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Licenciatura em Química Aplicada

Optimization of a pilot-scale acidogenic reactor using industrial food waste

Dissertação para obtenção do Grau de Mestre em Bioenergia

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ABSTRACT

Food wastes are essentially the organic material discarded, lost or degraded during the process of food processing or consumption and represent almost a third of all food produced.

The present work aims to optimise the valorisation of food wastes in order to produce bioplastics which is an interesting way to valorise this waste. The polyhydroxyalkanoates (PHA) are biopolymers naturally produced by microorganisms that is considered an important alternative to conventional fossil-based plastics since they have similar properties. However, PHA production costs still high, mainly due to the use of pure cultures and costly substrates.

The use of mixed microbial cultures and low-cost renewable feedstocks contribute to lower the PHA production costs. This process is divided in three steps: substrate acidogenic fermentation, culture selection and polymer accumulation. Acidogenic fermentation convert the food waste into volatile fatty acids that are used as substrate for the selection phase. Here, PHA accumulating bacteria are cultivated under selective conditions and accumulation is used for those selected bacteria producing the maximum PHA possible.

The following work focus on the first step, i.e. studying the impact of optimal operation conditions in terms of pH and dissolved hydrogen concentrations at acidogenic stage on fermentation products profiles. The variation of those profiles induces changes in the final bioplastic which ultimately could lead to new strategies to control the process in order of targeting a bioplastic with specific physical/thermal characteristic.

Keywords: Acidogenesis; upflow anaerobic sludge blanket (UASB); volatile fatty acids (VFAs); Hydrogen; mixed microbial consortia (MMC); polyhydroxyalkanoates (PHA)

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RESUMO

Os resíduos alimentares são essencialmente o material orgânico descartado, perdido ou degradado durante o processo de produção ou consumo de alimentos e representa quase um terço de todos os alimentos produzidos.

O presente trabalho tem como objetivo otimizar a valorização de resíduos alimentares, a fim de produzir bioplásticos, sendo esta uma interessante forma de valorizar esses resíduos. Os poli-hidroxialcanoatos (PHA) são biopolímeros produzidos naturalmente por microrganismos e são considerados uma alternativa importante aos plásticos convencionais de origem fóssil, pois possuem propriedades semelhantes. No entanto, os custos de produção de PHA ainda são altos, principalmente devido ao uso de culturas puras e substratos caros.

O uso de culturas microbianas mistas e matérias-primas renováveis de baixo custo contribui para reduzir os custos de produção de PHA. Esse processo é dividido em três etapas: fermentação acidogénea do substrato, seleção da cultura e acumulação de polímero. A fermentação acidogénea converte os resíduos alimentares em ácidos gordos voláteis que são usados como substrato na fase de seleção da cultura. Aqui, as bactérias acumuladoras de PHA são produzidas em condições seletivas e a acumulação é usada para as bactérias selecionadas para produzir o máximo de PHA possível.

O presente trabalho é focado na primeira etapa estudando o impacto das condições ideais de operação em termos de pH e concentrações de hidrogénio dissolvido nos perfis de produtos de fermentação. A variação desses perfis induz mudanças no bioplástico final, o que pode levar a novas estratégias para controlar o processo, a fim de atingir um bioplástico com características físicas / térmicas específicas.

Palavras-chave: Acidogénese; reator anaeróbio de fluxo ascendente; ácidos gordos voláteis (AGVs); hidrogénio; consórcio microbianos mistos (CMM); polihidroxialcanoatos (PHA)

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(C/N)	Carbon to nitrogen ratio
(COD)	Chemical oxygen demand
(DNA)	Deoxyribonucleic acid
(EGSB)	Biobed expanded granular sludge blanket
(Fd)	Ferredoxin
(FP)	Fermentation products
(FW)	Food waste
(H_2)	Molecular hydrogen
(HB)	Hydroxybutyrate
(HRT)	Hydraulic retention time
(HV)	Hydroxyvalerate
(MMC)	Mixed Microbial Consortia
(NADH)	Nicotinamide adenine dinucleotide
(OLR)	Organic loading rate
(OUT)	Operational taxonomic unit
(PHA)	Polyhydroxyalkanoates
(PHB)	Polyhydroxybutyrate
(PMF)	Proton-motive force
(SBR)	Sequencing batch reactor
(UASB)	Upflow anaerobic sludge blanket
(UFV)	Upflow velocity
(VFA)	Volatile fatty acids

1. Motivation

Food waste (FW) is nowadays a common type of waste found in municipal solid waste and represents one third of it (Elbeshbishy, Hafez, Dhar, & Nakhla, 2011). FW contains uneaten food and discarded food products during the stage of production, processing, retailing and consumption. The development of the economy and society, which is associated with an increment of population, generate every year 1.3 billion tons of municipal solid wastes and is expected to increase up to 2.2 billion tons in 2025 (Han, Fang, Liu, & Tang, 2016). Without treatment FW can cause the contamination of air, water and soil due to its odour, leachate and quick increment of microorganisms.

