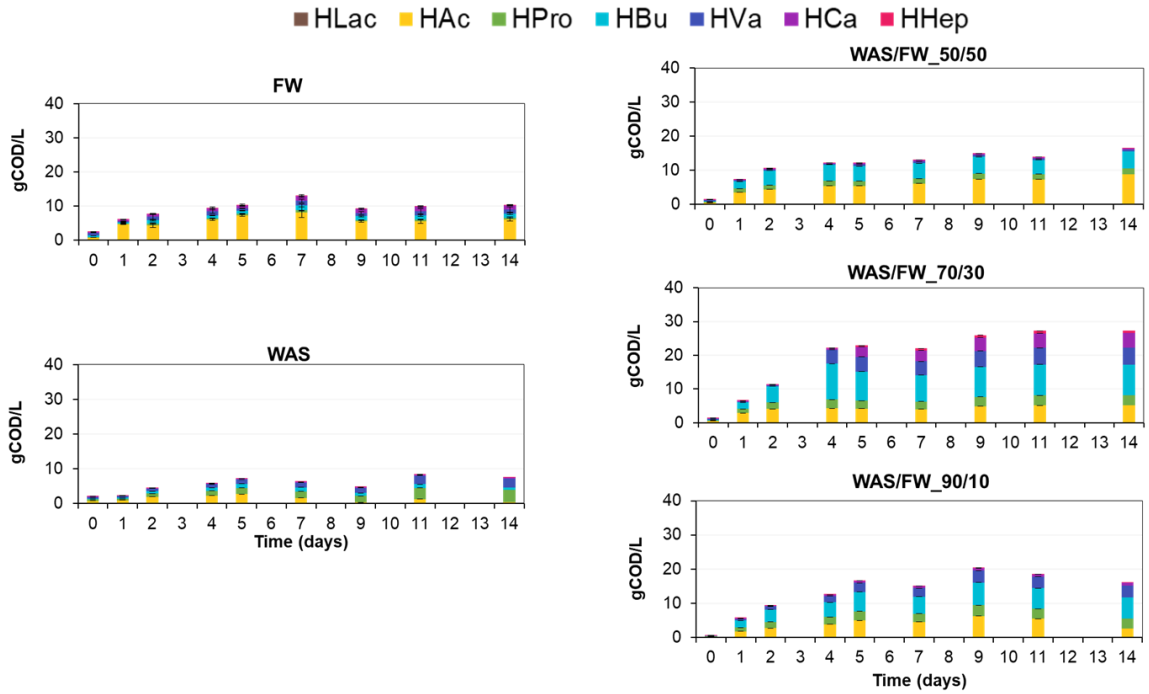

Figure 5.10. Evolution of the individual VFAs for each condition without autohydrolysis pre-treatment in Experiment 3. Error bars indicate the standard deviation.

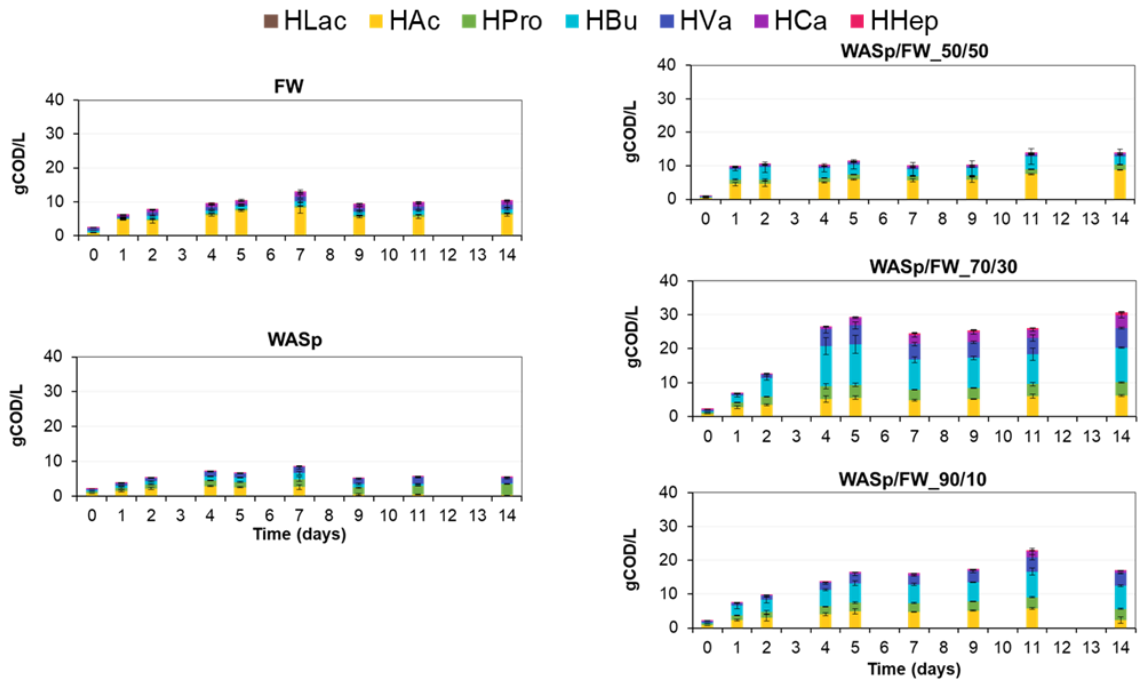

Figure 5.11. Evolution of the individual VFAs for each condition with autohydrolysis pre-treatment (WASp) in Experiment 3. Error bars indicate the standard deviation.

Regarding the WAS/FW co-fermentation with untreated WAS, the most remarkable difference with experiment 1 and experiment 2 was the poor performance of the WAS/FW_50/50 mixture (250 mgCOD/gVS), which was lower than the achieved by the WAS/FW_70/30 and the WAS/FW_90/10 mixtures (502 and 339 mgCOD/gVS, respectively). The lower yield of WAS/FW_50/50, when compared with the two previous experiments, can be related to the lower pH of the fermentation liquor (pH of 3.9). Accordingly, the fermentation profile of the WAS/FW_50/50 was different from the observed in the two previous experiments. Experimental results did not allow to elucidate the cause that led to the lower pH in experiment 3. However, the lower pH in experiment 3 could be due to (i) a lower WAS alkalinity, (ii) a higher FW or WAS biodegradability, and/or (iii) a different native microbial community.

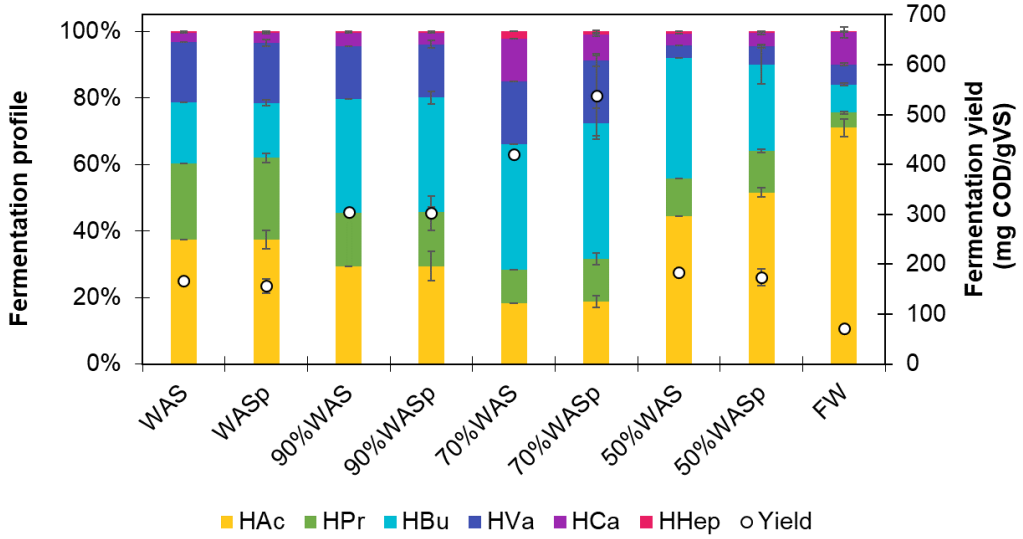

Figure 5.12. Fermentation profile in COD basis the maximum fermentation yield (day 5) in Experiment 3. Error bars indicate the standard deviation.

In Figure 5.9 and Figure 5.12 it can be observed that the co-fermentation mixtures carried out with WASp showed similar fermentation yields and fermentation profiles than the co-fermentation mixtures carried out with untreated WAS. These results indicate that WAS auto-hydrolysis pre-treatment may not be a suitable approach to enhance WAS/FW co-fermentation performance since the slight rate of improvement would hardly compensate for the higher process complexity and investment costs.

5.4. Conclusions

WAS and FW co-fermentation (50%, 70% and 90% WAS on VS basis) was investigated through batch testing under different experimental conditions to produce carboxylic acids. Results showed that the fermentation yields achieved under co-fermentation conditions were always higher than the obtained from both WAS and FW mono-fermentation. Co-fermentation yields increased as the proportion of FW in the mixture increased indicating that the improvement was primarily due to a higher FW degradation under co-fermentation conditions. Regarding the product profile, butyric acid was enriched in the mixture as the proportion of FW in the mixture increased and the concomitant pH decreased, to the detriment of acetic and propionic acid percentages. Experiments carried out with the addition of alkalinity showed that the

proportion of WAS in the mixture should be large enough to keep the pH above 5.0 to prevent fermenters inhibition and avoid the constant dosage of alkali. However, fermenters operational conditions should prevent the enrichment of acetic acid degraders immigrating with WAS. Finally, WAS/FW co-fermentation mixtures carried out with auto-hydrolysis pre-treated WAS resulted in minor kinetics improvements but did not improve the co-fermentation yields. Overall, these results showed that WAS/FW co-fermentation is an opportunity to boost fermentation yields while minimising the use of chemical reagents. The proportion between both substrates can be adjusted to tune the product profile based on the application requirements.

6. Impact of food waste composition on acidogenic co-fermentation with waste activated sludge

ABSTRACT

The impact of FW composition on co-fermentation performance was studied using WAS as main substrate. Experiments were carried out in mesophilic batch assays using the same mixture (70% WAS + 30% FW on VS basis) and no pH control. The first set of batch assays was carried out to assess the impact of each FW component, i.e., fruit, vegetables, pasta, rice, meat, fish, and cellulose. The results obtained for each component showed a distinct effect on the VFA yield and profile. The maximum VFA yield obtained was 502 and 442 mgCOD/gVS for WAS/Fish and WAS/Meat co-fermentation, respectively. The second and third set of batch assays were aimed to study the effect of protein to carbohydrate ratio and to evaluate the influence of different carbohydrates and protein sources. In the second experiment explored mixtures between fish and either fruit or cellulose, while in the third experiment explored mixture between meat and either vegetable or rice were carried out. In both experiments, the mixtures with a protein-rich substrate with different carbohydrates obtained similar yields. Specifically, the maximum production was about 500 mgCOD/gVS when the proportion of WAS/protein/carbohydrate was 70/20/10 being higher than WAS/protein and WAS/carbohydrate (70/30 on VS basis) co fermentation. These results show the importance of balancing the FW components to improve VFAs production. Finally, the PCA analysis showed that each WAS could be different but not influenced in the co-fermentation profile.

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6.1. Introduction

The EU Bioeconomy Strategy aims to manage natural resources sustainably and to reduce the dependence on non-renewable and unsustainable resources (European Commission, 2018a). The action plan of this strategy remarks the importance of developing and implementing new sustainable biorefineries to substitute fossil-based materials for bio-based, recyclable and biodegradable materials using organic wastes, residues and side streams (European Commission, 2018b).

Fermentation is a key biotechnology in most microbially-driven biorefineries schemes due to its capability to transform organic waste into easily assimilable organic compounds such as VFAs (i.e., acetic, propionic, butyric, valeric and caproic), lactic acid and alcohols (Annamalai et al., 2020; Puyol et al., 2017; Venkata Mohan et al., 2016). These fermentation products can be subsequently utilised as organic platform chemicals to produce biopolymers (Fradinho et al., 2019; Valentino et al., 2018), single cell protein (Allegue et al., 2021.; Capson-Tojo et al., 2020), medium chain fatty acids (Carvajal-Arroyo et al., 2021; Roghair et al., 2018), or to generate bioenergy (Abreu et al., 2019; Dahiya et al., 2015), among others.

Co-fermentation, the simultaneous fermentation of two or more waste, is an emerging strategy to increase the fermenters yield treating a single waste (mono-fermentation) (Perez-Esteban et al., 2022). Co-fermentation improves the fermentation yields by (i) increasing the organic loading rate, (ii) balancing macro- and micro-nutrients, (iii) diluting potential inhibitory and toxic compounds, (iv) increasing the buffer capacity, (v) improving rheological properties, and/or (vi) promoting an active microbial community (Fang et al., 2020; Peces et al., 2020; Perez-Esteban et al., 2022).

WWTPs are pioneering the paradigm change from treatment towards resource recovery. WWTP generate large amounts of WAS that is commonly diverted to anaerobic digestion for biogas and digestate production. However, biogas and digestate have a relatively low market value and a lower range of application than fermentation products (Dahiya et al., 2018). The acidogenic fermentation of WAS could be implemented in WWTP to produce VFAs for biological nutrient removal (N and P) as well as to support other more advanced and profitable biotechnologies (e.g.,

biopolymers production). However, WAS is characterised by low fermentation yields due to its poor biodegradability and low hydrolysis rate (Gonzalez et al., 2018; Gou et al., 2014; Peces et al., 2020; Xu et al., 2020b).

FW is the most studied co-substrate for WAS co-fermentation (Perez-Esteban et al., 2022). On the one hand, FW is a suitable co-substrate for WAS due to its high organic content and biodegradability. On the other hand, WAS is a suitable main substrate for FW due to its water content and buffer capacity. Most WAS-FW co-fermentation studies have focused on the impact of operational conditions such as pH, temperature, HRT and mixture composition. However, little attention has been given to other important parameters such as the impact of FW composition on VFA yield and product profile.

Strazzera et al. (2021) investigated the mesophilic mono-fermentation of the different fractions in food waste (i.e. protein, lipids, starch, cellulose, fruit and vegetable) at uncontrolled pH, pH 5.5 and pH 7.0. The highest fermentation yields were obtained from the protein-rich fraction (composed of cheese, tuna and beef) followed by the starch-rich fraction (composed of bread and pasta) and the fruit and vegetable fraction. The fermentation yield of the cellulose-rich fraction (composed lab-grade cellulose and paper) and lipid-rich fraction (olive oil) was negligible for all pH conditions. Regarding the pH, the highest yields were achieved at pH 7.0 followed by pH 5.5. At pH 7.0, the product profile of all fractions was dominated by butyric acid. The starch-rich and fruit and vegetable fractions also enriched propionic and acetic acid, whereas the protein-rich fraction also enriched valeric and acetic acid.

The fermentation of protein have not been studied as thoroughly as sugars and carbohydrates fermentation (González-Cabaleiro et al., 2015; Hoelzle et al., 2014; Zhou et al., 2018), although protein is a major constituent of most organic waste streams. Shen et al. (2017b) and Bevilacqua et al. (2020) evaluated the impact of different types of protein on acidogenic mono-fermentation performance. Shen et al. (2017b), who fermented tofu (plant protein) and egg white (animal protein) at 30 °C and pH 6.0, reported a higher fermentation yield for egg white than for tofu and a completely different product profile. Specifically, the tofu fermentation profile was dominated by acetic acid, while egg white presented an evenly distributed concentration of acetic, propionic, butyric and valeric acid. Bevilacqua et al. (2020), who fermented casein and

gelatin at 25 °C and circumneutral pH, also reported that different protein types result in different fermentation yields (higher for casein than for gelatin) and product profile. The dominant VFA in both fermenters was acetic acid; however, casein fermentation enriched butyric and propionic acid while gelatin fermentation enriched propionic and valeric acid.

These results indicate that FW composition has an impact on fermentation performance (yield and product profile). However, the relative importance of FW composition on the fermentation yield and product profile has not been fully elucidated. Furthermore, to the best of the authors' knowledge, the impact of FW composition has only been studied in FW mono-fermentation experiments and not under co-fermentation conditions. The capability to tune the product profile by adjusting the co-substrate composition is important for biorefinery applications since different biotechnologies may require different easily assimilable carbon compounds as platform chemical.

The goal of this study was to understand how FW composition (i.e., rice, pasta, meat, fish, fruit, vegetables, and cellulose) influences the yield and product profile of WAS-FW co-fermentation. First, the impact of each FW component was individually assessed. Second, the interaction between different FW components (fish-fruit & fish-cellulose) was evaluated under different proportions. Last, the interactions observed in the second set of experiments were validated using different set of FW components (meat-vegetables & meat-rice). All experiments were carried out using the same proportion between WAS and FW (70% WAS + 30% FW on VS basis).

6.2. Materials and methods

6.2.1. Substrates' origin

Thickened WAS collected in a municipal WWTP of the Barcelona Metropolitan Area (ca. 300,000 population equivalents) (Catalonia, NE Spain). After collection, it was stored in a fridge at 4 °C until use (maximum storage time of 3 days).

FW was formulated by mixing vegetables (30%), fruits (30%), carbohydrates (pasta and rice) (20%), meat (10%) and fish (10%) on wet-basis as detailed in Chapter 5. Synthetic FW was used to ensure FW reproducibility throughout the experiments as well as to better assess the individual impact of each component. The different components were: fruit (apple and banana), vegetable (Veg) (potato and onion), pasta (plain boiled pasta), rice (round-grain boiled rice), meat (canned ham), fish (surimi sticks) and microcrystalline cellulose (Cel). Microcrystalline cellulose was not present in the synthetic FW formulation, but it was used as a carbohydrate reference substrate. All ingredients were individually shredded with a kitchen blender where the minimum amount of deionised water was added to facilitate the particle size reduction. Table 6.1 the physico-chemical characteristics of the substrates used in each experiment.

Table 6.1. Substrates characterisation for each experiment. Results are expressed as average ± 95% confidence interval (n = 3).

	Sample	**TS** (gTS/L)	**VS** (gVS/L)	**VS/TS** (%)	**pH** -	**TAN** (mgN/L)	**VFA** (mgCOD/L)
Batch 1	WAS	38.0 ± 0.1	31.3 ± 0.1	82.4 ± 0.3	6.6 ± 0.1	113.0	371
	FW	159.9 ± 0.4	152.6 ± 0.7	95.4 ± 0.2	5.6 ± 0.1	20.3	1456
	Fruit	146.0 ± 1.4	131.3 ± 5.1	89.9 ± 2.8	4.6 ± 0.1	8.8	3426
	Veg	120.4 ± 3.9	111.6 ± 3.7	92.7 ± 0.1	5.5 ± 0.1	20.9	1584
	Pasta	191.5 ± 0.6	189.7 ± 0.6	99.0 ± 0.1	6.8 ± 0.1	-	-
	Rice	196.8 ± 1.3	195.5 ± 1.3	99.3 ± 0.1	7.5 ± 0.1	2.6	609
	Meat	193.1 ± 3.3	171.0 ± 3.4	88.6 ± 0.3	6.5 ± 0.1	76.9	504
	Fish	188.3 ± 3.2	176.5 ± 3.1	93.7 ± 0.1	6.6 ± 0.1	28.6	2268
	Cel	956.5 ± 2.1	953.6 ± 2.0	99.7 ± 0.1	-	-	-
Batch 2	WAS	40.6 ± 0.4	28.9 ± 0.3	71.1 ± 0.1	7.3 ± 0.1	99.0	659 ± 141
	Fruit	126.2 ± 8.3	121.9 ± 7.9	96.6 ± 0.4	4.4 ± 0.1	4.4	4250 ± 311
	Fish	188.0 ± 0.5	176 ± 0.2	93.6 ± 0.2	6.4 ± 0.1	26.6	10067 ± 776
	Cel	932.2 ± 1.3	930.5 ± 1.4	99.8 ± 0.1	-	-	-
Batch 3	WAS	43.2 ± 0.3	30.3 ± 0.3	70.2 ± 0.1	7.5 ± 0.1	73.9	366 ± 9
	Veg	140.9 ± 3.2	133.6 ± 2.4	94.9 ± 0.4	5.4 ± 0.1	30.0	816 ± 62
	Rice	170.4 ± 1.4	169.7 ± 1.5	99.6 ± 0.1	8.5 ± 0.1	2.3	276 ± 16
	Meat	164.3 ± 1.0	143.6 ± 1.1	87.4 ± 0.1	6.1 ± 0.1	103.4	850 ± 16

6.2.2. Co-fermentation experiment set-up

Co-fermentation batch experiments were carried out in 250 mL serum glass bottles (operating volume of 150 mL) under anaerobic conditions at mesophilic temperature. All WAS/FW co-fermentation experiments were carried out in triplicate using the same mixture, i.e., 70% WAS + 30% FW (on VS basis). No inoculum was added, hence the fermentation process relied on the native fermentative bacteria. The pH was not adjusted at the beginning nor during the experiment. After adding the substrates, each bottle was flushed with N_2 gas for 1 min (ca. 5 L/min) to achieve anaerobic conditions and sealed with a PTFE-butyl septum retained with a screwcap. Finally, the bottles were placed in a temperature-controlled incubator at 35 °C. Bottles were manually mixed by swirling each day and before each sampling event. Each fermentation experiment was run for 13 days and monitored by 8 sampling events. In each sampling event, 4 mL of sample were withdrawn with an 18G hypodermic needle connected to a 5 mL syringe (the total withdrawn volume represented about 20% of the initial volume). The samples were collected to analyse sCOD, VFAs, pH and TAN.

Three WAS/FW co-fermentation experiments were performed in this study, all of them using the same WAS and FW mixture proportion on VS basiso (70% WAS + 30% FW). However, FW composition varied based on the goal of each experiment detailed here:

Experiment 1: Co-fermentation of WAS with each FW component

The goal of these tests was to assess the impact of each FW component on co-fermentation performance (i.e., VFA yield, VFA profile). Accordingly, seven co-fermentation mixtures were tested: WAS/Fruit, WAS/Veg, WAS/Pasta, WAS/Rice, WAS/Meat, WAS/Fish and WAS/Cel. Two additional experiments were carried out (i) a WAS/FW co-fermentation and (ii) a WAS mono-fermentation control.