At the present, most of the FW is sent to landfills and incineration plants as final disposal points as well as a more traditional approach where FW is reutilized to feed animals and composted to produce fertilizers. However, those methods have some disadvantages, including the possibility of animals infections with untreated FW, the occupation of large quantities of valuable soil (Yan, Selvam, & Wong, 2014), the high disposal costs estimated as 88-144 US$ per ton (Pfaltzgraff et al. 2013) and the fact that

incineration is energy intensive and unstable due to the high moisture content of FW (Shen et al., 2013).

Traditional management solutions do not consider FW as a precious resource, until now, this waste only has been valued at 60-150 $ per ton as biomass for electricity production and 5-10 US$ per ton as biomass for compost production. However, it have a complex matrix of molecules such as starch, cellulose, hemicellulose, lignin, proteins, lipids and organic acids that potentially could be used in more sustainable economic ways, for example by being used for the production of bulk chemicals and bioplastics (Colombo et al., 2017; Uçkun Kiran, Trzcinski, Ng, & Liu, 2014).

In Europe, almost 87.6 million tons of FW are produced annually (FUSIONS, 2016), 38% of which is directly produced by the food manufacturing sector (Pfaltzgraff et al., 2013). European legislation have focused on avoiding FW landfilling by treating it through thermal processes (incineration) or by biological processes (anaerobic digestion and composting) using a separately collected organic fraction of municipal solid waste (Girotto, Alibardi, & Cossu, 2015).

This work is focus in apple pulp residues supplied by a Portuguese company (Sumol+ Compal Marcas S.A.) operating in food industry. This company works with different types of fruits and use them as base for juice production. The fruit is every year received by Sumol+Compal during every fruit harvesting season and processed in order to convert it to pulp and concentrates. This pulp cannot be all used at once to produce juice, because the production line is not capable to handle such a big amount of raw materials.

Sumol + Compal in order to manage the production level and to adjust it to the demands of the market, usually, store the excess of pulp in hermetic closed barrels until needed to a maximum of two years. However, by many different reasons, some of the containers had contaminations or leaks, which degrade the pulp and make it unusable for food application, resulting annually in more than 250 tons of pulp that had to be discarded and treated.

The present work aims to optimise the process of valorisation of these residues in order to produce bioplastics which is an interesting way to valorise this waste. The

chosen bioplastic will be composed by polyhydroxyalkanoates (PHA). Those are biopolymers naturally produced by microorganisms and are considered an important alternative to conventional fossil-based plastics since they have similar properties (Reis, Albuquerque, Villano, & Majone, 2011). The fact of being completely biodegradable and biocompatible makes PHA interesting to industry, however the high cost associated to production has been a barrier to expand its application to large scale. The fact of being usually produced using pure cultures and costly substrates, make the final PHA selling price very high, which is the main reason for failing in compete with conventional plastics.

There is a need for a better understanding about the first step in PHA production process, the acidogenesis using Mixed Microbial Consortia (MMC) and agro-industrial residues from a food industry as substrate. PHA production starts with a fermentation step, which produce volatile fatty acids (VFAs), the substrate used at the selection reactor where the MMC responsible to produce PHA grow. The final step is accumulation, where those bacteria are fed to its maximum capacity of producing/storing PHA.

This work pretends lower the costs by increasing the process productivity of acidogenic fermentation, by creating new operational strategies in order to control the composition and quantity of VFAs, which are used to produce biopolymers. Being able to predict and produce different VFAs compositions, will allow the production of biopolymer with different physical and chemical characteristics, since VFAs composition have a direct impact in monomers composition that ultimately affect the composition and characteristic of the final polymer.

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2. Literature review

2.1. FOOD WASTE

2.1.1. THE OPPORTUNITY SIZE

Nowadays, our society face problems such as resource depletion and waste accumulation, leading to escalation of raw material costs and increasingly expensive and restrictive waste disposal legislation. Most of the carbon-based compounds currently manufactured by the chemical industry derive from petroleum. The reduction of this resource leads to an increase of the price and makes crucial to find different sources of raw materials (Tuck, Pérez, Horváth, Sheldon, & Poliakoff, 2012).

A significant amount of FW comes from food supply chain, essentially by the organic material produced for human consumption that is discarded, lost or degraded primarily at the manufacturing and retail stages (Pfaltzgraff et al., 2013). The variety of food processes used in the food and drink industry make it inefficient, leading to a globally generation of wastes on a multi tonne scale every year (Pfaltzgraff et al., 2013).

In order to mitigate those problems, the research has been focused in new processes of biorefinery as it has an important role in the new concept of circular economy. European Union produces each year almost 90 million tonnes of FW, with 38% being directly produced by the food manufacturing sector (European Commission & Report, 2010). Food and Agriculture Organization of the United Nations estimated that every year globally, one third of the food aimed for human consumption ends up wasted (Blakeney, 2019).

The interest in wastes leads to new opportunities, with many new businesses starting to approach the use of these wastes. Is noticeable its potential when in 2012 begins a commercially viable venture that ships annually around 200,000 tonnes of household waste from Italy to Rotterdam to be used as feedstock for electricity generation in Dutch power plants with overcapacity.

2.1.2. A RESOURCE FOR HIGH ADDED-VALUE PRODUCTS

Most of wastes composed by biomass, such as food, wood, animal and agricultural wastes are generally composed by a complex and variable mixture of molecules and separation becomes a key issue due both of biological wastes and contaminants/extra-materials to be separated. Therefore, separation frequently involves organic solvents.

There is also known that FW contains or can be converted in lots of valuable molecules such as flavonoids, waxes, biopolymers, fatty acids and depending on the

efficiency, exists potential to explore the extraction of fine chemicals and other applications as shown in Figure 2.1.
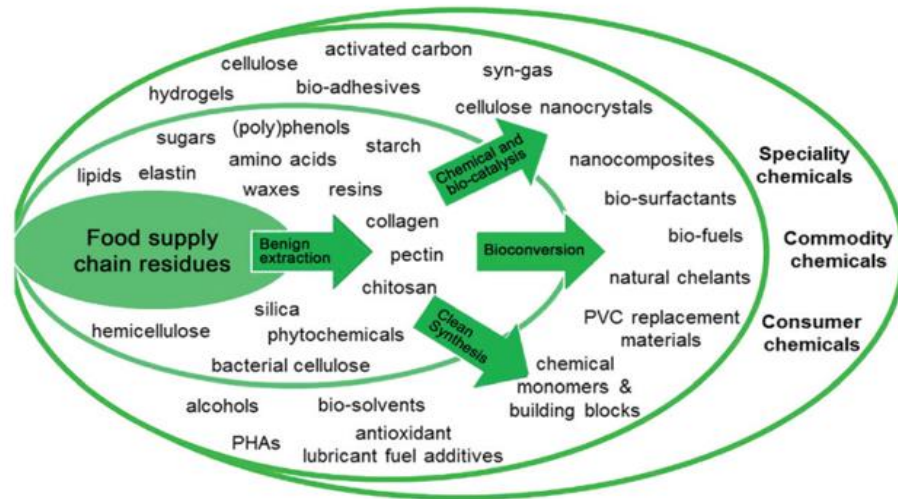
Figure 2.1 - Components present in food waste and their uses in common consumer applications, highlighting sectors of the chemical industry that could benefit from the use of such a renewable resource (Pfaltzgraff et al., 2013).

The direct use of FW for electricity generation, fuel production and animal feed, somehow represents a lost or underutilization of these chemical functionalities. As can be seen in Table 2.1, it was stated that the conversion of biomass waste to bulk chemicals, for example, was nearly 7.5 and 3.5 times more profitable than conversion to animal feed or transportation fuel respectively.

Table 2.1 - Approximate value and cost for different biomass applications (Tuck et al., 2012).