Experiment 2: Co-fermentation of WAS and fish with fruit or cellulose

The goal of the second set of batch tests was to explore the impact of FW composition on WAS/FW co-fermentation performance. Based on the results of the previous set of batch tests, different mixtures between fish (protein) and fruit or cellulose

(carbohydrates) were co-fermented with WAS to: (i) study the effect of FW composition, (ii) assess the importance of the protein-to-carbohydrate ratio, and (ii) determine the influence of the carbohydrates source. Specifically, WAS (main substrate) was co-fermented with either fish & fruit, or fish & cellulose. The tests carried out were (on VS-basis): WAS/Fish_70/30, WAS/Fruit_70/30, WAS/Cel_70/30, WAS/Fish/Fruit_70/20/10, WAS/Fish/Fruit_70/15/15, WAS/Fish/Fruit_70/10/20, WAS/Fish/Cel_70/20/10, WAS/Fish/Cel_70/15/15, WAS/Fish/Cel_70/10/20, and WAS mono-fermentation.

Experiment 3: Co-fermentation of WAS and meat with vegetables or rice

The goal of these tests was to validate the response observed in Experiment 2 using meat was used as protein source and vegetables or rice as carbohydrate source. The tests carried out in this third experiment were: WAS/Meat_70/30, WAS/Veg_70/30, WAS/Rice_70/30, WAS/Meat/Veg_70/20/10, WAS/Meat/Veg_70/15/15, WAS/Meat/Veg_70/10/20, WAS/Meat/Rice_70/20/10, WAS/Meat/Rice_70/15/15, WAS/Meat/Rice_70/10/20, and WAS mono-fermentation.

6.2.3. Analytical procedures and data analysis

TS, VS, sCOD, TAN were performed according to the Standard Method (APHA, 2017) as detailed in Section 3.3. pH was measured with a micro pH probe (PHEL-GB3-001) connected to a multi-meter. Individual VFAs concentration were determined using a gas chromatograph (GC 2010 plus, Shimadzu) equipped with a capillary column (see details on Section 3.3.7).

Principal component analysis (PCA) was used to elucidate the relationships between co-substrate composition and co-fermentation performance in reduced ordination space. The response variables were z-score standardised before PCA analysis to compare variables with different magnitudes. PCA analysis was carried out using the function prcomp() in RStudio (version 4.0.3).

6.3. Results and discussion

6.3.1. Co-fermentation of WAS with each FW component

Figure 6.1 shows the fermentation yield, pH and TAN concentration over the time of the co-fermentation mixtures and WAS mono-fermentation. All co-fermentation mixtures reached higher yields than WAS mono-fermentation (306 mgCOD/gVS). The highest fermentation yields were reached by protein-rich components, 502 and 442 mgCOD/gVS for WAS/Fish and WAS/Meat, respectively. WAS/Fish and WAS/Meat mixtures also displayed a slightly higher pH than the other co-fermentation experiments (values ~5.5), which can be attributed to the additional buffer capacity provided by TAN content from protein degradation. The TAN concentration at the end of the experiment was 2101 and 1524 mgN/L for WAS/Fish and WAS/Meat, respectively (Alibardi & Cossu, 2016; Dahiya et al., 2015; Strazzera et al., 2021).

The starch-rich mixtures, WAS/Pasta and WAS/Rice, showed a similar behaviour with a maximum VFA yield of 394 and 419 mgCOD/gVS, respectively. The pH of these mixtures was lower than that observed for protein-rich components, with values around 5.0. The WAS/Veg and WAS/Fruit mixtures yielded 432 and 350 mgCOD/gVS, respectively. The VFAs production of WAS/Fruit during the first 6 days was very low, which could be attributed to the inhibition of fermentative bacteria by low pH (Chen et al., 2007). The VFAs production from WAS/Fruit increased from the 6th day once the pH of the fermentation liquor was above 4.5. The mixture WAS/FW reached a VFA yield of 390 mgCOD/gVS, value that falls within the range obtained by the FW separate components (350 - 502 mgCOD/gVS). The co-fermentation of WAS/Cel, carbohydrate model substrate, reached a VFA yield of 350 mgCOD/gVS, probably due to its more complex composition (Strazzera et al., 2018; Yin et al., 2014).

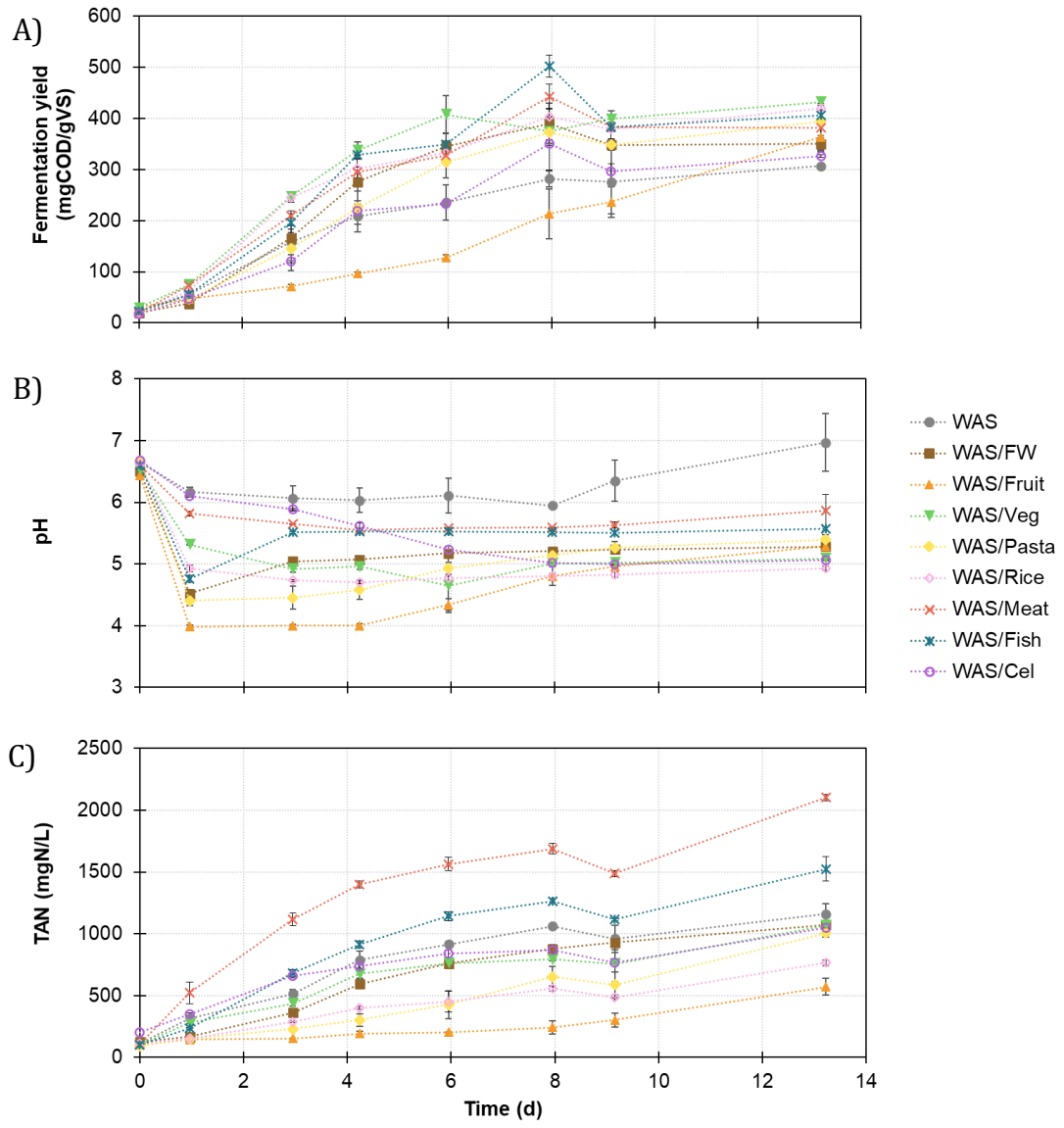

Figure 6.1. Evolution of the fermentation yield (A), pH (B) and TAN concentration (C) in Experiment 1. Error bars indicate the standard deviation.

Figure 6.2 shows the distinct clustering of each component depending on the VFA distribution of the fermentation liquor (in COD percentage). The results of the last four days of each bottle were selected to conduct the PCA, except for the WAS/Fruit and WAS/Cel mixtures, where the last three days were selected (see Figure 6.3 where VFA individual evolution for each test is represented). The PCA has shown that each mixture had a distinct cluster, indicating that each component yielded statistically different results. This fact implies that each co-substrate is a potential driver to VFA distribution on the WAS/FW co-fermentation. Based on their ordination space, the components

could be distributed in four groups: (i) WAS and WAS/Meat were driven by HAc, i-HBu, i-HVa and i-HCa, (ii) WAS/Rice, WAS/Pasta and WAS/Veg were driven by n-HBu and n-HVa (this group was located at the opposite side of the ordination space of the first group), (iii) WAS/Cel and (iv) WAS/Fish that is equally influenced by all variables located at the centre of the PCA. The co-fermentation of WAS/FW is located between WAS/Rice and WAS/Pasta, which means that this VFA profile resembles more the starch-rich substrates. The cluster formed by WAS/Fruit has not been included in any group because it had a high variability between samples considered as stationaries (days 8, 9 and 13) that have been considered stationaries, which can be related to its pH variations.

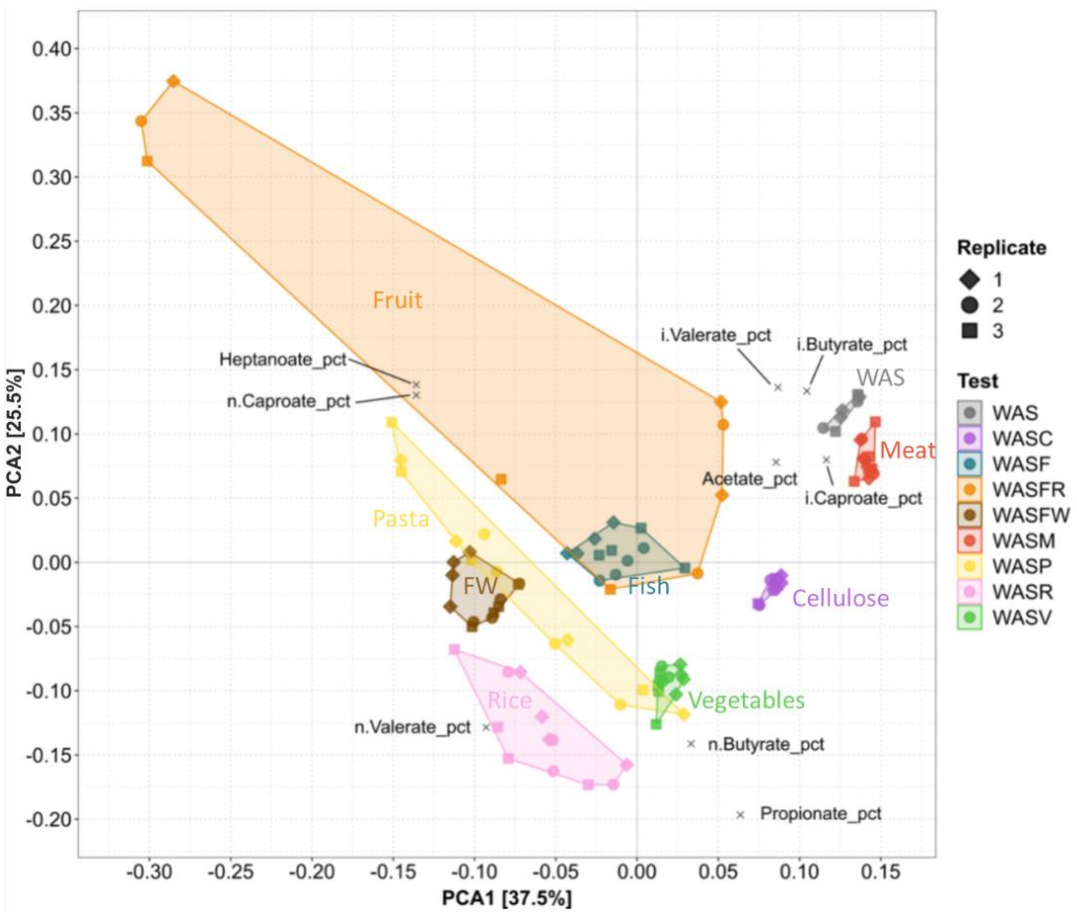

Figure 6.2. PCA plot summarises the results for all the fermentation conditions tested in pseudo-stationary stage in Experiment 1. All samples are for last four days except for the WAS/Fruit and WAS/Cel that are samples for last three days.

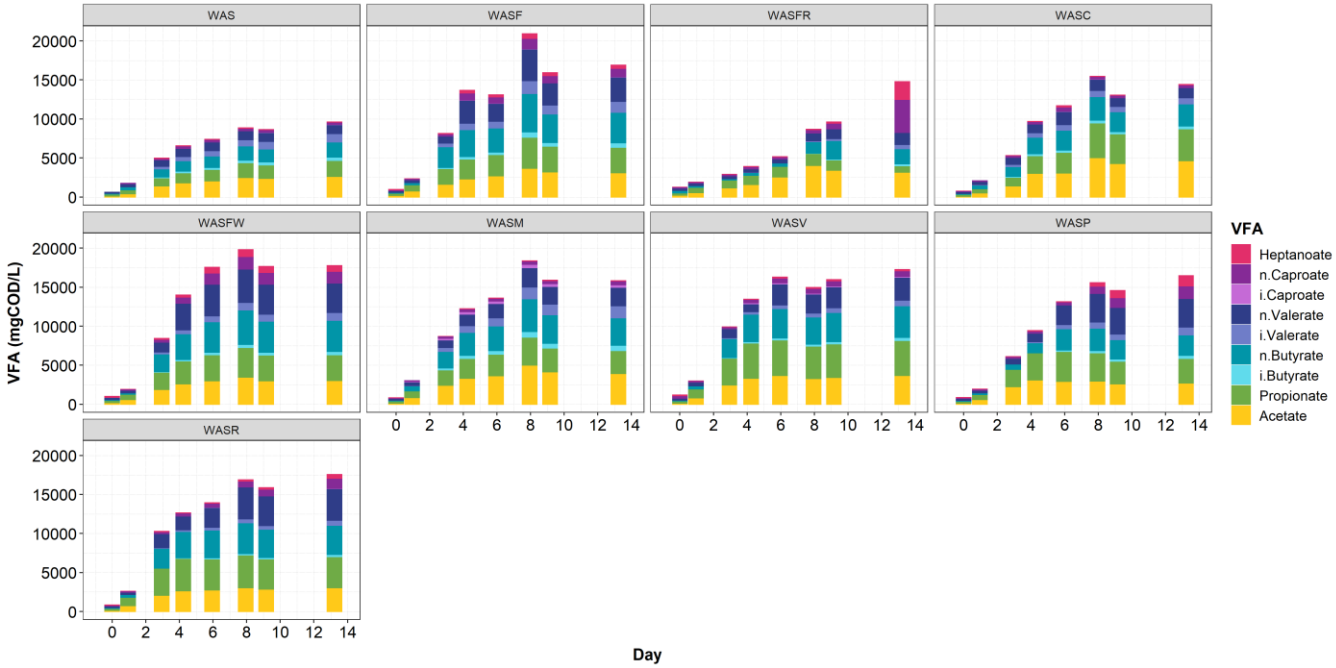

Figure 6.3. Evolution of the individual VFAs in Experiment 1. Error bars indicate the standard deviation.

Figure 6.4. shows the fermentation profile at the 8th fermentation day. The WAS mono-fermentation profile was characterised by acetic (29%), propionic (20%), n-butyric (19%) and n-valeric (11%) acids which is consistent with the reported results of Appels et al. (2011), Morgan-Sagastume et al. (2011) and Peces et al. (2020). WAS/Meat co-fermentation obtained a similar profile dominated by acetic (27%), n-butyric (22%), propionic (19%) and n-valeric acid (13%). These results are also consistent with previous publications where protein fermentation was correlated positively with the enrichment of butyric acid (Alibardi & Cossu, 2016). Even so, it is studied how different proteins affect in different way on the VFAs production and profile (Bevilacqua et al., 2020). Valeric acid accumulation is associated with protein fermentation as a result of the Stickland reaction between amino acids (Parawira et al., 2004; Yin et al., 2016). the accumulation of valeric acid has also been related to pH values between 4.0-5.5 (Feng et al., 2020; Wang et al., 2014). WAS/Fish co-fermentation was located at the center of the PCA, meaning that it is equally influenced by all variables considered, but obtained a profile dominated by n-butyric (23%), n-valeric (19%), propionic (19%) and acetic acid (18%). These results were similar to those obtained with WAS/Meat co-fermentation with n-butyric and n-valeric

acid as the main components. The main difference between both protein co-fermentation can be related to the different amino acids that compose these two substrates (Shen et al., 2017b; Strazzera et al., 2018).

WAS/Rice and WAS/Pasta were dominated by propionic (22-24%), n-valeric (22-23%) and n-butyric (18-23%) and WAS/Veg by propionic (27%), acetic (22%) and n-butyric (22%) acid. In previous publications, the fermentation of the carbohydrates led to butyric acid as the main VFA followed by propionic and acetic acid (Alibardi & Cossu, 2016; Strazzera et al., 2018; Yin et al., 2016). However, Albuquerque et al. (2007), who fermented sugar cane molasses in a continuous stirred tank at 30 ºC, reported that acetic and propionic acid concentrations decreased when the pH decreased from pH 7 to 5 while butyrate and valerate increased.

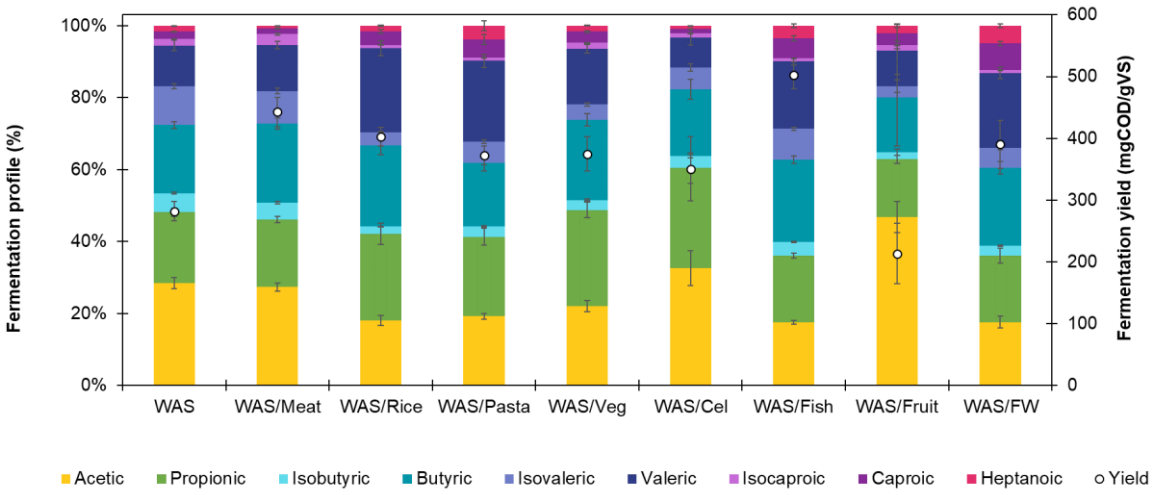

Figure 6.4. Fermentation profile in COD basis at the maximum fermentation yield (day 8) in Experiment 1. Errors bars indicate the standard deviation.

WAS/Cel co-fermentation was characterised by the accumulation of acetic acid content (33%) followed by propionic (28%) and n-butyric (19%) acids. This profile was similar to the reported by Garcia-Aguirre et al. (2017) and Bengtsson et al. (2008a) who obtained acetic, propionic and butyric when fermenting paper mill wastewater under mesophilic conditions at pH 5.5 and 6.0, respectively.

6.3.2. Co-fermentation of WAS and fish with fruit or cellulose

Figure 6.5 shows the evolution of the VFA yield, pH and TAN concentration of each condition tested. The WAS mono-fermentation was quite different from Experiment 1 with a lower yield (97 vs 307 mgCOD/gVS for Experiment 2 and Experiment 1, respectively). Moreover, the pH range during WAS monofermentation was a little higher (6.9-7.3) when compared to experiment 1 (5.9-7.0). Finally, the TAN concentration was also different with a maximum value of 708 mgN/L while in Experiment 1 was 1161mgN/L. The notable differences between the collected WAS used in Experiment 1 and Experiment 2 affect the co-fermentation mixtures yield comparison. For example, the maximum VFA yield obtained for WAS/Cel (350 and 275 mgCOD/gVS for Experiment 1 and 2, respectively) and WAS/Fish (502 and 363 mgCOD/gVS for Experiment 1 and 2, respectively) was lower in this set of experiments. However, in Experiment 2, the mixture WAS/Fruit_70/30 achieved a higher VFA yield (401 mgCOD/gVS) than in Experiment 1, which can be attributed to higher pH obtained throughout the experiment (5.0-5.5).

This second experiment comprised the simultaneous fermentation of three substrates in different proportions. The maximum yield was obtained by the mixture WAS/Fish/Fruit_70/10/20 (508 mgCOD/gVS) followed by WAS/Fish/Fruit_70/20/10 (464 mgCOD/gVS) and WAS/Fish/Fruit_70/15/15 (433 mgCOD/gVS) representing a maximum improvement of up to 39% compared to WAS/Fish and WAS/Fruit co-fermentation. Regarding the WAS/Fish/Cel mixtures, the maximum yield was very similar for the three mixtures, i.e., 447, 448 and 443 mgCOD/gVS for WAS/Fish/Cel_70/10/20, WAS/Fish/Cel_70/20/10 and WAS/Fish/Cel_70/15/15, respectively. The fermentation experiments with WAS, a carbohydrate-rich substrate and a protein-rich substrate, showed a 62% higher yield than the co-fermentation of WAS with one co-substrate rich in carbohydrates or rich in proteins. Consequently, these results indicate the importance of balancing the composition of the protein-to-carbohydrate ratio. Indeed, some authors have already reported that carbohydrate-rich substrates enhance the conversion of proteins increasing the VFAs produced in the mixture (Chen et al., 2013; Feng et al., 2009). Regarding pH, all

mixtures had a range between 5.0-5.5, except the WAS/Fish which had a higher value between 6.0-7.0, most likely due to the lower VFA yield recorded.