	Value (US$/t biomass)
Average bulk chemical	1000
Transportation fuel	200–400
Cattle feed	70–200
Generating electricity	60–150
	Cost (US$/t biomass)
Landfill	400

2.2. PHA – POLYHYDROXYALKANOATES

During many years, the plastic industry has been growing more than any other group of bulk materials and represents the third largest application for crude oil after the energy and transport sectors. The non-biodegradable nature of conventional plastic, due to high molecular mass and complex structure, represents nowadays one of the most visible and harmful type of environmental pollution with direct impact in water and soil quality. It is also the death cause to countless animals that get stuck in plastic residues or mistakenly ingesting it.

Concerns over the harmful effects caused by conventional plastics drive global scientific research to focus in bioplastics and biodegradable polymers as optional substitutes (Raza, Abid, & Banat, 2018; Reis et al., 2011).

2.2.1. DEFINITION AND CHARACTERISTICS OF PHA

Lemoigne, a French scientist first discover the biopolymer PHA, in *Bacillus megaterium* in 1925 (Chee, Yoga, Lau, Ling, & Abed, 2010), meanwhile there were found many more PHA bacteria producers (more than 90 genera), Gram-positive and Gram-negative under both aerobic and anaerobic conditions (Kim, Hyung, Moon, & Young, 2007).

PHA is a polyester composed by ester groups as part of the molecular chain. The biological synthesis is made by microbial fermentation that produces these biopolymers as lipid inclusions for energy storage in granular forms within the cytoplasm. Those granules range in size from 0.2 to 0.5 μm, with the core of polyester surrounded by either phospholipids or proteins as shown in Figure 2.2.
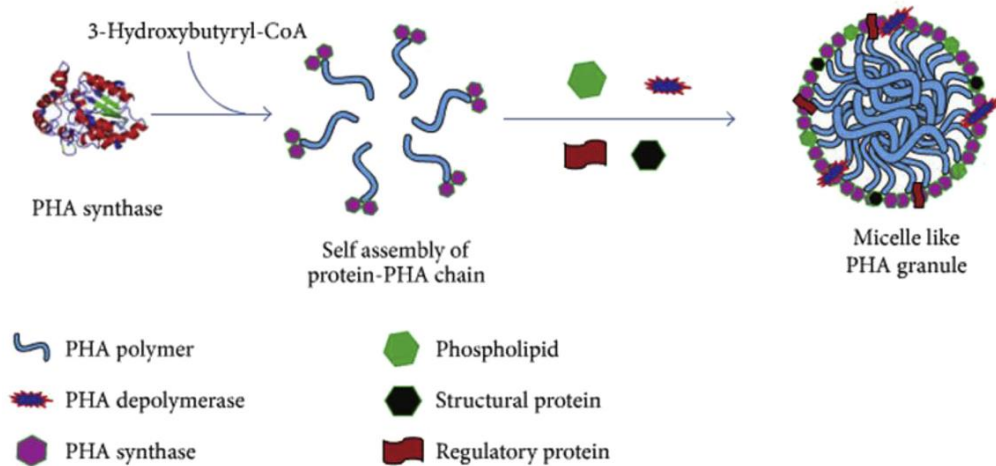
Figure 2.2 - PHA granule formation in microorganisms (Pakalapati, Chang, Show, Arumugasamy, & Lan, 2018).

PHA producer bacteria can be distinguish in two different groups, the first one composed by bacteria that requires a nutrient limitation such as phosphorus, nitrogen, oxygen or magnesium to accumulate PHAs and do not accumulate it during the growth phase, while the second group is composed by bacteria that do not require nutrients limitation and accumulate PHAs during the growth phase (Muhammadi, Shabina, Afzal, & Hameed, 2015).

2.2.2. CLASSIFICATION AND STRUCTURE OF PHA

PHA can be classified according to the number of carbons in the side chains. Short, medium or long chain length (scl, mcl and lcl, respectively). The scl-PHAs have less than 5 carbon atoms, mcl-PHAs have between 5 and 14 carbon atoms and lcl-PHAs have more

than 14 (Kunasundari & Sudesh, 2011). Those type of structures are represented in Figure 2.3 except for lcl-PHA which is less common and less studied.
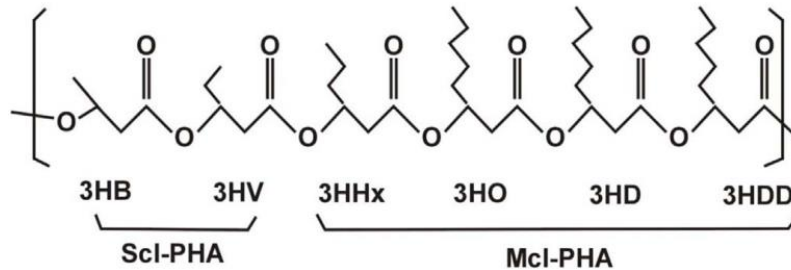
Figure 2.3 - Structure of PHAs with respect to classification, where 3HB=3-hydroxybutyrate, 3HV=3-hydroxyvalerate, 3HHx=3-hydroxyhexanoate, 3HO=3-hydroxyoctanoate, 3HD=3-hydroxydecanoate and 3HDD=3-hydroxydodecanoate (Raza et al., 2018).