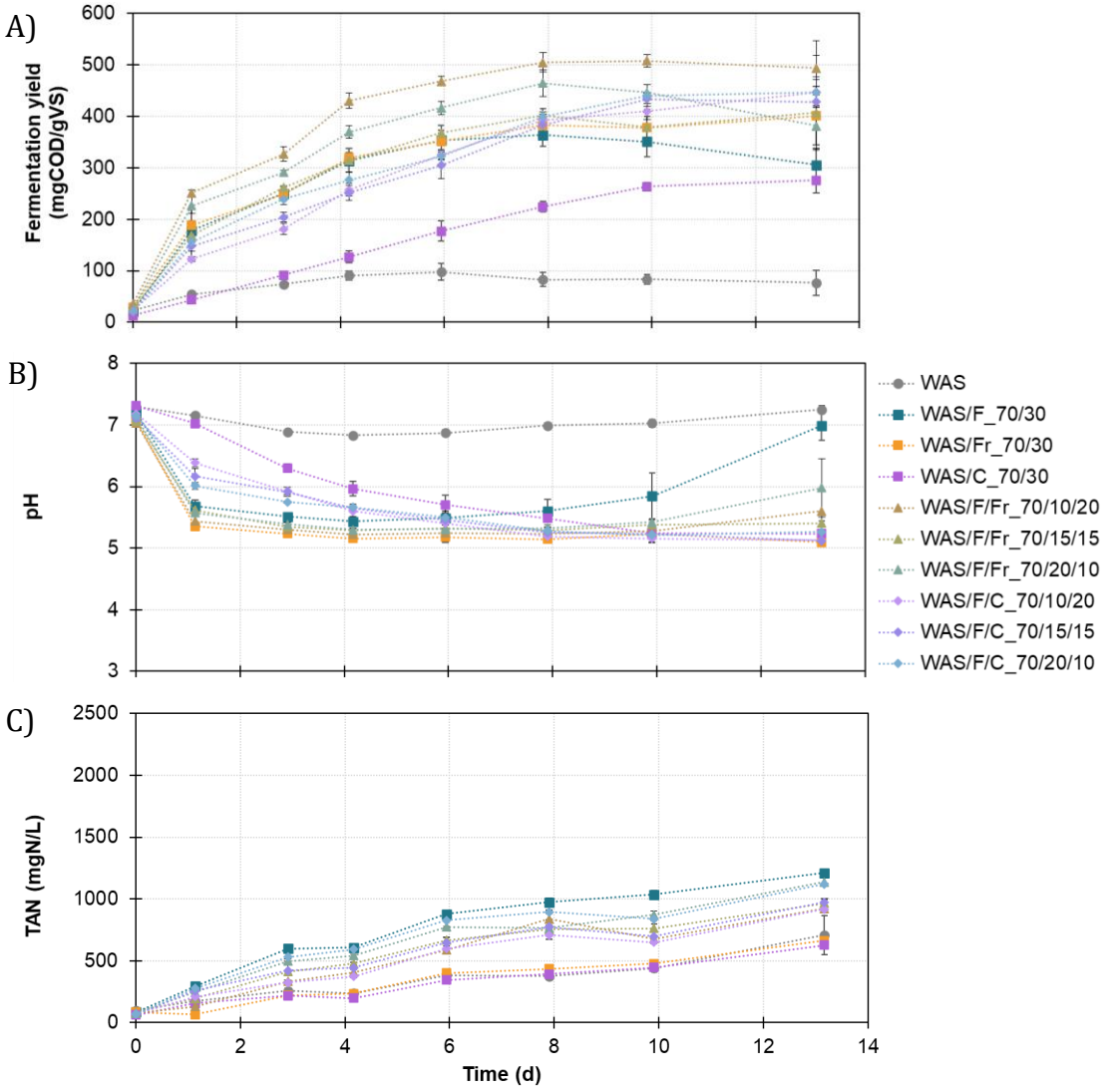

Figure 6.5. Evolution of the fermentation yield (A), pH (B) and TAN concentration (C) in Experiment 2. Error bars indicate the standard deviation.

The PCA analysis was performed using three samples of each condition in the pseudo-stationary stage (i.e. 8th,10th and 13th days) in samples without VFAs consumption, and (ii) 6th, 8th and 10th days (WAS/Cel and WAS/Fish/Cel). Figure 6.6 clearly shows how each co-fermentation mixture (WAS/Fish, WAS/Fruit and WAS/Cel) forms differentiated cluster revealing the influence of co-substrate on the VFAs distribution generated during the fermentation. The mixtures with two co-substrates are located between their co-fermentation mixtures in an orderly way. On the one hand, the

mixture WAS/Cel is enriched in HAc and HPr, unlike WAS/Fruit which is on the opposite side, which is dominated by n-HBu and n-HVa. As in Experiment 1, the mixture WAS/Fish is in the centre of the ordination space indicating that is equally influenced by all variables. It is worth noting that mixtures with fish and cellulose (WAS/Fish/Cel) are located between WAS/Cel and WAS/Fish considering the percentage of each substrate. That is WAS/Fish/Cel_70/20/10 is closer to WAS/Fish, WAS/Fish/Cel_70/10/20 is closer to WAS/Cel and, finally, WAS/Fish/Cel_70/15/15 is between two others). The same pattern is observed by mixtures with fish and fruit which are situated between WAS/Fish and WAS/Fruit in an orderly way. Finally, the mono-fermentation of WAS is located on the other side indicating that the response of the co-fermentation mixtures is not notably influenced by the WAS.

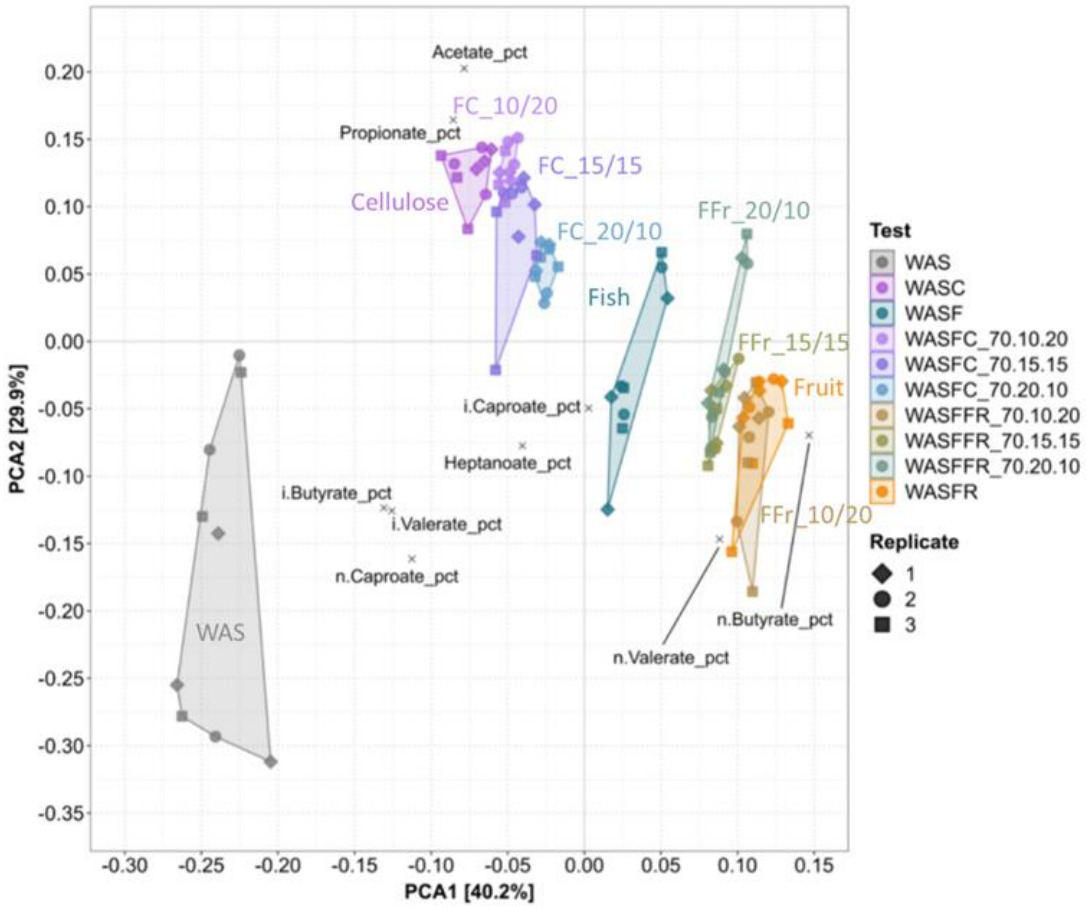

Figure 6.6. PCA plot summarises the results for all the fermentation conditions tested in pseudo-stationary stage in Experiment 2.

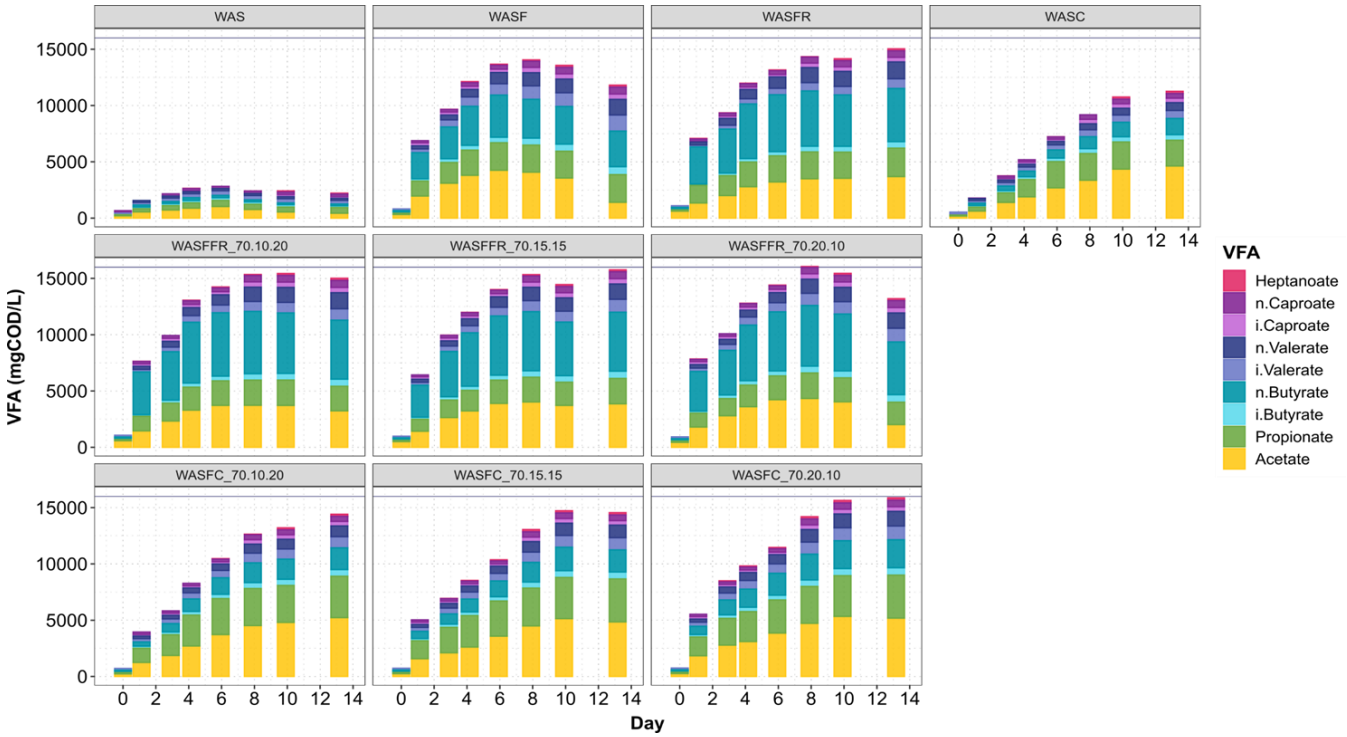

Figure 6.7. Evolution of the individual VFAs in Experiment 2. Error bars indicate the standard deviation.

The fermentation profile at the maximum fermentation yield (day 8) of co-fermentation of Experiment 2 is illustrated in Figure 6.8. The co-fermentation of WAS/Cel was dominated by acetic (37%) and propionic acid (32%), while WAS/Fruit was dominated by n-butyric acid (38%). On the other hand, the WAS/Fish co-fermentation (protein source) was dominated equally by acetic (31%) and n-butyric acid (27%). The mixtures of WAS/Fish/Cel were dominated by acetic (34-36%) and propionic acid (26-31%) with higher percentages. The concentration of acetic and propionic acid increased as the proportion of cellulose in the mixture increased. A similar response was observed in mixtures with WAS/Fish/Fruit which were dominated by n-butyric acid (37-40%); the maximum concentration was obtained in the mixture WAS/Fish/Fruit_70/10/20.

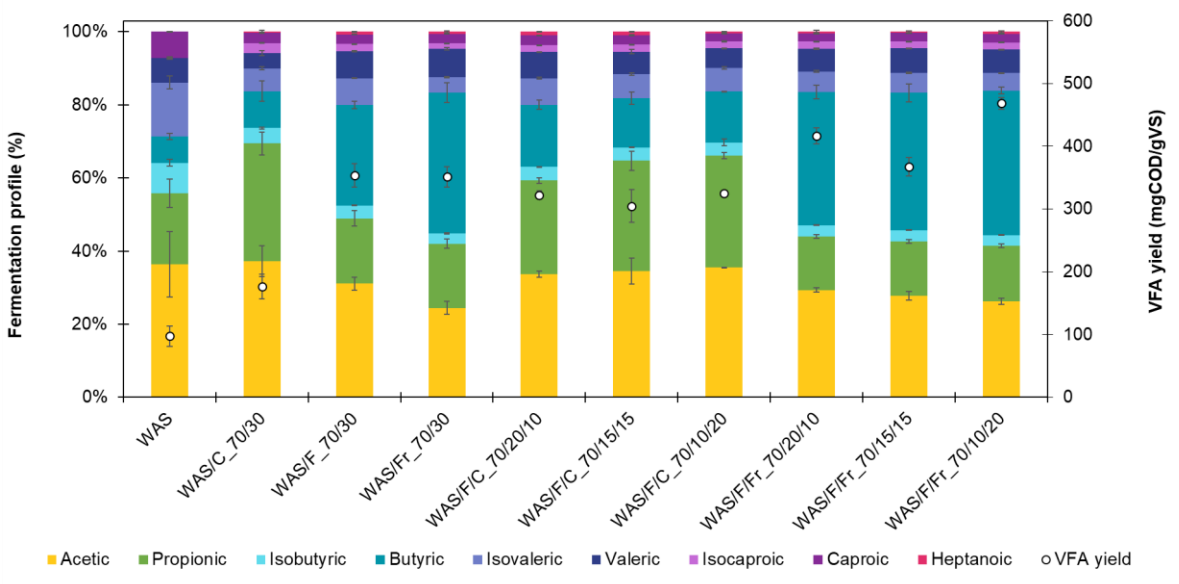

Figure 6.8. Fermentation profile in COD basis at the maximum fermentation yield (day 8) in Experiment 2. Errors bars indicate the standard deviation.

Experiment 2 results show that co-fermentation of mixtures of protein and carbohydrates with WAS achieve higher VFA values than only co-ferment protein or carbohydrate. Based on the differences observed from fruit or cellulose as carbohydrate source, these results show that not only the composition is important but also the type of substrate used. Co-fermentation of WAS with cellulose promotes the production of acetic and propionic acid while the co-fermentation of WAS with fruit promotes butyric and valeric acid.

6.3.3. Co-fermentation of WAS and meat with vegetables or rice

Figure 6.9 shows the VFA yield, pH and TAN concentration for each mixture as in previous experiments. WAS mono-fermentation was similar to the one in Experiment 2 with a maximum VFA yield of 162 mgCOD/gVS with a pH between 7.0-7.5 and final TAN concentration of 1249 mgN/L. The maximum fermentation yield of carbohydrate-rich substrates was quite similar to Experiment 1 with a maximum VFA yield of 430 mgCOD/gVS for WAS/Veg and 452 mgCOD/gVS for WAS/Rice with pH around 5.5-5.7. Nevertheless, the protein-rich mixture of WAS/Meat obtained a lower VFA yield (373 mgCOD/gVS) compared to Experiment 1.

The mixtures of WAS/Rice/Meat obtained similar values of maximum VFA yield, with values of 459, 414 and 476 mgCOD/gVS for WAS/Rice/Meat_70/20/10, WAS/Rice/Meat_70/15/15 and WAS/Rice/Meat_70/10/20, respectively. These results were quite similar than WAS/Rice yield (452 mgCOD/gVS). The mixtures composed by WAS/Veg/Meat obtained higher values than WAS/Veg or WAS/Meat with a maximum yield of 461, 503 and 519 mgCOD/gVS for WAS/Veg/Meat_70/20/10, WAS/Veg/Meat_70/15/15 and WAS/Veg/Meat_70/10/20, respectively. These results show an improvement of up to 39% compared with WAS/Meat co-fermentation and 21% compared with WAS/Veg co-fermentation. In both mixtures, the maximum yield was obtained when protein composition was higher than carbohydrate composition (protein/carbohydrate 20/10, on VS basis). This fact reinforces the importance of balance carbohydrate-to-protein ratio to improve the conversion to VFAs.

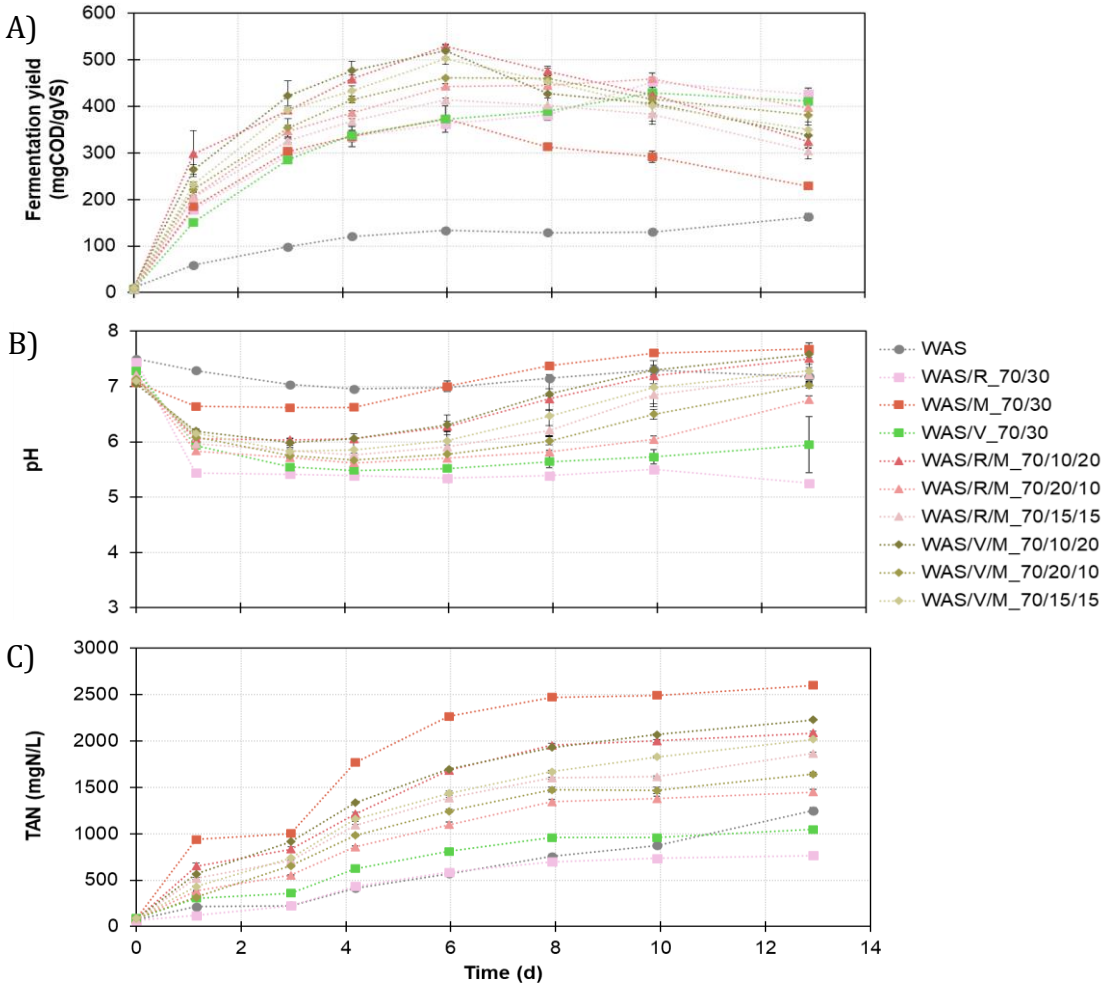

Figure 6.9. Evolution of the fermentation yield (A), pH (B) and TAN concentration (C) in Experiment 3. Error bars indicate the standard deviation.

PCA plot was performed using pseudo-stationary stage which corresponds to 4th, 6th, 8th and 10th days (see Figure 6.10 and 6.11). As in Experiment 2, WAS control mono-fermentation had a differentiated cluster. This fact reinforces the idea that WAS does not have a direct influence on the composition of mixtures combining two different co-substrates. The WAS/Meat is enriched by HPr, i-HBu, and i-HVA, while WAS/Rice and WAS/Veg were enriched by n-HBu, n-HCa and HHep. Moreover, the carbohydrate co-fermentation of rice and vegetables with WAS obtained similar results with very little statistical difference. A similar behavior was obtained in Experiment 1 (see Figure. 6.3) with the same co-substrate response: the mixtures of WAS/Meat/Rice and WAS/Meat/Veg are located between WAS/Meat and WAS/Rice or WAS/Veg in an orderly way.

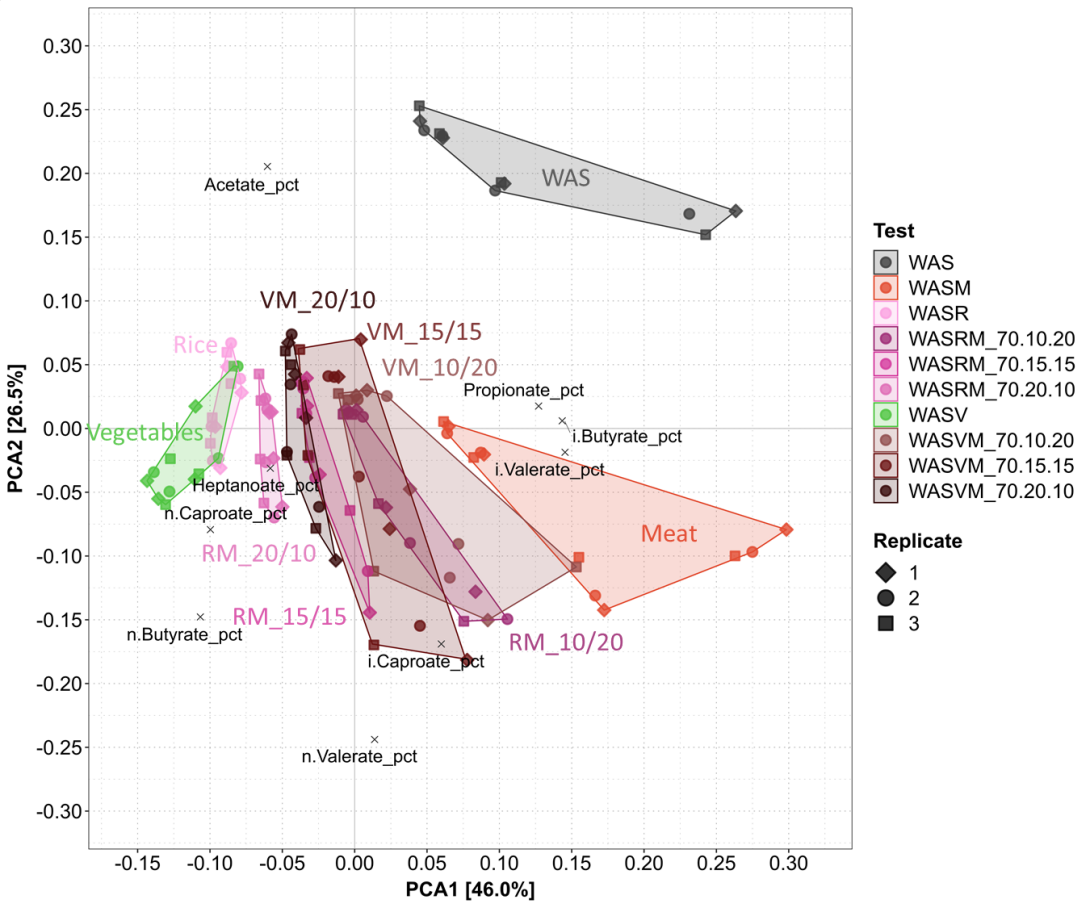

Figure 6.10. PCA plot summarises the results for all the fermentation conditions tested in pseudo-stationary stage in Experiment 3. Samples used were for days 4, 6, 8 and 10.

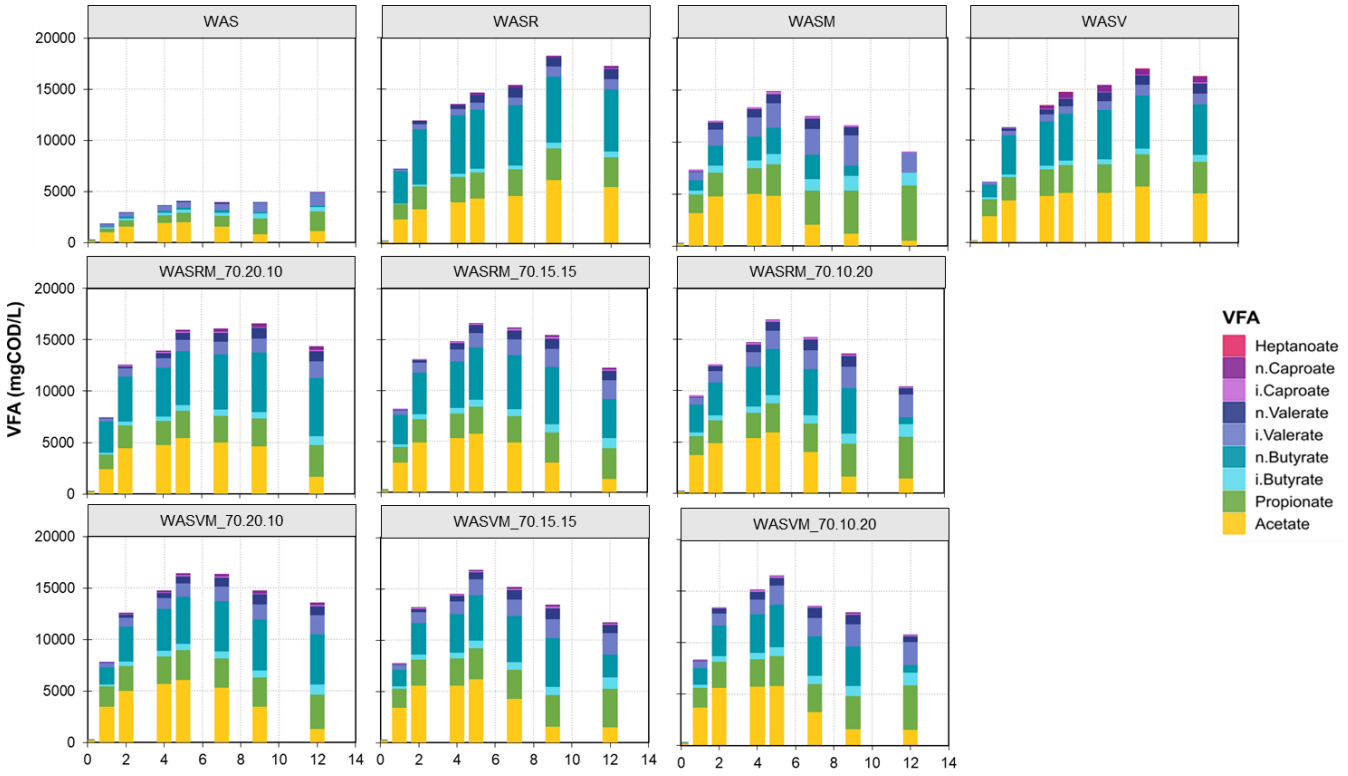

Figure 6.11. Evolution of the individual VFAs in Experiment 3. Error bars indicate the standard deviation.

Finally, Figure 6.12 about the fermentation profile in the maximum production day (6th) seems to not show significant differences between de co-fermentation mixtures. The WAS mono-fermentation was primarily composed of HAc (50%) and HPr (23%). The co-fermentation mixtures were dominated by HAc and n-HBu with a major proportion of n-HBu (39%) in WAS/Rice co-fermentation and i-HVa (16%) in WAS/Meat. Moreover, the mixtures WAS/Meat/Rice and WAS/Meat/Veg were very similar in this maximum production day with a HAc (34-37%) and HPr (30-35%) as main acids.

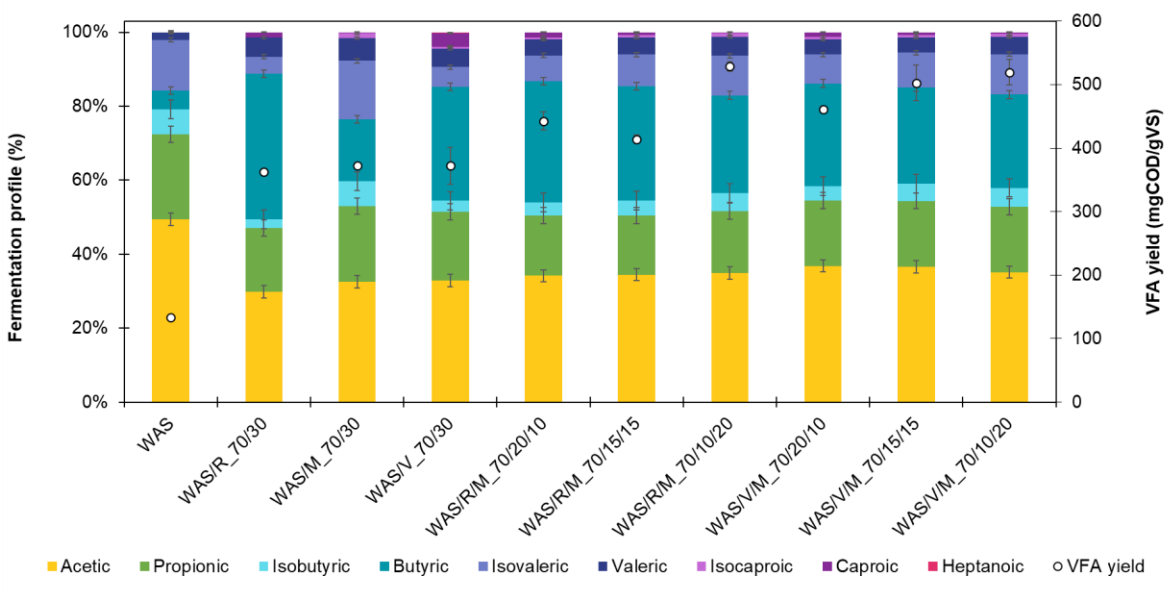

Figure 6.12. Fermentation profile in COD basis at the maximum fermentation yield (day 6) in Experiment 3. Errors bars indicate the standard deviation.

6.4 Conclusions

WAS and FW co-fermentation (70% WAS + 30% FW on VS basis) to produce VFAs was investigated through mesophilic batch test. The results obtained confirmed that each component had a distinct effect on the VFA yield and profile. The maximum VFA yield was obtained on WAS and protein co-fermentation with 502 and 442 mgCOD/gVS for fish and meat co-fermentation, respectively. Regarding the VFA profile, four groups were differentiated: (i) WAS and WAS/Meat enriched with HAc, i-HBu, i-HVa and i-HCa, (ii) WAS/Rice, WAS/Pasta and WAS/Veg enriched in HBu and n-HVa, (iii) WAS/Cel enriched with HAc, HPr and HBu, and (iv) WAS/Fish with VFAs equally distributed. Furthermore, the first set of experiments showed that the FW distribution was highly influenced by carbohydrates (rice and pasta) and less for the proteins (meat and fish). Hence, the experiments mixing the protein-rich and with carbohydrate-rich substrates demonstrated the importance to balance protein-to-carbohydrate ratio obtaining an improvement of up to 39% in the VFAs yields than when WAS was co-fermented with protein-rich or carbohydrate-rich substrates alone. These experiments demonstrated that the fermentation yield is improved when the components are balanced in the mixture. Although each WAS had different initial characteristics that directly affected

the mono-fermentation and mixtures yields, the PCA analysis has demonstrated that the use of different WAS did not have a direct influence on the fermentation profile of the mixtures.

7. Effect of the organic loading rate on the acidogenic co-fermentation of waste activated sludge and food waste

ABSTRACT

Most studies of co-fermentation were carried out in batch mode although it has limitations to differ from continuous fermenters without reflecting the influence of operational parameters such as the OLR or the HRT. This study was performed to test the feasibility of WAS and FW co-fermentation in a continuous reactor at different OLR. This process was carried out in a 5L lab-scale reactor at 35 ºC operated for 160 days. The OLR was changed when steady-stable conditions were achieved during 3 HRT equivalents under each operating period. Four OLR (9, 11, 14 and 18 gVS/(L·d)) were tested by increasing the FW influent flowrate and maintaining the WAS influent flowrate, which led to a variation of the VS proportion of the FW/WAS mixture (80/20, 35/65, 50/50 and 60/40 %). WAS used came from two different origins in each OLR (WAS_A and WAS_B). As a general trend, as OLR increased, the VFA yield increased but only for the collected WAS with higher buffer capacity. In the first stages (OLR 9 and 11 gVS/(L·d)), the VFA yield was very low, which was attributed to anaerobic biomass immigration of WAS as well as the circumneutral pH obtained that favoured methanogenic archaea activity. In these periods, propionic acid was the main VFA obtained and acetic acid represented less than the 10% (COD basis) of the produced VFAs. When the OLR was increased to 14 gVS/(L·d), the VFA yield started to increase obtaining values between 100-300 mgCOD/gVS with a concomitant pH drop until 5.7 and a VFA profile enriched in butyric and valeric acids. Finally, at OLR 18 gVS/(L·d), the maximum fermentation yield was obtained (475 mgCOD/gVS), but only for the WAS with higher alkalinity, being acetic and butyric acids the main VFAs produced. Microbial community analyses are ongoing to validate the interpretation of these results.

7.1. Introduction

The substantial population growth and urbanization have elevated the uncontrolled waste generation with the depletion of non-renewable resources requiring a change from end-of-pipe waste treatments to resource recovery schemes (Puyol et al., 2017). Hence, the WWTPs need to be transformed into biorefineries where waste streams are conceived as valuable resources of energy, chemicals, nutrients, and water (Nghiem et al., 2017; Pikaar et al., 2020; Strazzera et al., 2018).

Conventional WWTPs usually include an AD unit to valorise the thickened WAS in the form of biogas and soil amendment for agriculture when possible. Currently, acidogenic fermentation is gaining more attention to transform organic wastes into easily assimilable compounds such as VFAs, alcohols and other carboxylic acids (e.g., lactic acid or succinic acid) (Agler et al., 2011; Dahiya et al., 2018), that are platform products with higher market values than biogas and a wider range of applications (Abreu et al., 2019; Bahreini et al., 2021; Ramos-Suarez et al., 2021; Valentino et al., 2018). Even so, the WAS acidogenic fermentation is still limited by its low biodegradability due to its proteinic nature that limits the hydrolysis step despite its high organic content (Gonzalez et al., 2018; Nghiem et al., 2017; Xu et al., 2020b). To improve these limitations, co-fermentation stands as a new approach by (i) increasing the OLR (ii) balancing nutrients (e.g., C/N ratio), (iii) diluting inhibitory and toxic compounds, (iv) improving buffer capacity, (v) promoting synergistic effects, and (vi) treating different wastes simultaneously in the same facility obtaining better yields (Feng et al., 2020; Peces et al., 2020; Wu et al., 2016).

FW is characterised by a high concentration of organic matter, moisture content and good biodegradability (Ren et al., 2018). The anaerobic fermentation of FW achieves better VFA yields than WAS acidogenic fermentation. Specifically, VFA yields for FW fermentation range between 50-400 mgCOD/gVS (Jiang et al., 2013; Li et al., 2018; Lim et al., 2008a) while VFA yields for WAS fermentation range between 10-250 mgCOD/gVS depending on operational parameters (Chen et al., 2007; Guo et al., 2015; Ma et al., 2017; Peces et al., 2020). Even so, the FW fermentation is limited by its high content of lignocellulosic compounds (Strazzera et al., 2018) and by its low pH that led to acidogenic fermentative bacteria inhibition. Nevertheless, FW stands as an

ideal co-substrate with high organic matter content and excellent biodegradability but with a lack of buffer capacity that will be provided by WAS which allows maintaining pH above inhibitory levels (pH > 5.0) (Fang et al., 2019). Several studies have been carried out to test WAS and FW co-fermentation obtaining very promising results, but most of them have been conducted in batch mode (Chen et al., 2013; Feng et al., 2011; Li et al., 2014, 2021; Ma et al., 2017; Moretto et al., 2019; Vidal-Antich et al., 2021; Wu et al., 2016; Zhao et al., 2016). However, the batch mode has limitations that differ from continuous fermenters because it cannot reflect the long-term influence of the operational parameters such as HRT or OLR. Moreover, these parameters affect directly or indirectly the process parameters such as pH, alkalinity, or concentration of inhibitory compounds that could affect the product profile and microbial community (Perez-Esteban et al., 2022). Even so, the microbial ecology of mixed culture fermentation is still not fully understood and could be used as a strategical tool to drive VFAs production (Jankowska et al., 2017; Llamas et al., 2022).

The results of the batch test are highly influenced by the starting microbial community without allowing the evaluation of the acclimation to different operational conditions and substrate composition as it occurs in continuous operation. Hence, the development of a specialized microbial community, the selective pressure and the immigration of the microorganisms could be completely evaluated in batch tests (Perez-Esteban et al., 2022). Even so, in continuous experiments, the initial microbial community is evolving and adapting over time with the operational conditions and increasing the inhibition tolerance, which could lead to higher fermentation yields. Additionally, acetic acid could be consumed using WAS as the main substrate by methanogens presence in the fermentation liquor to produce biogas (Ma et al., 2017; Vidal-Antich et al., 2021). Nonetheless, WAS also introduces denitrifying bacteria, PAOs or or sulphate-reducing bacteria which are VFAs consumers (Nierychlo et al., 2020). Hence, the constant bacteria immigration with WAS could prevent acetic acid accumulation in continuous mode (Wu et al., 2016) increasing the stochasticity of the fermenters' microbial community assembly (Vrieze et al., 2020; Yuan et al., 2019). Furthermore, most studies are limited to using one single WAS or WAS of one origin without considering the WAS role in the fermentation yield, fermentation profile and microbiology community.

Therefore, co-fermentation literature is still inconclusive and further research is required to control WAS and FW co-fermentation in continuous reactors. For instance, the existing literature does not clearly elucidate (i) the long-term effect of OLR and HRT on continuous co-fermentation, (ii) the role of the WAS type on co-fermentation, (iii) the study of the immigration communities, and (iv) the importance of the microorganisms' adaptation in continuous mode on WAS/FW co-fermentation. Hence, the aim of this study is to investigate how WAS and OLR impact on mesophilic co-fermentation performance (yield and profile) at an HRT in the range of 2.9-3.5 days. Samples were collected to also evaluate the underpinning microbiological activity, but the analysis of these samples is still ongoing.

7.2. Materials and methods

7.2.1. Organic substrates

The organic substrates used in this study were thickened WAS and FW. The WAS has been collected from a secondary settler tank of two different WWTPs from the metropolitan area of Barcelona (Spain): (i) WAS_A from conventional A/O process with 4,000 population equivalents, and (ii) WAS_B from WWTP with MBR and 300,000 population equivalents. After collection, WAS was conserved in a refrigerator at 4 ºC until its use. Moreover, WAS was diluted with deionized water to adjust the VS content at 25 gVS/L. Synthetic FW was formulated to assure reproducibility avoiding the high heterogeneous composition of the real FW composition during the entire experimental work. Specifically, the FW contained on a wet weight basis: vegetables (30%), fruits (30%), carbohydrates (20%), meat (10%) and fish and seafood (10%) based on previous studies (Vidal-Antich et al., 2021) (for further details, see Chapter 5, Section 5.2.1). The ingredients used were found in the supermarket all year-round to ensure the reproducibility. FW was shredded with a kitchen bender (MMB66G5M, Bosch) for 3-4 min and diluted with deionized water to adjust the VS content between 150-200 gVS/L (Jiang et al., 2013; Zhang et al., 2016). After preparation, FW was conserved in a refrigerator at 4 ºC until being used (maximum 3 days). All substrates were characterised in terms of TS and VS, pH, alkalinity, sCOD, TAN and VFAs and

alcohols content. The chemical-physical characterisation of the organic substrates is summarised in Table 7.1.

No inoculum was added in the acidogenic fermenters because the fermentation process relied on endogenous microorganisms present in WAS.

7.2.2. Reactors' set-up

Two identical jacketed glass reactors with a working volume of 4L were operated in semi-continuous mode for WAS/FW co-fermentation (reactor A) and WAS mono-fermentation (reactor B, as control) at mesophilic conditions (35 ± 1 ºC). Fermenters were equipped with thermostatic bath and mechanical stirrer at 100 rpm as has been detailed in Section 3.1.2. The WAS fermenter (reactor B) was run for 160 days at HRT of 3.5 days and OLR at 7.14 gVS/(L·d) as control. The other reactor (reactor A) was started as WAS fermenter for 28 days until achieving steady-state conditions during 3 HRT equivalent. After the steady operation (day 28), the reactor was fed with WAS and FW mixture to study the effect of OLR increasing on co-fermentation. Specifically, the co-fermentation experiment was divided into four periods where the OLR was increased from 9.0 to 18.0 gVS/(L·d), with a corresponding decrease of HRT (from 3.38 to 2.89 days), while increasing the FW influent flowrate and maintaining the WAS influent flowrate as is shown in Table 7.2. Each period was characterised by an increment of OLR with a decreasing of HRT using two different WAS (WAS_A and WAS_B). Moreover, each period was operated for a minimum of 8 HRT equivalent cycles. Furthermore, the pH was not adjusted either at the beginning or during the experiment. The effluent was collected before feeding to analyse TS and VS content, pH, alkalinity, TAN, VFAs and sCOD (three times per week). Moreover, microbiological samples were taken into sterilized Eppendorf tubes and frozen at -20 ºC for their analysis (results not available yet).

Table 7.1 Substrates characterisation for each collection period. FW results are referred to average of all FW preparations. Results are expressed as average ± 95% confidence interval (n = 3).

Sample	**Type**	**TS**	**VS**	**VS/TS**	**pH**	**Alkt**	**sCOD**	**TAN**	**VFAs**
Unit		(gTS/L)	(gVS/L)	(%)	-	($gCaCO_3$/L)	(gCOD/L)	(mgN/L)	(gCOD/L)
WAS	A	3.04 ±0.01	2.28 ±0.01	75.04±0.80	7.42 ± 0.14	2.7 ±0.2	0.7 ±0.1	86.6±6.1	0.1 ±0.1
	B	3.29 ±0.01	2.44 ±0.01	74.12±0.74	7.42 ± 0.02	2.8 ±0.6	1.3 ±0.3	93.2±2.0	0.2 ±0.1
	A	3.32 ±0.01	2.58 ±0.01	79.66±0.28	7.19± 0.49	2.0 ±0.1	0.6 ±0.1	152.1±16.1	0.1 ±0.1
	B	3.46 ±0.06	2.44 ±0.05	70.51±0.25	7.72± 0.09	2.9 ±1.0	0.3 ±0.1	52.5±16.0	0.1 ±0.1
	A	3.34 ±0.01	2.53 ±0.01	75.67±0.74	7.39± 0.31	2.9 ±0.2	0.8 ±0.1	116.5±30.6	0.1 ±0.1
FW		17.48 ±1.60	16.98 ±1.29	97.14±0.20	5.54 ±0.12	-	77.5 ±7.0	36.3 ±2.1	4.4 ±0.7

Table 7.2. Summary of the operational conditions of the WAS acidogenic fermentation and WAS/FW co-fermentation.