The main general structure is shown in Figure 2.4, which can have more than 150 different congeners of PHA (B. Zhang, Carlson, & Srienc, 2006). This monomers can be straight, branched, saturated, unsaturated and also aromatic structures (Witholt & Kessler, 1999).
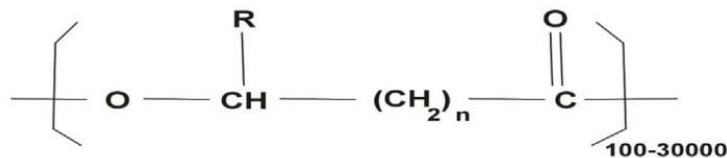
Figure 2.4 - General structure of polyhydroxyalkanoates as reported by Ojumu (Ojumu, Yu, & Solomon, 2004).

2.2.3. FERMENTATION PROCESS

The conditions required to PHA biosynthesis are an important factor to develop cultivation techniques useable in large scale production. Batch and fed-batch fermentations are commonly used in industrial fermentations processes, with fed-batch, allowing a better control of substrate and nutrients concentrations inside the reactor. This helps preventing substrate inhibition, resulting in better control, which is usually

translated in a higher cell concentration and more productivity in terms of products. However, fed-batch results in long downtime between batches, which increase the operational costs (Yu, 2007).

A two-stage cultivation method is the most employed technique initially adopted by ICI to produce P(3HB-3HV) at industrial scale (Byrom, 1987). This method is still used nowadays without big changes. The first stage is where bacteria are cultivated until achieve a pre-determined cell mass concentration, without applying nutrients limitation.

The cultivated cells move then to the second stage where occurs a feeding phase with carbon substrate that is limited in phosphorus and nitrogen. This condition induces cells to produce PHA as much as possible and is known as accumulation step, followed by a step of extraction and purification of the produced biopolymer.

2.2.4. MIXED MICROBIAL CONSORTIA (MMC) FOR PHA PRODUCTION

Generally, the use of monoseptic cultures to produce PHA is associated to high operational costs. Dispensing major sterility conditions and using less expensive equipment and control, MMC can be adapted to use inexpensive and complex substrates, such as industrial and domestic wastes maintaining the higher capability to accumulate PHA.

Nowadays, there is an increasing effort in developing the use of MMC instead of monoseptic processes using pure cultures. Generally, MMC approach a common nature principle of selection and competition, imposing what can be called "evolutionary engineering". Instead of resorting in genetic or metabolic engineering, this approach benefits from selective pressure on a desired metabolism of a microbial consortium by imposing appropriate feeding and cultivation conditions in the bioreactor, letting the system to be engineered (Reis et al., 2011; Rhu, Lee, Kim, & Choi, 2003).

As mentioned before, PHA is a bacterias' reserve compound that is naturally produced under specific conditions in a cyclic feast-famine regime. These regimes consist in repeatedly alternating between abundance (feast phase) and absence of substrate (famine phase). During the famine phase, the microorganisms that cannot store carbon

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and energy reserves end up dying due to the lack of feed, which ultimately leads to an efficient process to select PHA-storing microbial populations. Only microorganisms naturally prepared to survive these harsh conditions, can prosper on the reactor. The microbial culture that ends up remaining on the process will be greatly composed by PHA-accumulating bacteria, because during the famine phase, these bacteria fall back on its PHA reserves to stay alive.