Reactor	**Substrate/s**	**Phase**	**Operational period**	**OLR**	**HRT**	**WAS flowrate**	**FW flowrate**	**FW/WAS**
Units			(days)	(gVS/(L·d))	(days)	(L/d)	(L/d)	(on VS basis, %)
Reactor A	WAS	0	1-159	7.14	3.50	1.14	-	0/100
Reactor B	WAS	0	1-28	7.14	3.50	1.14	-	0/100
	WAS/FW	1	29-65	9.00	3.38	1.14	0.04	20/80
	WAS/FW	2	66-97	11.00	3.26	1.14	0.09	35/65
	WAS/FW	3	98-125	14.00	3.09	1.14	0.15	50/50
	WAS/FW	4	126-159	18.00	2.89	1.14	0.24	60/40

7.2.3. Analytical procedures

TS, VS, sCOD, TAN and alkalinity analysis were performed following the Standards Methods for the examination of Water and Wastewater (APHA, 2017) as detailed in Section 3.3. The pH was measured using a pH probe (Basic 20 pHmeter, CRISON) connected to an automatic titrator (pH Burette 24, CRISON). Finally, VFAs and alcohols were determined using gas chromatography with the specifications explained in Section 3.3.7 and were converted into COD equivalents using stoichiometric conversion factors.

7.3. Results and discussion

7.3.1. Start-up with WAS mono-fermentation

Both reactors started the operation working as replicates on phase 0 at HRT 3.5 days and OLR at 7 gVS/(L·d) with WAS as a single substrate for 28 days (see Figures 7.1, 7.2 and 7.3) achieving a steady performance. The effluent of both reactors was characterised by a TS content of 3.28 ± 0.14 % and VS content of 2.44 ± 0.12 % throughout the start-up with a neutral pH (around 7.0).

Regarding the WAS mono-fermentation, the maximum yield obtained was 55 mgCOD/gVS with a corresponding VFAs concentration of 1400 mgCOD/L. This low production might be related to the protein as the main component of WAS with low carbohydrates levels that are needed to carry out the bioconversion (Zhao et al., 2016). This fact was demonstrated by Feng et al. (2009) who studied the effect of carbohydrate addition at WAS using rice as a carbohydrate model to achieve a C/N ratio around 20/1 that was more suitable for microorganisms and benefit the VFAs production. These authors obtained a VFAs production of 61.4 and 101.4 mgCOD/gVSS at pH 7.0 and 8.0 using WAS and 406.3 and 520.1 mgCOD/gVSS with the rice addition at pH 7.0 and 8.0 demonstrating the beneficial effect of carbohydrate addition to regulate the C/N ratio.

As shown in Figures 7.2 and 7.3, from day 8, , the total VFAs concentration ranged 1000-1400 mgCOD/L and the acetic acid concentration started to decrease from

745 mgCOD/L to 102 mgCOD/L probably consumed by methanogenic archaea which may proliferate at neutral pH. Yuan et al. (2015) studied the long-term effect of different pH (4.0, 10.0 and uncontrolled) on WAS acidogenic fermentation at 30 ºC for over 90 days obtaining a SCFAs accumulation of 1721.4, 114.2 and 58.1 mgCOD/L for pH 10.0, pH 4.0 and uncontrolled pH, respectively. Moreover, the sequencing at last day samples revealed ratios of archaea to bacteria of 1:41, 1:16 and 1:9 for pH 10.0, 4.0 and uncontrolled without acidogenic bacteria detection on uncontrolled pH (6.9 – 7.4) as probably occurs in this study. Nonetheless, WAS also introduces denitrifying heterotrophic bacteria, PAOs or sulphate-reducing bacteria which are VFAs consumers (Nierychlo et al., 2020).

Regarding the fermentation profile, both reactors were mainly composed by acetic and propionic acid, followed by valeric acid (see Figure 7.2 and 7.3). This profile was in accordance with previous studies where WAS was fermented at 35 ºC in batch test as control without any pre-treatment (He et al., 2016; Pang et al., 2020; Wang et al., 1999; Yang et al., 2015). Specifically, these authors tested various methods to improve the hydrolysis of the WAS fermentation with rhamnolipid pre-treatment at alkaline pH (He et al., 2016), with NaCl addition which deteriorates the structural properties of WAS (Pang et al., 2020) or combining freezing/thawing with *Geobacillus* sp. pre-treatment (Yang et al., 2015). In all cases, the pre-treatments are beneficial to improve the WAS hydrolysis and better VFA yields are obtained. This fact also reinforces the importance to improve the limiting step of WAS fermentation to achieve better production.

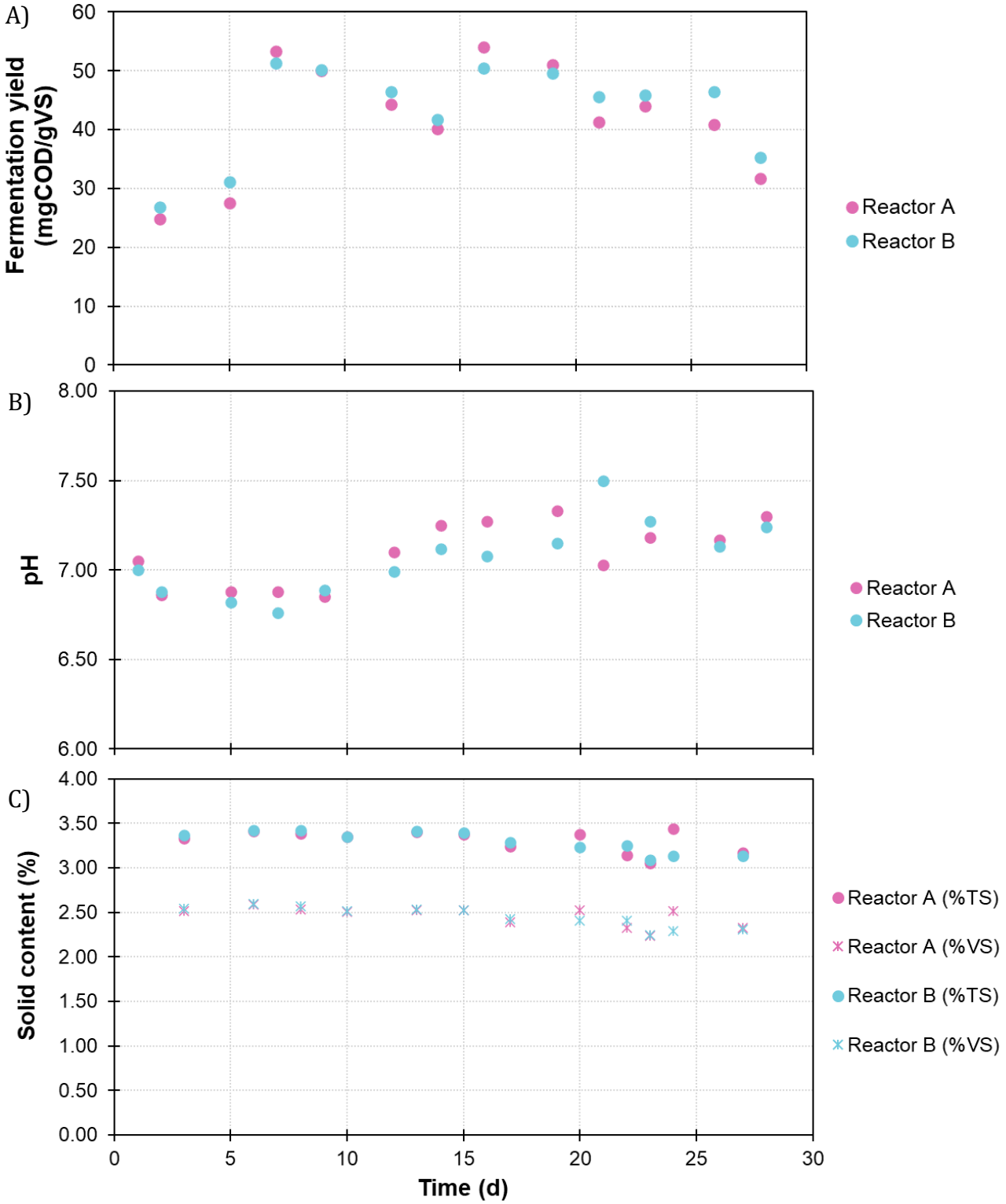

Figure 7.1. Evolution of both mono-fermentation reactors for 28 days in phase 0. (A) Fermentation yield, (B) pH and (C) solids contents.

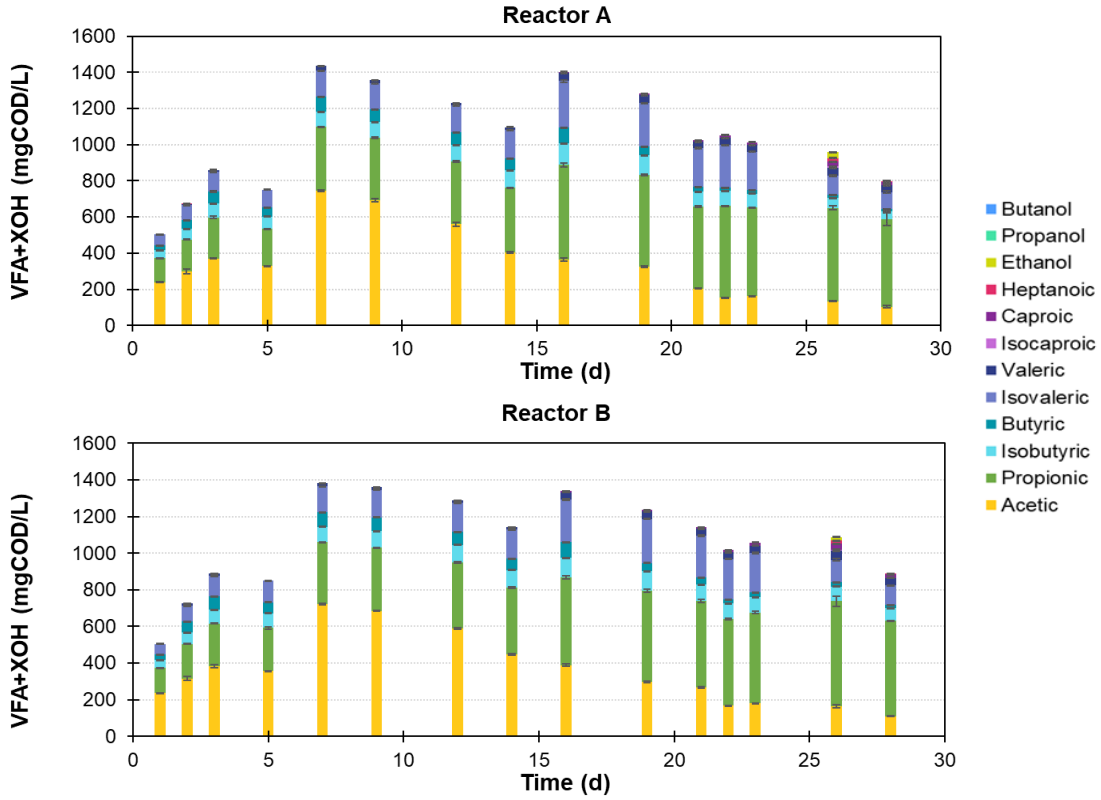

Figure 7.2. Evolution of the concentration of fermentation products (volatile fatty acids and alcohols (XOH)) in reactor A (top) and reactor B (bottom).

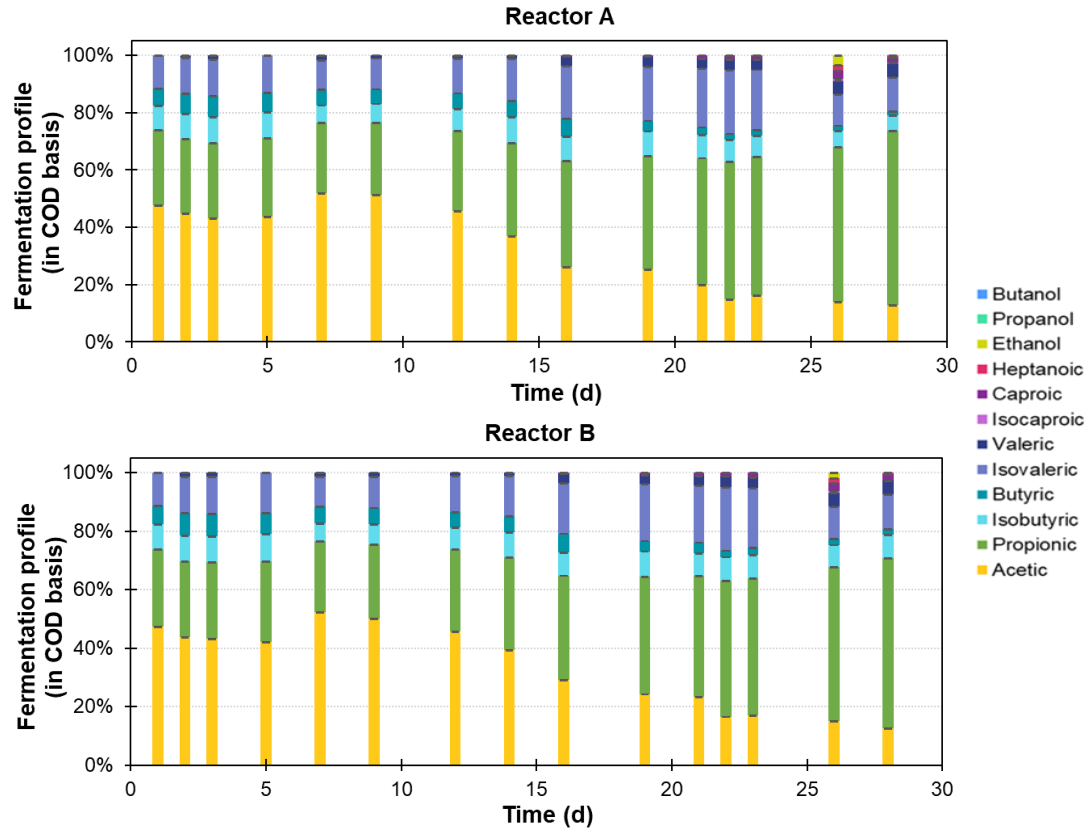

Figure 7.3. Evolution of the fermentation products distribution of the fermentation profile for reactor A (top) and reactor B (bottom).

7.3.2. Co-fermentation at OLR 9-11 gVS/(L·d)

When it was demonstrated that both reactors worked as replicates and after 3 HRT equivalents on steady stage performance, reactor A started to operate as a co-fermenter fed with a mixture of WAS and FW (from the 29th day), and reactor B continued working only with WAS as a mono-fermenter. From then, the operation of the co-fermenter reactor was divided into four stages rising the OLR from 9 to 18 gSV/(L·d) by increasing the FW influent flowrate and maintaining the WAS influent flowrate, with a consequent HRT decrease from 3.38 to 2.89 days (see details in Table 7.2). Each stage was operated for a minimum period of 8 HRT equivalent cycles and achieved a new steady-state within 3-4 HRT due to the collected WAS variation in each phase. Table 7.3 summarises the data of operation conditions and process average performance of the co-fermenter at steady-state conditions for each phase with each WAS collection period (WAS_A and WAS_B).

As a general trend, the OLR rise increased the fermentation products concentration and lowered the pH on the co-fermenter as Fig. 7.4 shows. Even so, the co-fermentation reactor operated with a similar tendency at OLR 9 and 11 gVS/(L·d) with neutral pH (between 6.7-7.1), low fermentation yields (41-103 mgCOD/gVS) and fermentation products concentration (1209-3549 mgCOD/L) although FW concentration was higher in phase 2 (FW/WAS ratio of 20/80 and 35/65 for phase 1 and 2). Consequently, the sCOD was 2.9-3.2 gCOD/L on phase 1 and higher on phase 2 (4.5-6.3 gCOD/L). Moreover, the process parameters such as TS, VS, alkalinity, and TAN were quite similar than in steady-state with a slight increase in TS and VS content due to the increase of FW content in the influent without significant differences between both WAS (WAS_A and WAS_B). Regarding the fermentation profile on COD basis, the predominant component was propionic acid (53-76%), followed by valeric (9-26%) and butyric acids (5-17%) in both phases. Even so, a notable difference in fermentation profile was obtained in WAS_A of phase 2 (OLR 11 gVS/(L·d)). As Figure 7.5 shown, the second WAS used on Period 2 (WAS_A) yielded a higher VFAs production (although the same operational conditions were applied) and the fermentation broth was enriched by valeric (26% vs 17%) and butyric acids (17% vs 6%), with a decrease in the proportion of propionic acid (from 74 to 53%) in the VFAs produced.

Table 7.3. Operational conditions and process performance for each phase with each WAS on co-fermenter effluent at steady state conditions (average ± standard deviation).

		Phase 1		Phase 2		Phase 3		Phase 4	
		WAS_A	**WAS_B**	**WAS_B**	**WAS_A**	**WAS_A**	**WAS_B**	**WAS_B**	**WAS_A**
Operational conditions									
OLR	(gVS/L·d)	9.00	9.00	11.00	11.00	14.00	14.00	18.00	18.00
HRT	(d)	3.38	3.38	3.26	3.26	3.09	3.09	2.89	2.89
FW/WAS	(%, on VS)	20/80	20/80	35/65	35/65	50/50	50/50	60/40	60/40
Process performance									
pH	-	7.0 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	6.6 ± 0.1	6.1 ± 0.1	6.5 ± 0.1	4.5 ± 0.3	4.5 ± 0.1
TS	(%)	2.7 ± 0.2	3.3 ± 0.1	3.0 ± 0.1	3.3 ± 0.1	3.3 ± 0.2	3.7 ± 0.3	4.5 ± 0.1	4.9 ± 0.6
VS	(%)	2.0 ± 0.20	2.4 ± 0.1	2.2 ± 0.1	2.6 ± 0.1	2.7 ± 0.2	2.7 ± 0.2	3.4 ± 0.1	4.1 ± 0.6
VS/TS	(%)	73.7 ± 0.6	73.9 ± 0.2	73.6 ± 0.3	78.3 ± 0.1	79.9 ± 0.8	73.8 ± 0.2	76.7 ± 0.5	82.9 ± 1.4
Alk_T	($gCaCO_3/L$)	3.4 ± 0.3	4.2 ± 0.1	3.7 ± 0.1	2.8 ± 0.1	3.4 ± 0.3	3.4 ± 0.2	1.8 ± 0.2	0.9 ± 0.3
TAN	(mgN/L)	485.2 ± 3.4	409.1 ± 69.2	541.4 ± 2.3	390.4 ± 40.7	651.4 ± 43.5	452.3± 102.3	269.9 ± 26.2	n.d.
sCOD	(gCOD/L)	3.2 ± 0.6	2.9 ± 0.3	4.4 ± 0.2	6.3 ± 0.2	12.9 ± 0.6	9.6 ± 0.6	26.0 ± 1.3	24.3 ± 1.4
Fermentation yield	(mgCOD/gVS)	82.7 ± 2.8	40.9 ± 3.1	69.3 ± 10.2	102.6 ± 27.8	227.7 ± 6.4	172.7 ± 14.6	439.0 ± 51.4	211.3 ± 12.7
VFAs+Alcohols	(gCOD/L)	1.8 ± 0.2	1.2 ± 0.1	2.3 ± 0.2	3.5 ± 0.8	9.5 ± 0.2	7.1 ± 0.8	21.6 ± 1.0	10.0 ± 0.5
Acetic	(%)*	4.8 ± 0.6	9.4 ± 2.5	3.7 ± 0.6	1.9 ± 0.2	2.7 ± 1.4	1.6 ± 0.4	20.3 ± 0.8	55.9 ± 1.4
Propionic	(%)*	77.6 ± 1.4	74.7 ± 2.3	73.5 ± 3.3	53.1 ± 2.9	22.2 ± 1.4	32.7 ± 5.5	7.0 ± 0.3	0.0 ± 0.0
Butyric	(%)*	5.1 ± 0.3	6.1 ± 1.1	5.6 ± 1.1	16.7 ± 1.6	28.9 ± 1.5	23.0 ± 4.0	42.4 ± 1.5	3.1 ± 0.1
Valeric	(%)*	12.5 ± 1.0	8.9 ± 0.8	16.6 ± 1.8	25.9 ± 2.4	39.6 ± 0.8	33.4 ± 1.4	13.2 ± 1.3	1.7 ± 0.0
Caproic	(%)*	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	1.2 ± 0.7	5.1 ± 0.7	5.3 ± 1.5	9.1 ± 2.4	6.0 ± 0.5
Heptanoic	(%)*	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.5 ± 0.1	1.4 ± 1.1	4.0 ± 1.8	2.0 ± 0.3	0.9 ± 0.8
Ethanol	(%)*	0.0 ± 0.0	0.9 ± 1.5	0.0 ± 0.0	0.6 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 1.1	32.5 ± 1.8
Butanol	(%)*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Propanol	(%)*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

* Fermentation products are reported in COD basis.

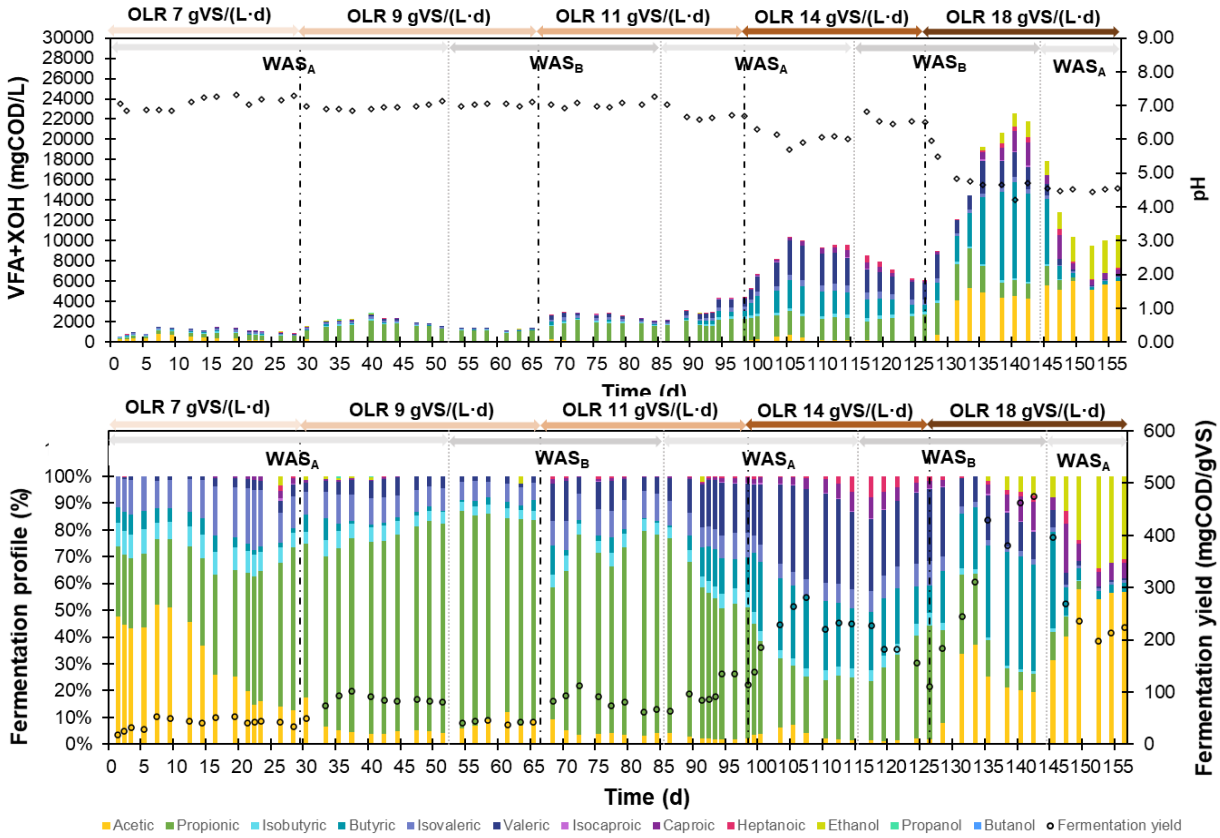

Figure 7.4. Evolution of fermentation products concentration (expressed in COD basis) and the effluent pH of the co-fermentation reactor (top). Fermentation products distribution in the effluent (COD basis) and fermentation yield (mgCOD/gVS) of the co-fermentation rector (bottom).

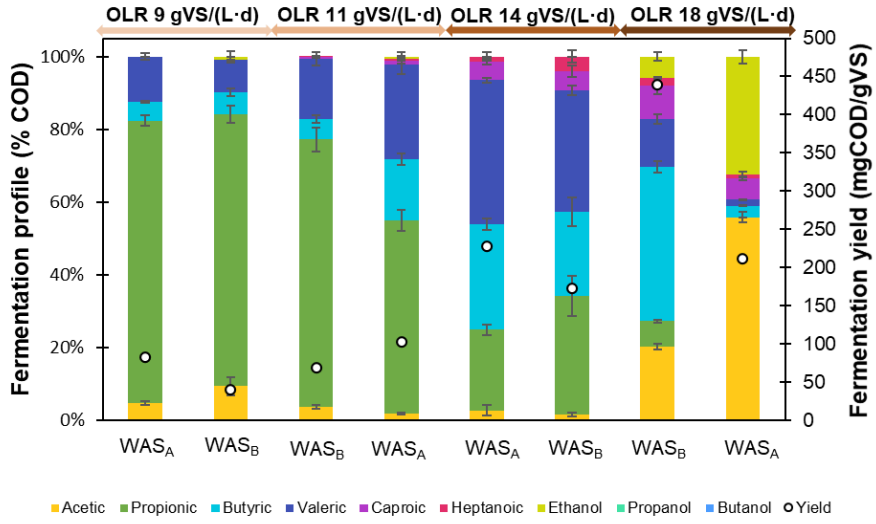

Figure 7.5. Summary of fermentation products distribution (COD basis) and fermentation yield on each period with each WAS.

Regarding the VFAs production, it was observed that fermentation yield and profile were affected throughout the fermenter operation. As observed in Figures 7.4 and 7.5, in phases 1 and 2 (OLR 9 and 11 gVS/(L·d)), the fermentation yield was very low, and the distribution was characterised by propionic acid as the main acid with a very low acetic acid concentration at neutral pH. Probably, the start-up with sole WAS at pH near 7.0 promoted the methanogenic archaea proliferation and dominance, even with the relatively short HRT and SRT applied (3.5 days). Yet, the proliferation of methanogens has been recorded at HRT as low as 2 days (Fernando-Foncillas & Varrone, 2021; Ho et al., 2014; Long et al., 2014; Peces et al., 2021). Moreover, Nierychlo et al. (2020) and Dueholm et al. (2021) studied the bacterial communities in WAS and found the presence of denitrifying heterotrophic bacteria, sulphate-reducing bacteria and PAOs. All of them are VFAs consumers and could be responsible of the reduced VFAs content. While FW was added in phases 1 and 2, the lower FW flowrate compared to WAS flowrate was insufficient to lower the pH and to allow the acidogenic fermentation bacteria to growth (see Table 7.2). Consequently, the methanogenic archaea wash-out was not achieved and the acetic acid consumption also prevented the accumulation of butyric acid, which could be expected during WAS and FW co-fermentation (Garcia-Aguirre et al., 2019; Moretto et al., 2019). Jie et al. (2014) explored the VFAs production and the bacterial community structure from excess sludge (ES) at different pH (i.e., 5, 6, 7, 8, 9, 10, 11, 12 and without pH adjustment) on batch mode obtaining low VFAs production at pH 5.0-8.0. Jie et al. (2014) checked that VFAs produced in neutral conditions were consumed by methanogens. Moreover, the sequencing and phylogenetic analysis demonstrated that methanogens appear in all ES samples. However, the methanogenic archaea presence in our reactor must be confirmed and demonstrated with ongoing microbial analyses to provide consistent conclusions. Regarding the propionic acid dominance, Feng et al. (2009, 2011) and Zhao et al. (2016) obtained propionic acid as the main VFAs on WAS and rice and WAS and FW co-fermentation, respectively, at pH 6.0-9.0 although obtaining major acetic acid concentrations than obtained in our study. This fact might be attributed to the propionic-type fermentation that is the most common carbohydrate fermentation when pH was greater than 6.0 which leads to propionic acid with some valeric acid production (Ren et al., 1997). Moreover, these results are consistent with previous studies of WAS and FW co-fermentation on batch mode, that demonstrated the

prevalence of propionic acid as the main VFAs as is proportion increased on the WAS/FW mixture (Vidal-Antich et al., 2021) (see Chapter 5).

7.3.3. Co-fermentation optimisation at OLR 14-18 gVS/(L·d)

On Phase 3 (OLR 14 gVS/(L·d)), notable changes in the process parameters were observed compared to Phase 1 and Phase 2 (see Table 7.3 and Figures 7.4 and 7.5). As a general tendency, the total and volatile solids concentration slightly increased due to the high FW content (FW/WAS 50/50, on VS content) on the influent as occurs with sCOD (9.7-12.9 gCOD/L) for Phase 3. Due to higher FW addition, the pH started to decrease with a concomitant alkalinity reduction (see Figure 7.6).

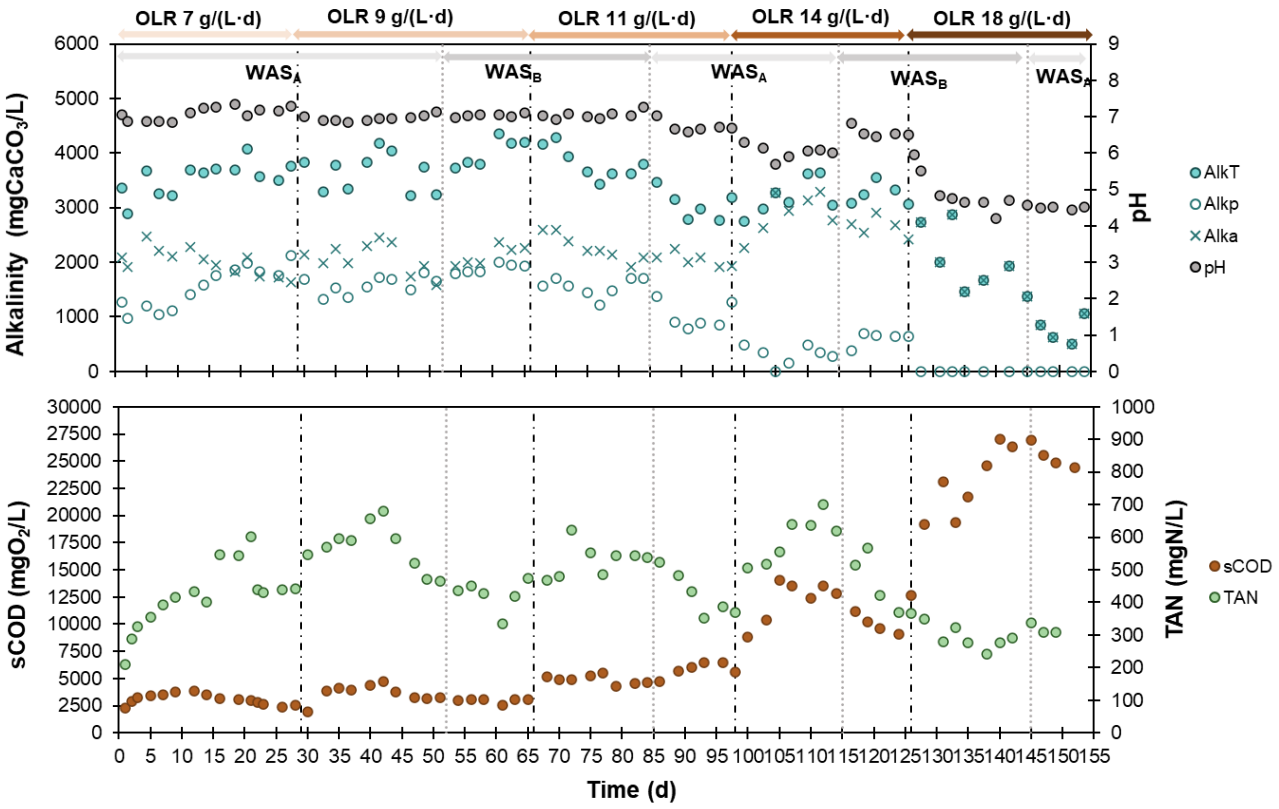

Figure 7.6. Summary of operational parameters throughout the periods: Alkanity and pH (top) and sCOD and TAN concentrations (bottom).

An important increase in the fermentation products generation was observed in this third stage, where a maximum fermentation products concentration of

10350 mgCOD/L was obtained and was quite stable using both collected WAS (WAS_A and WAS_B). More concisely, the average fermentation yield was 228 and 170 mgCOD/gVS in the steady-stage for WAS_A and WAS_B, respectively (see Figure 7.4). Consequently, when OLR increased from 11 to 14 gVS/(L·d) using the same WAS (WAS_A), the pH dropped from 6.7 until 5.7 due to VFAs production (see Figure 7.6). Moreover, the partial alkalinity decreased from 850 $mgCaCO_3$/L using WAS_A on the last day of Phase 2 until 300 $mgCaCO_3$/L on Phase 3 (see Figure 7.6). This lack of partial alkalinity, together with the slightly acidic pH on the co-fermenter, lead to an increase in the VFA yield and a change in the fermentation products profile.

More specifically, valeric increased from 26% on Phase 2 to 40% on Phase 3 being the main acid, followed by butyric acid that increased from 17% to 29% and propionic acid which highly decreased from 53% to 22% for WAS_A (see Figure 7.5). Although propionic acid proportion was lower than Phase 2, the propionic acid concentration was maintained around ~2gCOD/L as in Period 2. Hence, the proportion was lowered by the production of other acids as butyric and valeric acids that were not produced in Phase 2 (see Figure 7.4). Valeric acid production was related with the protein degradation via Stickland reaction (Garcia-Aguirre et al., 2017; Jankowska et al., 2017; Zhou et al., 2013). At day 115 the sludge was changed (from WAS_A to WAS_B) providing more partial alkalinity (650-700 $mgCaCO_3$/L) and a slightly increase on the pH (~6.5) (see Figure 7.6), that affected the VFAs proportion with a decrease on valeric acid and butyric acids production. Independently of the WAS used (WAS_A or WAS_B), at an OLR of 14 gVS/(L·d) (Phase 3), the butyric and valeric acid concentration increased and the propionic concentration remained stable (~2gCOD/L) with respect to Phase 2 results. Both sludges (WAS_A and WAS_B) achieved similar profiles and fermentation yields at different pH values (6.0 and 6.5), although better results were recorded in this period when working with WAS_A (lower alkalinity). It is important to highlight that the reported pH is measured in the effluent, before feeding the reactor. Consequently, the pH inside the reactor could vary between feeding and discharge events due to the VFAs generation inside the reactor that lows the pH (Capson-Tojo et al., 2018). Specifically, the average pH of the mixture FW/WAS used as feed applying either WAS_A or WAS_B were 6.4 ± 0.6 and 6.9 ± 0.3, respectively, and these differences of pH and alkalinity could also affect the observed VFAs production and profile.

Finally, the last phase with OLR 18 gVS/(L·d) obtained the maximum fermentation products concentration of 22560 mgCOD/L on the 140th day with a lower pH value of 4.2 and a higher sCOD (24-26 gCOD/L). During this step, the co-fermenter obtained an average yield of 440 mgCOD/gVS for WAS_B and 211 mgCOD/gVS for WAS_A. With both sludges, the pH inside the reactor was acidic (4.5) without partial alkalinity (0 $mgCaCO_3/L$). Even so, the influent using WAS_B was characterised by a larger total alkalinity (2996-4050 $mgCaCO_3/L$) and pH (6.9-7.7) than influent using WAS_A (1262-3038 $mgCaCO_3/L$ and pH 5.3-6.8). As stated before, a gradient of pH could be found inside the reactor affecting the fermentation profile obtained. Consequently, the operation with WAS_B (higher alkalinity and pH) achieved a better fermentation yield and profile characterised by high butyric acid proportion (42%) followed by acetic acid (20%), valeric acid (13%) and caproic acid (9%) (see Figure 7.5). These butyric acid accumulation accompanied by a pH decrease was expected when FW increases on the mixture based on previous short-term results (Vidal-Antich et al., 2021). Moreover, it is well known that butyric dominance in fermentation profile can be related to the pH because their accumulation is favoured at pH between 4.0 and 5.5 (Fang et al., 2020; Lu et al., 2020; Ren et al., 2007; Wang et al., 2014). Moreover, Albuquerque et al. (2007) reported that acetic and propionic concentrations were lower when pH value decreased from 7.0 to 5.0, while butyric and valeric concentrations increased as occurs in Phase 3.

This high OLR related to a high FW proportion, lead to a pH decrease that finally inhibited the methanogenic production achieving acetic acid accumulation that was not possible throughout the other phases. However, the last WAS used (WAS_A) characterised by a lower alkalinity, lead to low fermentation yields and promoted the accumulation of acetic acid (56%) and ethanol (32%) although the effluent pH of the reactor was similar to the one obtained using WAS_B (Figures 7.5 and 7.6). Therefore, this profile was determined by ethanol-type fermentation which is favoured at a pH of about 4.5 producing ethanol and acetic acid (Fang et al., 2020; Ren et al., 1997).

The concentration of fermentation products and sCOD increased as OLR increased obtaining a maximum fermentation yield during OLR 18 gSV/(L·d), but only when the buffer capacity of WAS was sufficient to sustain VFAs production. The increasing of OLR can be used to accumulate VFAs and further stimulate chain elongation (De Groof

et al., 2020). However, it was reported that OLR higher than 13 gTS/(L·d) could cause a destabilisation of the FW fermentation process. In this way, Lim et al., (2008a) studied the effect of the three OLR (5, 9 and 13 gTS/(L·d)) working at mesophilic temperature (35 ºC), controlled pH (5.5) and HRT of 8 days with an increase on VFAs concentration (from 13 to 30 gVFA/L) with increasing OLR. However, at OLR of 13 gTS/(L·d) the operation of the reactor was unstable due to its high OLR which turns the fermentation liquor very viscous and leading to accumulating unutilized solids in the reactor that decreased the VFA yield (from 0.34 to 0.29 gVFA/gVS at OLR 5 and 13 gTS/(L·d), respectively). The same trend was observed by Jiang et al., (2013) who fermented FW at 35 ºC, HRT of 5 days and controlled pH (6.0) with OLR of 5, 11 and 16 gTS/(L·d). At OLR 16gTS/(L·d), the VFAs concentration increased until day 12 when declined sharply for 5 days. Unlike these studies, the co-fermenter worked correctly at OLR 18 gVS/(L·d) in this study with uncontrolled pH probably attributed to the microorganisms' adaptation increasing the OLR on stepwise mode. This fact was consistent with Llamas et al., (2022) who decreased the HRT from 10 days to 2 days comparing stepwise reduction and direct HRT application demonstrating that stepwise decreasing allowed microbial system adaptation to an stressful situation.

Finally, it seems that the use of different sludge could be relevant only when working at high OLR. This fact might be related to the importance of the WAS buffer capacity which allows maintaining the pH above inhibitory levels (pH >5.0) during the co-fermentation performance using FW (Cabbai et al., 2016; Zhang et al., 2017). Consequently, when the reactor operates at high OLR, high FW was added to the fermenter (FW/WAS ratio of 60/40 on VS basis) and the buffer capacity of the WAS could be not enough to maintain the pH above 5.0 and fermentation bacteria might be inhibited (Moretto et al., 2019; Xing et al., 2020).

Even so, it is important to take into consideration the importance of carrying out a microbiology analysis to confirm all these facts. Hence, these analyses are ongoing and will be very important in the interpretation of the results.

7.4 Conclusions

WAS and FW co-fermentation using different OLR (9, 11, 14 and 18 gVS/(L·d) were studied on a semi-continuous reactor at mesophilic conditions (35 ºC) to produce VFAs and alcohols. As a general trend, results show that an increase in OLR leads to a higher fermentation yield with a lower effluent pH as FW proportion is risen. On WAS mono-fermentation, the VFAs production was very low probably due to the methanogenic archea proliferation which consumes acetic acid at neutral pH, affecting the first steps of co-fermentation. Phase 1 and Phase 2 with OLR 9 and 11 gVS/(L·d) obtained low yields with propionic acid as the main VFAs working at neutral pH (no acetic acid production was recorded probably related to biomass immigration with WAS and methanogens' adaptation). However, when the OLR increased to 14, higher fermentation yields were promoted at acidic pH (6.0), with propionic, butyric, valeric acids as the main VFAs produced. Finally, at 18 gVS/(L·d) the fermentation yield was higher (440 mgCOD/gVS) at pH 4.5 and the VFAs profile was affected by the sludge characteristics leading to acetic acid accumulation. Hence, the use of WAS of different origins was reflected only when high OLRs were applied. Finally, the ongoing microbial community analyses will be key to clarify the mechanisms of VFAs production and to validate the hypothesis performed to discuss the results.

8. Study of a sequencing batch reactor for the selection of polyhydroxyalkanoates accumulating cultures

ABSTRACT

The start-up and performance of a SBR for the selection of PHA-storing microorganisms was evaluated for more than 200 days combining aerobic feast/famine and uncoupled carbon and nitrogen feeding strategies. The SBR was fed with a VFA-rich solution (53.1% acetic, 21.3% propionic, and 25.6% butyric on COD basis) in the feast phase and with a nitrogen-rich solution in the famine phase. During the operation of the selection SBR working at OLR between 2.0 and 2.8 gCOD/(L·d), VFAs were completely depleted during the feast phase with a total SBR cycle length less than 17%, suggesting a successful selection of PHA-storing microorganisms. The biomass selected on the SBR was fed into an accumulation reactor where pulses of VFA-rich feeding were progressively added to maximize its PHA content reaching a PHA content around 44-46% (on SS basis) regardless of the OLR applied in the selection SBR. The recovered PHA was mainly composed of PHB since the even-chain VFA were predominant in the synthetic solution. Overall, the results of this study demonstrated that combining feast/famine with uncoupled carbon and nitrogen feeding strategies allowed improving the selection of PHA-storing microorganisms and increasing the maximum PHA content of the selected biomass.

Part of this chapter was presented as poster and oral communication in:

Start-up of sequencing batch reactor for the selection of polyhydroxyalkanoates accumulating cultures by means of a carbon and nitrogen decoupling strategy. *8th International Conference on Sustainable Solid Waste Management*, Thessaloniki, Greece, June 2021.

8.1. Introduction

Anthropogenic activities are leading to a rapid natural resources' depletion, which is accompanied by a huge production of residual streams. In this context, the recovery of high value-added products from waste is gaining attention to promote the circular economy concept and minimize the consumption of non-renewable raw materials (Dahiya et al., 2018; Fernández-Domínguez et al., 2020). Therefore, WWTPs are being conceived as RRFs (also known as biorefineries) to obtain resources within a circular economy scenario. These RRFs combine different technologies to produce energy, biofuels, and chemicals from biomass using integrated conversion processes (Moncada et al., 2016; Vinardell et al., 2020).