Figure 2.5 shows pictures captured with a scanning electron microscope (SEM) that shows bacteria selected under feast and famine regimes. At the right side after being appleyd a cut it is shown the PHA granules inside the bacterium.
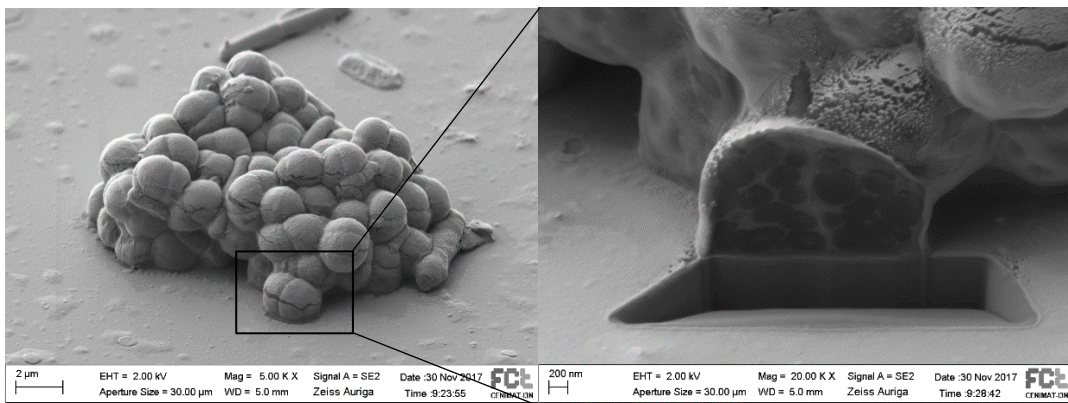
Figure 2.5 – PHA-accumulating bacteria on left side and PHA granules on the right, observed under scanning electron microscope.

Despite the fact this approach using MMC shows promising results, it cannot simply substitute the production using pure cultures, because MMCs consists of different PHA-producing species and each of them accumulate PHAs with different molar masses, distinct molar distribution, crystallinity and monomeric composition, which do not allow the production of highly uniform PHA required for fine applications, such as medical uses. This diversity in PHA composition is however described to be more associated with differences in feedstock than properly on biodiversity variations inside the selected MMC (Koller, Maršálek, de Sousa Dias, & Braunegg, 2017).

The physical and mechanical properties of the polymer depend directly on their monomer composition. For instance, the polyhydroxybutyrate (PHB) is a very brittle

polymer and crystalline with limited final applications. As can be seen in Table 2.2 more flexible polymers can be obtained by incorporating other monomers, such as hydroxyvalerate (HV), in the polymer chains, enlarging the range of possible applications.

Table 2.2 - Comparison of polymer properties due to its monomers composition (S. Y. Lee, 1996).

Polymer	Melting temp. (°C)	Young's modulus (GPA)	Tensile strength (MPa)	Elongation to break (%)	Notched izod impact strength (J/m)
P(3HB)	179	3.5	40	5	50
P(3HB-co-3HV)					
3 mol% 3HV	170	2.9	38	-	60
9 mol% 3HV	162	1.9	37	-	95
14 mol% 3HV	150	1.5	35	-	120
20 mol% 3HV	145	1.2	32	-	200
25 mol% 3HV	137	0.7	30	-	400
Polypropylene	170	1.7	34.5	400	45
Polystyrene	110	3.1	50	-	21

This can be accomplished by using mixtures of VFA containing acetate, propionate, butyrate and valerate rather than single carbon sources. In mixed culture PHA production, acetic and butyric acids favour the production of the monomer 3-hydroxybutyrate (3HB) whereas propionic and valeric acids promote the synthesis of 3-hydroxyvalerate (3HV) (Lemos, Serafim, & Reis, 2006). Other organic acids, such as lactate is described as favouring the production of HB (Jiang, Marang, Kleerebezem, Muyzer, & van Loosdrecht, 2011). Ethanol, an alcohol commonly produced in fermentation, shown a trend to be used as 3HB precursor due some previous tests realized by BIOENG group.

However, at the time of publication of this work, it was not found any conclusive report about the role of ethanol in PHA production.

Related to substrates, a MMC capable to produce PHA can be fed with pure substrates as acetate, which achieve good yields and specific productivity, however, complex mixtures as wastewater, food waste, sugar cane molasse or oils effluents are also been used, resulting in lower yields that still need to be optimized. These mixtures can be directly applied to the PHA-producing reactor or firstly fermented to VFAs. However, most wastes and feedstocks contain several organic compounds that are not equally suitable for PHA productions. Carbohydrates such as glucose, starch or cellulose end up being used to produce glycogen instead of PHA which affects the process lowering the PHA production.

Since the PHA composition is highly ruled by the VFA profile used as substrate and the microbial population present, which, in turn, is also affected by the feed composition, this will require a better control in the fermentation phase, because the polymer has to fulfil the requirements for envisaged the typical processing techniques as melt extrusion, film blowing or injection moulding.