PHAs are biodegradable polymers that can be produced in biorefineries by microorganisms under growth-limiting conditions (Akaraonye et al., 2010). These biopolymers are polyesters with mechanical properties similar to petroleum-based plastics. The main difference is that this material is biodegradable, bio-compostable, and can be synthetised from renewable carbon sources. Two groups of homopolymers can be distinguished in the PHA, namely, PHB and PHV, and its combination produces PHBV, which is characterised by high flexibility and good mechanical properties when high HV contents are present (Albuquerque et al., 2011).

A wide range of strategies has been reported in the literature to produce PHA. Several industries have used PMCs to produce biopolymers (Sabapathy et al., 2020). Nevertheless, the use of PMCs requires a high cost due to the necessity to work under sterile conditions (Mannina et al., 2020). Conversely, the use of MMCs is gaining attention for PHAs production since it reduces the operational costs avoiding the sterilization needed for PMCs (Albuquerque et al., 2011). Therefore, the production of PHA using MMCs is expected to be the most common strategy to produce PHA in biorefineries.

Three steps need to be performed to produce PHA using MMC treating organic wastes: (i) acidogenic fermentation, (ii) PHA-storing microorganisms' selection, and (iii) accumulation of PHA (Reis et al., 2011). Acidogenic fermentation is the process in which waste is fermented by anaerobic microorganisms breaking down the organic matter into easily assimilable compounds (e.g., VFAs and alcohols) (Kleerebezem et al.,

2015). The VFAs produced during this stage are used as a carbon source for the selection of PHA-storing microorganisms in the second stage, where one or more selective forces are used to select these microorganisms. Finally, the selected biomass enriched in PHA-storage microorganisms is fed into an accumulation reactor to maximise the PHA content (Moretto et al., 2020).

Several strategies are studied to select PHA-storing microorganisms such as feast/famine and uncoupling the carbon and nitrogen feeding (Serafim et al., 2008), usually in SBRs. Specifically, the feast/famine which consists in the alteration of external carbon source availability and scarcity during the SBR cycle has been widely used (Huang et al., 2018). In the feast stage, the external carbon source is consumed by microorganisms and stored as intracellular PHA. In the famine stage, the intracellular PHA is consumed by the biomass to grow. In addition to this strategy, uncoupling carbon and nitrogen feeding could be used to enhance the selection of PHA-storage microorganisms (Lorini et al., 2020; Nguyenhuynh et al., 2021; Silva et al., 2017). Hence, the carbon source is fed at the beginning of the feast phase to promote the intracellular PHA accumulation, while the external nitrogen source is fed at the beginning of the famine phase to promote the growth of biomass using the intracellular PHA (Oliveira et al., 2017). The ratio between feast/famine duration for PHA production has been widely evaluated by several studies concluding that should be less than 0.2 to enhance a good selection of PHA-storage microorganisms (Albuquerque, 2010b; Dionisi et al., 2007; Hao et al., 2018). Nevertheless, more research is needed to evaluate the long-term selection of PHA-storing microorganisms under a carbon and nitrogen uncoupled feeding.

This investigation aims to start-up and evaluate the performance of a bioreactor (>200 days) to select PHA-storage microorganisms using both feast/famine and carbon and nitrogen uncoupling feeding strategies using a SBR fed with a synthetic solution of VFAs and nitrogen-rich solution during feast and famine stages, respectively. Furthermore, the biomass selected at this stage was used on an accumulation batch reactor to determine the PHA storing capacity of the selected biomass.

8.2. Materials and methods

8.2.1. Selection of the PHA-storing microorganisms

8.2.1.1. Experimental set-up and operation

The SBR used to select the PHA-storing biomass was described in Section 3.1.3. The operation took place at 30 ºC, which is a suitable temperature for the selection of PHA-storing microorganisms (Colombo et al., 2017).

The SBR cycles had a total length of 6h with 7 different stages: (i) the external carbon source (i.e., VFAs) was fed to the SBR at 2.40 L/h in anaerobic conditions (15 min); (ii) aerobic reaction with air supply and agitation when the microorganisms consume the carbon source and store internal PHA into the cells (150 min); (iii) the selected biomass, with a maximum PHA content, was purged from the SBR at 3.12 L/h (3 min); (iv) a nitrogen-rich solution was feed to the reactor to allow the microorganisms' growth at 1.32 L/h under aerobic conditions (4 min); (v) the internal PHA accumulated during the feast stage was consumed for biomass growth under aerobic conditions (150 min); (vi) the biomass was settled by turning off the agitation and air supply (30 min), and (vii) the treated effluent was discharged until the desired working volume (2.31L) (see Figure 8.1). It is important to highlight that these lengths were implemented to temporise the pumps, and the feast/cycle duration ratio was dependant on the microorganisms' adaptation.

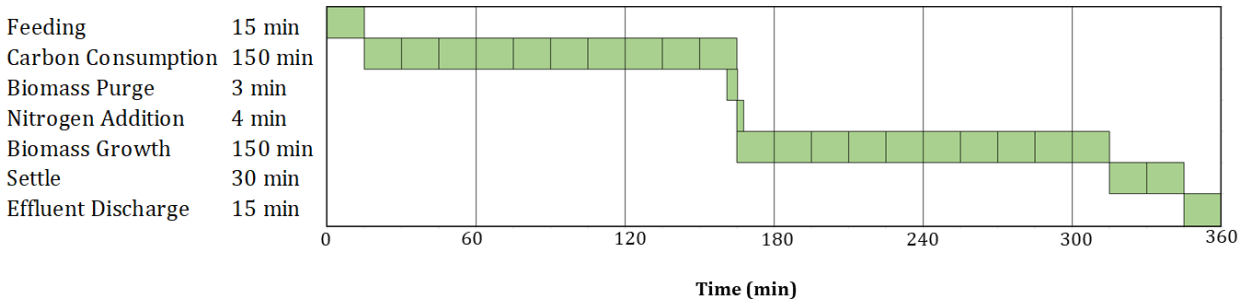

Figure 8.1. Distribution of the SBR stages during the SBR cycle duration.

The SRT and the HRT were set as 4.81 and 1.25 days, respectively, based on Oliveira et al. (2017) results. The operation was divided into three periods depending on the applied OLR, nitrogen loading rate (NLR) and feast/total cycle time ratio. Period I and Period II were characterised by an OLR of 2.0 gCOD/(L·d) with a C/N ratio of 27.7 and 20.8 gCOD/gN, respectively. In Period III, the OLR was increased to 2.8 gCOD/(L·d) and the C/N ratio was set at 29.2 gCOD/N, aiming to adjust the N dosage to obtain an effluent without nitrogen. These C/N ratios were similar to the adjusted by Lorini et al. (2020) who established a C/N ratio of 33.4 gCOD/N to avoid nitrogen limitation on the famine phase. The operating conditions used are summarized in Table 8.1.

Table 8.1. Operational conditions of the selection SBR reactor for three periods.

Parameter	Units	Period I	Period II	Period III
Days of operation	-	1-117	118-169	170-209
HRT	days	1.25	1.25	1.25
SRT	days	4.81	4.81	4.81
Feast/cycle	% time	>20%	<17%	<17%
OLR	gCOD/(L·d)	2.0	2.0	2.8
VFAs feed	gCOD/L	2.5	2.5	3.5
Influent acetic acid	% COD	53.1	53.1	53.1
Influent propionic acid	% COD	21.3	21.3	21.3
Influent butyric acid	% COD	25.6	25.6	25.6
NLR	mgN/(L·d)	72.0	96.0	96.0
C/N ratio	gCOD/gN	27.7	20.8	29.2

8.2.1.2. Synthetic feed and inoculum used in the selection SBR

The synthetic wastewater used contained a mixture of acetic acid (53.1 % of COD), propionic acid (21.3 % of COD), and butyric acid (25.6 % of COD), representing the proportion between these acids obtained in the fermentation effluent of a previous study when treating OFMSW (Dosta et al., 2018) (see Table 8.1). Two OLRs were used on the PHA-storage microorganisms' selection: (i) 2.0 gCOD/(L·d) for Period I and II and (ii) 2.8 gCOD/(L·d) for Period III. Consequently, the VFAs concentration in the synthetic feeding was 2.5 gCOD/L in Periods I and II, whereas it was set at 3.5 gCOD/L during Period III to maintain the HRT at 1.25 days. Macronutrients and micronutrients were also added to the synthetic feeding using the trace solution proposed by Dapena-Mora et al. (2004) (see Table 8.2). The pH was adjusted to 6.5 using 1.5 g/L of $NaHCO_3$.

Table 8.2. Macronutrients and micronutrients concentration in the synthetic wastewater of this study.

Macronutrients

Compound	Units	Value
K_2HPO_4	g/L	0.58
KH_2PO_4	g/L	0.23
$MgSO_4 \cdot 7H_2O$	g/L	0.09
$CaCl_2 \cdot 2H_2O$	g/L	0.07
EDTA	g/L	0.02

Micronutrients

Compound	Units	Value
$FeCl_3 \cdot 6H_2O$	mg/L	1.50
H_3BO_3	mg/L	0.15
$CuSO_4 \cdot 5H_2O$	mg/L	0.03
KI	mg/L	0.03
$MnCl_2 \cdot 4H_2O$	mg/L	0.12
$Na_2MoO \cdot 2H_2O$	mg/L	0.06
$ZnSO_4 \cdot 7H_20$	mg/L	0.12
$CaCl_2 \cdot 2H_2O$	mg/L	0.12

The nitrogen supply was prepared separately using NH_4Cl to uncouple the nitrogen addition from the carbon source. Different NH_4Cl concentrations were used during the famine phase achieving two NLR: (i) 74 mgN/(L·d) for Period I and (ii) 96 mgN/(L·d) for Periods II and III.

The SBR was inoculated with 200 mL of WAS (12.3 gTSS/L; VSS/TSS of 74%) from a municipal WWTP of the Barcelona metropolitan area (Spain) with 350,000 population equivalents. Once the WAS was characterised, it was diluted until achieving an initial TSS of 2 gTSS/L to inoculate the SBR.

8.2.2. PHA accumulation tests

The accumulation reactor used to maximize the PHA content of the selected biomass was previously described in Section 3.1.4. The operation was performed by multiple pulse-feeding strategy (batch mode) to obtain the maximum PHA content on the microorganisms previously selected on the SBR (Conca et al., 2020; Valentino et al., 2020). Hence, to carry out an accumulation test, 800 mL of purged biomass from Period II and III of the SBR was added to the reactor. Then, 100 mL of the synthetic VFA-rich solution with the same VFAs proportion but twice COD as the selection reactor was added by pulses (5 and 7 gCOD/L for Periods II and III, respectively). Every pulse was added to the accumulation reactor when VFAs was totally depleted. This fact was observed with DO rise above 6.0 mgO_2/L. Nitrogen was not added to the accumulation

assays because the PHA accumulation is promoted without allowing biomass growth. Namely, each test takes 7 hours with 5 carbon source pulses.

8.2.3. Analytical methods

TSS, VSS and TAN were analysed in accordance with the Standards Methods for the examination of Water and Wastewater (APHA, 2017) as detailed in Section 3.3. The VFAs were analysed using gas chromatography (see details on Section 3.3.7). The analysis of PHA content was performed following the protocol of Lanham et al. (2013) as described in Section 3.3.9.

8.3. Results and discussion

8.3.1. Selection SBR operation

Figure 8.2 shows the evolution of the parameters in the selection reactor throughout the three operational periods. Moreover, Table 8.3 shows the average values with the minimum and maximum values in brackets of the parameters monitored in Figure 8.2. The VFAs concentration on the purged biomass was very low for all periods indicating that this carbon source was consumed during the feast phase to store intracellular PHA. The variations in the VFAs concentration observed in Figure 8.2. after the feast stage were mainly attributed to the fact that microorganisms tended to produce biofilm in the porous stones where the dissolved oxygen was supplied. Consequently, the oxygen transfer to the mixed liquor was reduced and the PHA accumulation rate was limited. To avoid this fact, the porous stones were replaced when the VFAs concentration was higher than 50 mgCOD/L at the end of the feast stage to ensure the aerobic conditions to achieve working under ADF strategy.

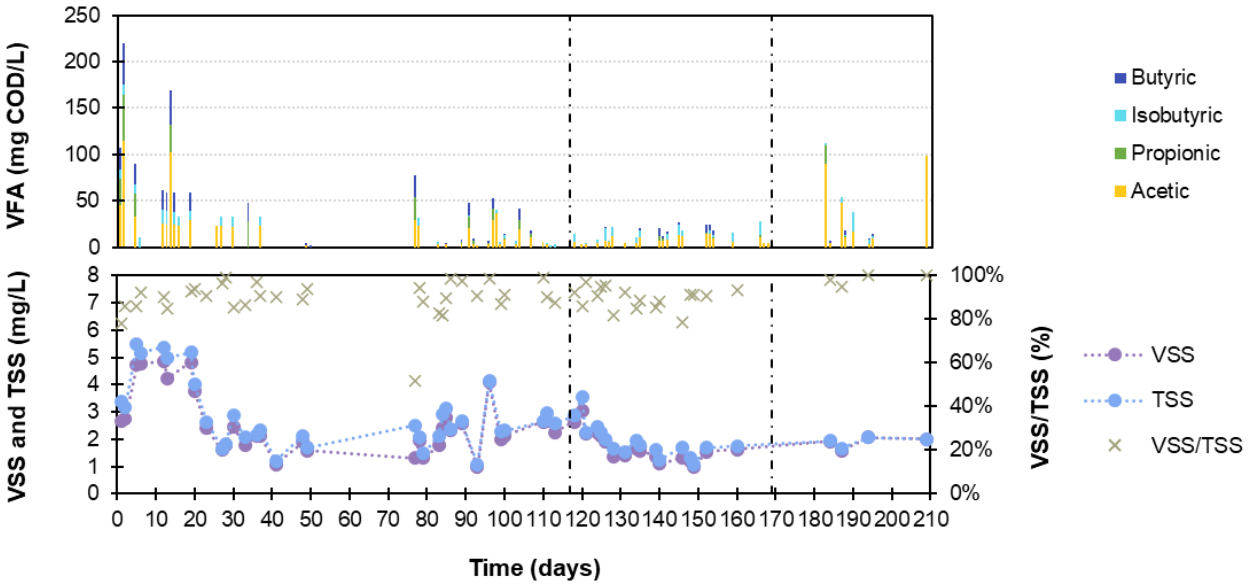

Figure 8.2. Evolution of VFAs (top) and VSS, TSS and VSS/TSS ratio in the biomass purge stream of the selection SBR.

As Table 8.3 shows, the TSS and VSS were evaluated throughout the selection process achieving higher concentrations in Period I due to the inoculation from diluted WAS that contains higher TSS and VSS content. Moreover, the VSS concentration fluctuated and settleability was hindered due to the presence of filamentous bacteria (Wen et al., 2012). This type of microorganisms is present in the reactors where nutrients are limited (Cardete et al., 2018) as occurred in Period I where nitrogen was totally depleted before the end of the famine phase. Consequently, the nitrogen concentration was increased in Period II to reach a better selection of PHA-accumulation microorganisms and to avoid the presence of filamentous bacteria. Consequently, from Period II, the TSS and VSS concentration remained stable around 1.9-2.0 gTSS/L and 1.7-2.0 gVSS/L. Nevertheless, the rise in nitrogen feeding caused that nitrogen was not totally depleted at the end of the famine phase and the growth of non-PHA storing microorganisms inside the reactor was not completely prevented. Therefore, the OLR was increased from 2.0 to 2.8 gCOD/(L·d) in Period III, and a higher VSS concentration was recorded although the PHA content remained between 5-13% under these experimental conditions. The higher OLR was preferred since keeping a reasonably high concentration of active biomass in the system is important to (i) maximise the

amount of PHA recovered within the PHA-accumulating microorganisms purge and (ii) improve the economic competitiveness of the system.

Finally, it is important to take into account that PHA content was not analysed in the first step because the reactor does not work in optimised conditions with VFAs peaks on the effluent probably due to the low DO control by biofilm formation in the porous stones. Consequently, the PHA content was analysed from Period II.

Table 8.3. Average (minimum – maximum) values of the operating parameters in the selection SBR.

Parameters	Units	Period I	Period II	Period III
OLR	gCOD/(L·d)	2.0	2.0	2.8
TSS	g/L	2.88 (1.08 – 5.48)	1.92 (1.07 – 3.55)	2.01 (1.66 – 2.39)
VSS	g/L	2.57 (0.98 – 4.86)	1.73 (0.98 – 3.44)	2.00 (1.58 – 2.35)
VSS/TSS	%	89 (52 – 99)	89 (79 – 97)	97 (95-98)
VFAs concentration (sludge purge)	mgCOD/L	55 (2 – 219)	23 (4-82)	41 (6-136)
VFAs removal (treated effluent)	%	>99	>99	>99
PHA percentage	% (on SS basis)	n.a	9 (6-10)	8 (5-13)

n.a: data not available

8.3.2. SBR cycle analysis

Figure 8.3 shows the pH, DO, VFAs, TAN and PHA profile for a representative SBR cycle of Period III on day 194. The TSS and VSS content during this cycle were 2.39 gTSS/L and 2.45 gVSS/L. DO concentration was close to 0 mgO_2/L during the feast stage (A-B), which indicates that the biological oxygen uptake rate was limited by the DO transfer rate to the mixed liquor. Furthermore, the low oxygen concentration during the feast stage indicated that VFAs were consumed and stored as PHA by PHA-storing microorganisms (Wang et al., 2017). Hence, the sharp increase in the DO concentration indicates that VFAs were totally consumed in the reactor (see Figure 8.3, approximately at 100 min). Unlike the feast stage, the DO consumption rate was lower during the famine phase since DO was consumed by the PHA-storing microorganisms using the intracellular PHA rather than by using an external carbon source. As is shown in Figure 8.3., the famine phase (C-D) was longer than the feast phase representing a feast/cycle ratio of 0.17, which is a suitable value to create an internal selection pressure for a correct selection of PHA-storing microorganisms (Dahiya et al., 2018; Lanham et al., 2013).

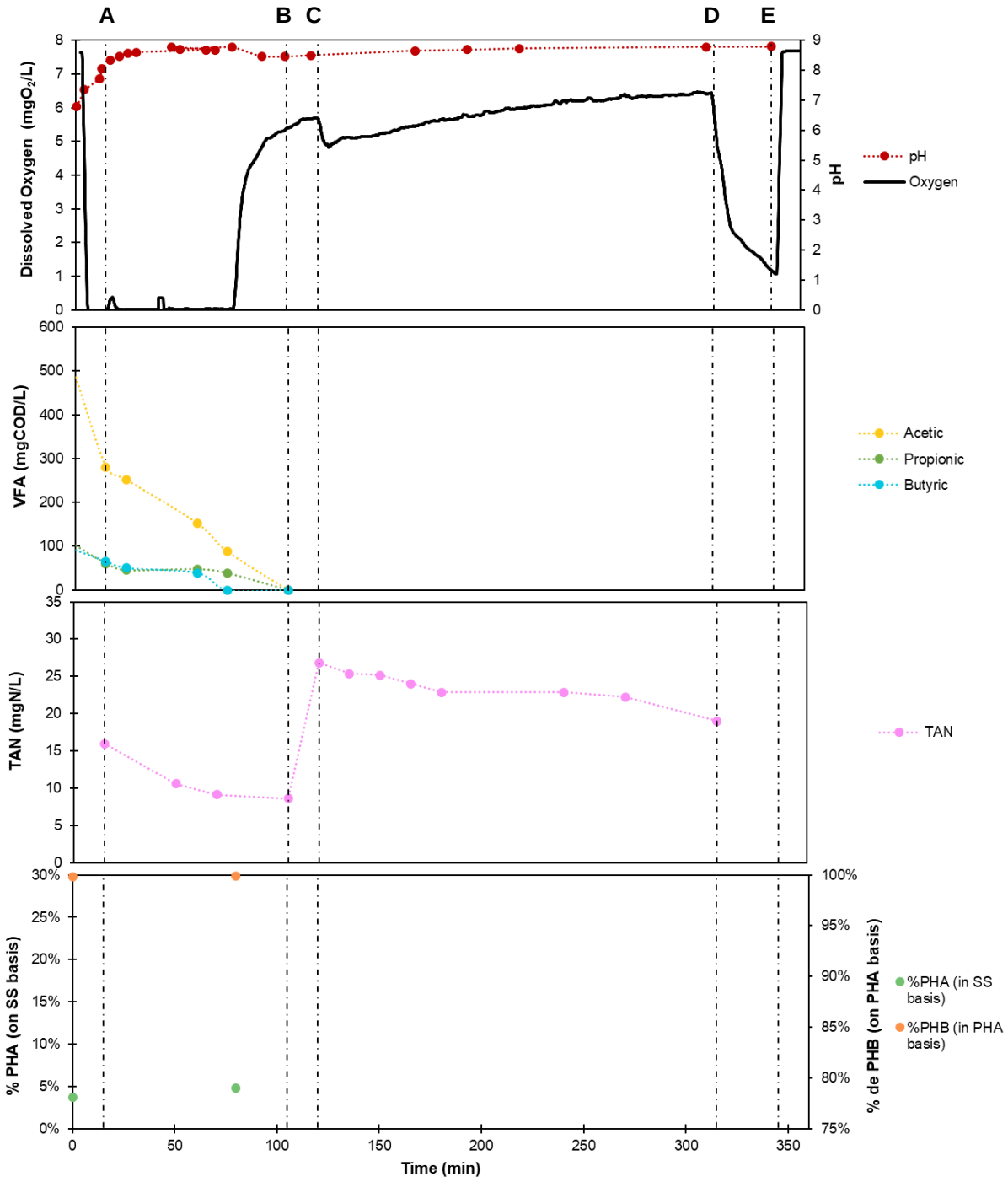

Figure 8.3. DO, pH, VFA, TAN, and PHA profiles for a representative SBR cycle of Period III (Operation day 194). (A = feed addition; B = biomass purged; C = ammonia addition; D = sedimentation; E = effluent discharge).

Regarding Figure 8.3, it can also be observed that the VFAs concentration in the biomass purged was 0 mgCOD/L for this cycle, which means that VFAs were completely depleted during the feast phase (A-B). Acetic acid was consumed at a higher rate than

propionic and butyric acids probably due to its lower molecular weight. Consequently, it is suggested that acetic acid was the primary driver for PHA production, which agrees with results reported by Wijeyekoon et al. (2018).

Moreover, the PHA content was measured at the beginning of the cycle and in the purged biomass (B). As Figure 8.3 shows, the PHA content on the biomass was increased during the feast stage from 5% on SS at the beginning until 15% during this cycle. These results agree with other studies that obtained similar contents (9% in TSS basis) in the purged biomass from the selection SBR under different cycle distribution (two settles) and carbon feed based on sugars (Ahmadi et al., 2020). The PHB content was the main component in PHA (~100%) since the VFAs with an even number of carbons (i.e., acetic and butyric acids) were predominant in the synthetic feeding favouring the formation of HB polymer (Fradinho et al., 2014; Wang et al., 2018).

Ammonium concentration was not totally depleted after the famine phase of this cycle indicating that these working conditions could not completely prevent the non-PHA-storage microorganisms' growth during the feast stage. Furthermore, in some previous SBR cycles, the VFAs were not completely removed before the end of the feast phase as has been explained before (see Figure 8.3). Hence, the nitrogen fed to the famine phase was higher resulting in sufficient nitrogen in the feast phase for biomass growth instead of PHA accumulation. Probably, the main reason was the biofilm generation in the porous stones which decrease the oxygen transfer into the SBR affecting the PHA-storage microorganisms' selection. Moreover, further research will be needed to better control the oxygen transfer rate into the mixed liquor and to adjust the C/N ratio to completely remove the nitrogen at the end of the famine stage enhancing the PHA-storage microorganisms' selection and productivity.

8.3.3. PHA accumulation tests

Different accumulation assays were carried out with the biomass purged when VFAs were completely depleted on selection reactor. This biomass was characterised by a high abundance of microorganisms able to further accumulate PHA inside their cells. Figures 8.4 and 8.5 show two representative accumulation tests for Period II and Period III, respectively.

The first accumulation test was performed with the biomass of Period II, which contained a TSS and VSS concentrations of 1.79 gTSS/L and 1.70 gVSS/L with a VSS/TSS ratio of 96%. In this specific assay, the accumulation reactor was fed with a VFA-rich solution containing a VFAs concentration of 5 gCOD/L (double than VFAs fed at SBR of Period II) with the same proportion of acetic, propionic and butyric acids (see Section 8.2.1.2). The VFAs pulses were performed when the DO concentration on the reactor increased (~7 mgO_2/L) indicating that PHA-storage microorganisms already consumed the external VFAs. Specifically, five pulses were added to the accumulation reactor at 0.2, 1.9, 2.3, 3.4 and 4.8 h (see Figure 8.4).

The DO concentration slightly decreased when the VFA-rich stream was fed to the accumulation reactor since the PHA-storing microorganisms used the DO to accumulate intracellular PHA using the external VFAs. Moreover, the VFAs consumption rate was decreasing after each VFAs pulse since the capacity of microorganisms to store intracellular PHA also decreased as a result of the higher among of intracellular PHA after each VFAs pulse (Table 8.4). It is important to take into account that biomass settling was carried out before the third and fifth pulses leading to a sudden decrease in the DO concentration because agitation and oxygen supply were switched off.

Figure 8.4. shows that microorganisms were able to store the VFAs as intracellular PHA with a PHA content in the biomass from 9.76 ± 4.03 % PHA on SS basis at the beginning until 44.09 ± 4.81 % PHA on SS basis at the end of the accumulation tests. Moreover, as expected, PHA content was reported at 90% of total PHA being the main compound, which is in accordance with the results obtained in the selection SBR.

Table 8.4. The average speed of VFAs degradation of each pulse in the accumulation test performed on biomass purged from the selection SBR working under 2 gCOD/(L·d) (Operation day 169).

Pulse	**Initial PHA** (% on SS)	**Initial PHB** (% PHA basis)	**Final PHA** (% on SS)	**Final PHB** (% PHA basis)	**Average VFAs degradation rate** (mgCOD/(min·L))	**Time spent** (min)
1	8	94	10	92	8.73	46
2	10	92	-	-	8.02	48
3	-	-	26	94	5.89	63
4	26	94	-	-	4.34	89
5	-	-	44	94	3.87	101

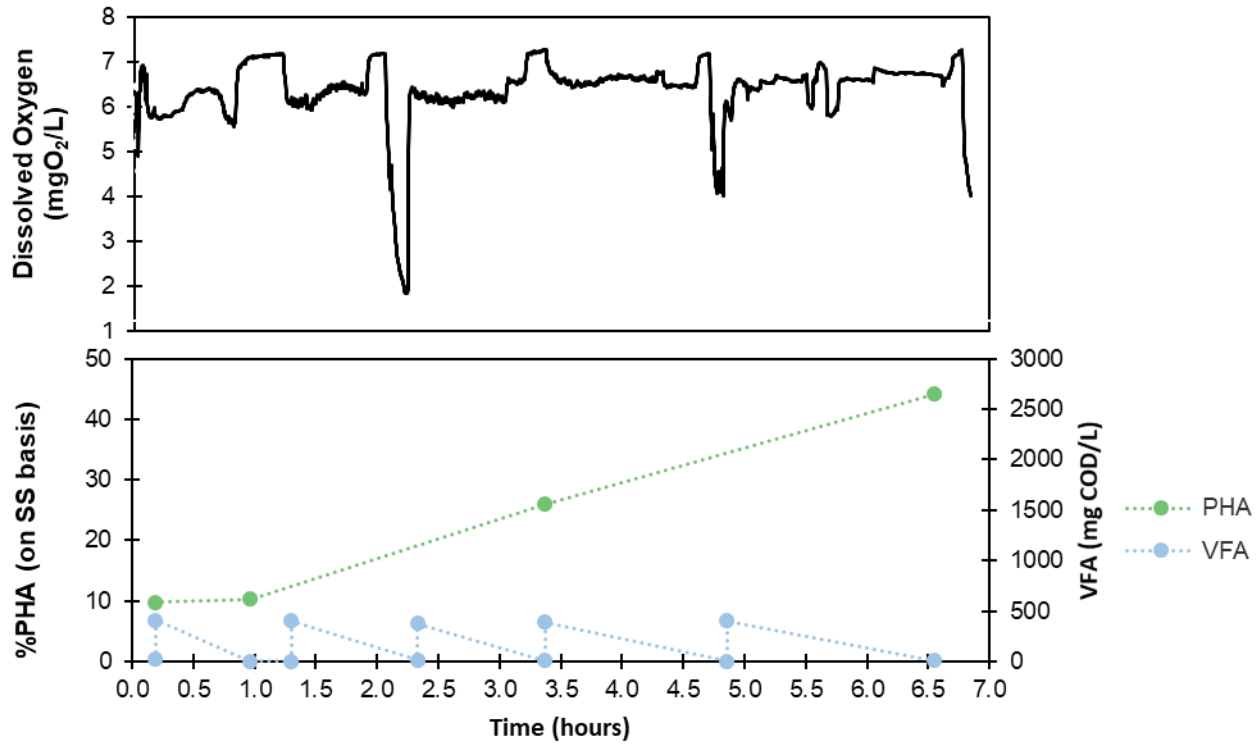

Figure 8.4. Accumulation test for Period II of the selection SBR (Operation day 169). The VFA-rich solution contained a VFAs concentration of 5 gCOD/L (acetic, propionic, and butyric acids).

Another accumulation test was carried out with the biomass purged of the SBR on Period III which TSS and VSS concentration of 1.84 gTSS/L and 1.81 gVSS/L and a VSS/TSS ratio of 98%. In this test, the accumulation reactor was fed using a VFAs concentration of 7 gCOD/L (double than VFAs fed at SBR of Period III) being higher than the VFAs used on the previous accumulation test. The proportion of acetic, propionic, and butyric acids was the same again. In this test, five VFAs pulses were performed to the accumulation reactor at 0.1, 1.9, 3.1, 4.4 and 6.8 h (see Figure 8.5).

The results showed a similar trend than in the first accumulation test as the DO concentration decreased after the VFA-rich solution was added to the reactor as expected. Unlike the first accumulation test, the DO concentration did not experience a sharp decrease because biomass settling was not performed during the test. The VFAs consumption rate was faster in the two first pulses than in the other ones with a progressive decline until reaching a sharp decrease in the last spike. This could explain that the microorganisms nearly reached their maximum PHA content (Table 8.5).

Table 8.5. The average speed of VFAs degradation of each pulse in the accumulation test performed on biomass purged from the selection SBR working under 2.8 gCOD/(L·d) (Operation day 197).

Pulse	**Initial PHA** (% on SS)	**Initial PHB** (% PHA basis)	**Final PHA** (% onSS)	**Final PHB** (% PHA basis)	**Average VFAs degradation rate** (mgCOD/(min·L))	**Time spent** (min)
1	9	95	20	90	8.25	54
2	20	90	29	94	8.41	51
3	29	94	38	91	5.54	78
4	38	91	39	94	2.68	144
5	39	94	46	93	0.33	96

Figure 8.5. shows that PHA content in the biomass increased from 9.2 ± 5.5 at the beginning until to 46.1 ± 1.0 % PHA on SS basis at the end of the accumulation test. The PHA content in the biomass at the first accumulation test (44 %) was very similar that the obtained on the second accumulation test (46 %) although a higher VFAs load was applied in the selection and accumulation reactors. This fact implies that a 40 % of OLR increase during the selection of PHA-storage microorganisms did not have a high impact on their storage capacity. Villano et al. (2014) reached a similar final PHA concentration in the accumulation reactor with a percentage of 46 %, although a higher OLR and longer cycle length than in the present study were applied. Nonetheless, a higher OLR would demand higher oxygen supply rates or a longer duration of the selection SBR cycles to satisfy a feast/famine ratio below 20%. Nevertheless, Lorini et al. (2020) obtained a PHA content in the selection reactor as high as 0.53 gPHA/gVSS which was higher after the accumulation test (0.70 gPHA/gVSS) working with an OLR up to 12.5 gCOD/(L·d) and feast/famine and uncoupled carbon and nitrogen feeding. On the other hand, Campanari et al. (2014) reached PHA values of 30% using real feed without uncoupling carbon and nitrogen feeding. Furthermore, the implementation of the uncoupling carbon and nitrogen feeding strategy could improve the selection of microorganisms with their PHA storage capacity.

Overall, these results show that the PHA-storing organisms were able to accumulate over 40% of PHA (on SS basis) using multiple pulse-feeding strategies, which is close to the threshold PHA content required to make the recovery of bioplastics commercially viable.

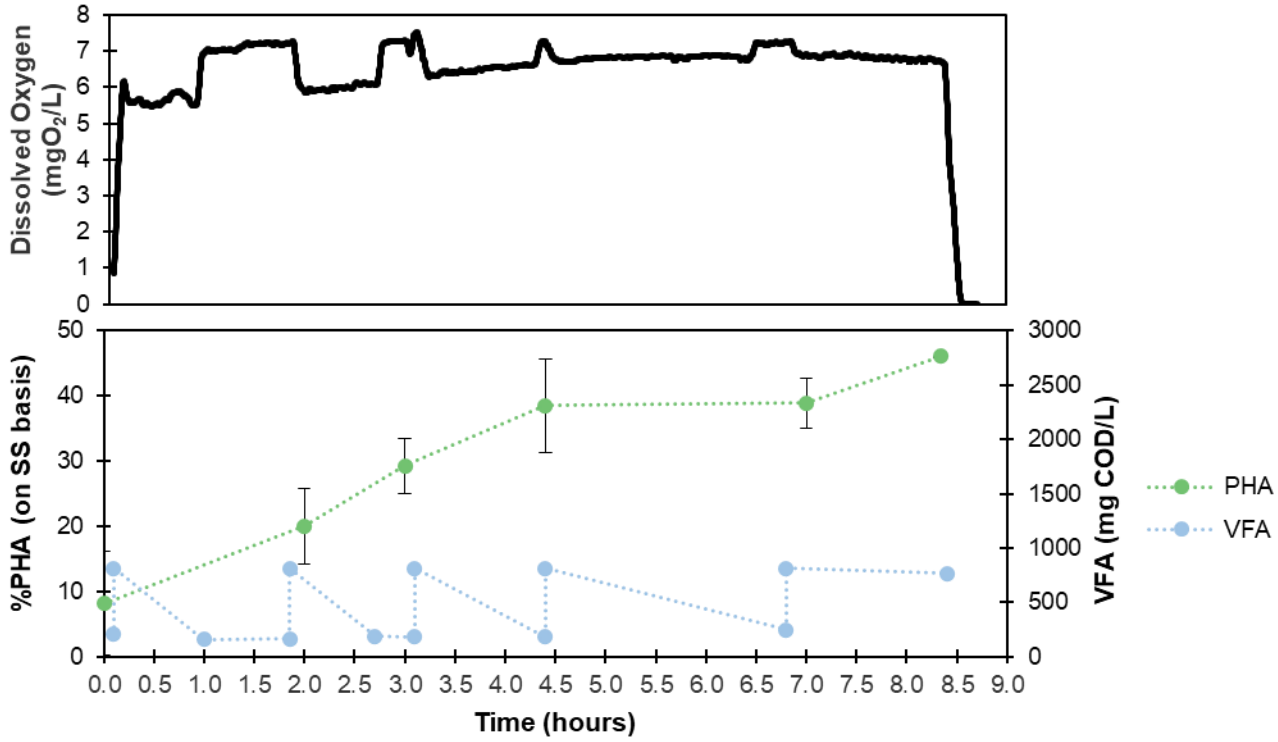

Figure 8.5. Accumulation test for Period III of the selection SBR (Operation day 197). The VFA-rich solution contained a VFAs concentration of 7 gCOD/L (acetic, propionic, and butyric acids).

8.4. Conclusions

The performance of an SBR for the selection of PHA-storing microorganisms was evaluated (>200 days) trying to combine feast/famine and an uncoupled carbon and nitrogen feeding strategy at an OLR of 2.0 and 2.8 gCOD/(L·d) and COD/N ratio of 20.8-29.2 gCOD/gN on three operational periods. The results showed that the strategy applied led to a good selection for PHA-storing microorganisms, despite the supplied nitrogen was not completely removed at the end of the SBR cycle. An increase of the OLR lead to higher production of selected microorganisms, but this biomass reached PHA contents between 44 and 46% (on VSS basis) in the accumulation reactor regardless of the applied OLR in the selection SBR. The results also showed that PHB was the main component in PHA because the synthetic feed was rich in even-chain VFAs. Overall, the combined effect of feast/famine periods and uncoupled carbon and nitrogen feeding allowed obtaining a good selection of PHA-storing microorganisms,

able to increase its PHA content above 40% during accumulation assays. Further experimentation will be needed to better control the oxygen transfer rate to the mixed liquor and to adjust the C/N ratio to completely remove the nitrogen source at the end of the famine phase, thus enhancing the PHA accumulating organisms' selection and productivity.

9. General conclusions and recommendations

In this chapter, the general conclusions of this thesis and recommendations for future work are summarised.

9.1. General conclusions

In this thesis, the FW acidogenic fermentation, and WAS and FW acidogenic co-fermentation has been evaluated as promising key biotechnology on the biorefineries to produce PHA as a final product. Hence, the main conclusions extracted from this study are compiled and discussed in this section.

Referring to Chapter 4 - Volatile fatty acids production from food waste under different working pH

- The maximum VFA yield obtained on the batch test was in the pH range between 6.0 and 9.0 with values of 13.2-16.2 gCOD/L and acetic acid as the main VFA obtained (33-48%), followed by butyric acid (16-20%) and caproic acid (23-38%).
- The extreme pH conditions tested (pH 4.0 and 11.0) achieved lower VFA concentrations of 6.1 and 8.0 gCOD/L, respectively, with acetic as the main VFA.
- On semi-continuous mesophilic fermenters, high differentiation between acidic pH (6.0) and alkaline pH (9.5-10.0) conditions was observed with a VFA profile dominated by acetic acid (34-44%) and caproic acid (33-43%) on the acidic reactor, and acetic acid (achieving up to 85%) as the main component on the alkaline reactor.
- pH adjustment could be an excellent strategy to tune the VFA profile depending on the preferences of the final use of the fermentation broth.

Referring to Chapter 5 - Assessing the potential of waste activated sludge and food waste co fermentation for carboxylic acids production.

- The fermentation yields obtained under co-fermentation conditions were always higher than those obtained from WAS and FW mono-fermentation.
- When the pH value was above 5, the co-fermentation yield raised as the proportion of FW increases on the mixture and, indicating that the improvement was primarily due to the higher FW degradation on the mixture.
- Butyric acid was enriched in the fermented product as the proportion of FW in the mixture increased and the concomitant pH decreased. Moreover, the percentage of acetic, and propionic acids in the VFAs obtained decreased as the WAS decreased on the mixture.
- The addition of alkalinity to FW (30 $gNaHCO_3$/kg) was not enough to have a notable effect on the fermentation yield nor the fermentation profile of the WAS/FW_70/30
- The amount of FW in the co-fermentation mixture should be limited to keep the pH above 5.0. Higher proportions of FW are possible but at the expense of constantly dosing external alkali chemicals, which should be considered in the techno-economic analysis. Note that the pH is not only affected by the mixture composition but also by the operational conditions of the fermenter.
- WAS auto-hydrolysis pre-treatment resulted in minor VFAs production kinetics improvements but did not enhance the co-fermentation VFA yield.
- Co-fermentation is an excellent option to boost the fermentation yield without external chemicals addition. Moreover, the proportion of both substrates can be adjusted to tune the product profile.

Referring to Chapter 6 - Impact of food waste composition on acidogenic co-fermentation with waste activated sludge.

- The results of fermentation batch assays performed in this study demonstrated that each FW component (i.e., fruit, vegetables, pasta, rice, meat, fish and cellulose) had a statistically distinct effect on the VFA profile and yield during its co-fermentation with WAS.

- The maximum VFA yield were recorded when WAS was co-fermented with protein-rich organic wastes, such as fish and meat, reaching 502 and 442 mgCOD/gVS, respectively.
- The importance to balance the protein-to-carbohydrate ratio to improve the VFA yield production was demonstrated obtaining higher yields when the co-fermentation was carried out using WAS with a mixture of proteins and carbohydrates than WAS with only proteins or only carbohydrates.
- Although several batch tests were performed with WAS of different origins and different initial characteristics, PCA analysis indicated that the use of the different WAS selected in this study on co-fermentation mixtures did not have a direct influence on the fermentation VFA profile.

Referring to Chapter 7 - Study of the organic loading rate increasing on the acidogenic co-fermentation of WAS and FW.

- The semi-continuous operation with WAS (25 g VS/L) mono-fermentation at mesophilic conditions with an HRT of 3.5 days lead to a working pH around neutrality and low VFA yields around 50 mgCOD/gVS, probably related to the proliferation of methanogens (microbial community analysis pending).
- For FW and WAS co-fermentation, an OLR of 9-11 gVS/(L·d) resulted in lower VFA yields when compared to 14-18 gVS/(L·d). Furthermore, for 9-11 gVS/(L·d), propionic acid was the main VFA and acetic acid concentration was below 0.1 gCOD/L, which is hypothesized to have been converted to biogas due to methanogens' adaptation throughout the process at an operating pH near neutrality.
- At OLR 14 gVS/(L·d), a descent of the working pH at values near 6 was observed, as well as an increase in the VFA yields (namely 230 mgCOD/gVS) and an accumulation of both butyric and valeric acids.
- The highest fermentation yield (440 mgCOD/gVS) was recorded at OLR 18 gVS/(L·d), where low pH values could have inhibited methanogens, leading to a rise in acetic acid concentration.
- The effect of using WAS of different origins was only detected when working at the higher OLR conditions (14-18 g VS/(L·d)), since its alkalinity content was a

key parameter to prevent that VFA production would result in an undesired sudden pH drop to highly acidic values.

Referring to Chapter 8 - Study of a sequencing batch reactor for the selection of polyhydroxyalkanoates accumulating cultures.

- The PHA-storing microorganisms were successfully selected on the selection reactor using the strategy applied despite the supplied nitrogen was not completely removed at the end of the famine stage.
- When higher OLR was applied, more production of selected microorganisms was obtained and reached a similar PHA content in the accumulation reactor (between 44 and 46% on VSS basis).
- The PHA produced was highly enriched in PHB, which is attributed to the composition of the synthetic feeding used, that was enriched in even-chain VFAs.
- Further experimentation will be needed to better control the oxygen transfer rate to the mixed liquor and to adjust the C/N ratio to completely remove the nitrogen source at the end of the famine phase.

9.2. Future recommendations

For further research, the following recommendations are proposed:

Regarding acidogenic fermentation:

- Microbiology analyses are recommended in all acidogenic fermentation experiments to extract more conclusions and facilitate the process understanding. In fact, research focused on the analysis of microbiology in samples from the long-term effect of OLR in WAS co-fermentation (Chapter 7) is being performed to better understand the results obtained.
- pH is a key parameter to control the acidogenic fermentation process. Hence, it could be interesting to do a screening of pH on WAS and FW co-fermentation batch test. Consequently, the best conditions should be studied in

semi-continuous mode adjusting the pH using external chemical reagents and analyzing the microbial community developed.

- The WAS and FW co-fermentation could be compared under psychrophilic, mesophilic, and thermophilic regimes that could highly affect the hydrolysis and acidogenesis steps.
- Real FW from food wholesale markets could be co-fermented with WAS to assess the effect of FW seasonality on the fermentation process performance, especially on VFA yields and profile.

Regarding PHA production:

- The carbon and nitrogen uncoupled feeding strategy should be adjusted to assure the total nitrogen depletion at the end of the famine phase. A nitrification inhibitor could be also added to the synthetic feeding to prevent nitrification to take place in the system or, alternatively, NO_2^--N and NO_3^--N could be monitored, thus confirming that nitrogen depletion is only related to the PHA-storing microorganisms.
- The use of a real fermentation effluent for PHA production could be a very interesting point to consider. In this way, it would be important to pre-treat the fermentation effluent to remove or recover nitrogen as a previous step to the feeding of this stream to the selection reactor using both feast/famine and carbon and nitrogen decoupling strategies.
- Further research are required to better control the oxygen transfer rate to the mixed liquor and to adjust the C/N ratio to completely remove the nitrogen source at the end of the famine phase.

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