2.3. ANAEROBIC DIGESTION

Anaerobic Digestion (AD) is a multi-stage biological process for decomposition and stabilization of organic matter in absence of molecular oxygen. AD involves a variety of microorganisms such as the hydrolysing and acid forming community, syntrophic oxidizers and methane generating archaeal community. All types of microorganism present in AD reactors have their own specific physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Yuchen Liu & Whitman, 2008).

The AD process could be divided in four different stages, beginning with the microbial hydrolysis, hydrolytic bacteria and fungi break down insoluble complex molecules such as proteins, fats, carbohydrates and other biodegradable polymers, resulting in a release of amino acids, fatty acids and sugars. Hydrolytic bacteria are

phylogenetically diverse but mostly are from two phyla, *Bacteroidetes* and *Firmicutes* (Venkiteshwaran, Bocher, Maki, & Zitomer, 2015).

Hydrolysis is followed by acidogenesis where fermentative bacteria transform sugars and other monomeric organic products into organic acids, alcohols, carbon dioxide, hydrogen and ammonia. Most species of acidogenic bacteria belong to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi* and *Actinobacteria*. Under these phyla, can be commonly identified different genera, *Clostridium*, *Bacillus*, *Bacteroides*, *Proteiniphilum*, *Desulfovibrio*, *Geobacter*, *Chloroflexus* and *Mycobacterium* (Cai, Liu, Li, Gao, & Qin, 2016).

The third step of AD is acetogenesis. While acetate, formate, methyl compounds, hydrogen and carbon dioxide can be directly used by methanogenic microorganisms, other compounds such as butyrate, propionate, lactate and ethanol produced in the acidogenesis step, need further biodegradation by a group of syntrophic bacteria. Syntrophic acetogens usually include *Syntrophobacter*, *Pelotomaculum* and *Smithella* associated with propionate degradation, *Syntrophus*, *Syntrophomonas* and *Syntrophothermus* responsible for the oxidation of butyrate and other fatty acids (Cai et al., 2016; Venkiteshwaran et al., 2015). Syntrophic acetogenesis is thermodynamically unfavourable under standard conditions, depending on a partnership with methanogens, which maintain a low hydrogen (H_2) partial pressure (p_{H2}) and reduce the concentration of acetate and formate (Stams & Plugge, 2009).

Methanogenesis is the last step in AD and is composed by four main pathways for methane (CH_4) production. Acetoclastic methanogens use acetate to produce CH_4 and carbon dioxide (CO_2); hydrogenotrophic methanogens utilize H_2 or formate to reduce CO_2 to CH_4; methylotrophic methanogens metabolize methyl compounds to produce CH_4; lastly, syntrophic partnerships of acetate-oxidizing bacteria and hydrogenotrophic methanogens convert acetate to CH_4 using H_2 and CO_2 (Leng et al., 2018).

Hydrogenotrophic methanogens are essential to promote electron flow in the AD, being able to scavenge H_2/Formate to low levels and promote syntrophic acetogenesis.

2.3.1. UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR

A commonly reactor used in AD process is the upflow anaerobic sludge blanket (UASB) reactor. It has been widely used as a wastewater treatment technology, with estimated use of 80% of overall anaerobic wastewater treatment (Lettinga, van Velsen, Hobma, de Zeeuw, & Klapwijk, 1980). Its applications are in the treatment of industrial wastewater, essentially with contaminants such as alcohols, carbohydrates and aromatic pollutants (H. H P Fang, Liang, Zhang, & Liu, 2006; Li, P. Fang, Chen, & Chui, 1995).

As schematically represented in Figure 2.6, a UASB reactor consists of a built-in gas-liquid-solid separator able to retain the biomass that is capable of aggregate in granules due to the long solid retention times (H. H.P. Fang, 2000).
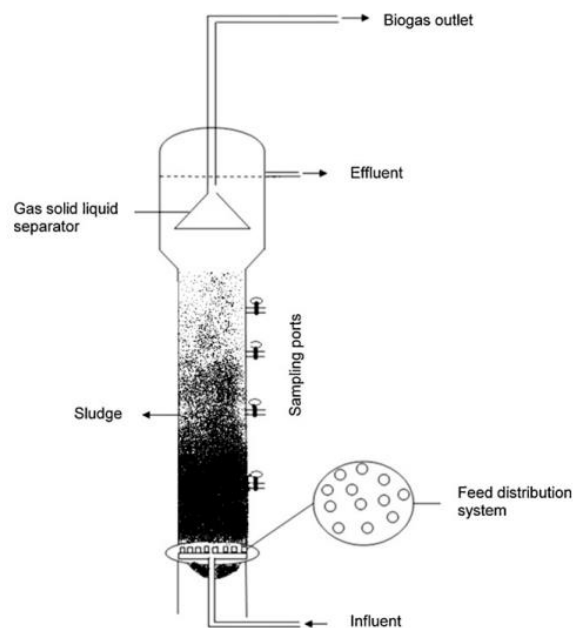
Figure 2.6 - Schematic of an upflow anaerobic sludge blanket reactor (Abbasi & Abbasi, 2012).

Biogranules have some big advantages over suspended biomass in mixed liquor. A sludge bed of biogranules may contain a higher concentration/density of active biomass, reaching concentration levels of 50g/L of volatile suspended solids (VSS)

(Herbert H.P. Fang & Chui, 1993). Biogranules also settle better, due to their larger sizes/mass, a very important factor to prevent a wash out of the biomass, which would lead to a reactor failure. This better settling property allow higher hydraulic loading rates, and a better separation between the liquid and the solid phase, therefore enable higher process efficiency.

Compared to disperse microorganisms, granules can withstand high gas and liquid shear stress without disintegrating and provide a higher resistance to toxins and disturbances in the process (Schmidt & Ahring, 1996)

The formation of sludge biogranules is important to support active biofilms and also provide the buoyancy and the settleability necessary to enable very vigorous granule–liquid contact in the reactor which also reduce inter-species mass transfer limitation between syntrophic groups (Abbasi & Abbasi, 2012). In UASB reactors, the granule formation slowly happens when the reactor, operated under appropriate conditions, is seeded with anaerobic sludge and fed with an organic substrate with a flow in upward direction through the sludge (Show, Wang, Foong, & Tay, 2004).

The use of granular sludge reduces the amount of biomass in the liquid effluent (Parawira, Murto, Zvauya, & Mattiasson, 2006), which is important when exists a need to use it in a following step, for example to be fed as substrate for PHA production process.

2.3.2. MORPHOLOGY OF BIOGRANULES

Biogranules are dense particles normally about 1-3 mm in size, consisting in a mixture of symbiotic anaerobic microorganisms (H. H.P. Fang, 2000). A typical granule has millions of organisms per gram of biomass, and each granule is a functional unit itself, capable of degrade organic matter. Due to its composition in different microorganisms, granules are very useful to degrade/treat complex organic mixtures (ex: wastewater), which is not possible for individual species of micro-organism (Zheng, Angenent, & Raskin, 2006). This microbial diversity allows the operation of bioprocesses under non-sterile conditions with no risk of strain degeneration.

A layered structure is commonly proposed to describe the structure of a common anaerobic biogranule. As shown in Figure 2.7, the granule has a central core of

acetoclastic methanogens, surrounded by a layer of hydrogen- or formate-producing acetogens and hydrogen- or formate-consuming methanogens. The external layer is composed by bacteria that hydrolyse and acidify complex organic matter (Guiot, Arcand, & Chavarie, 1992; Yu Liu, Xu, Yang, & Tay, 2003).
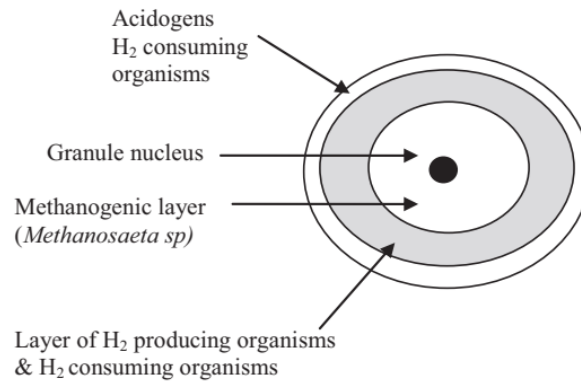
Figure 2.7 - The proposed layered structure of UASB granules. Adapted from (McHugh, Carton, Mahony, & O'Flaherty, 2003).

2.3.3. ROLE OF SPECIFIC FACTORS IN THE FORMATION, MAINTENANCE AND PERFORMANCE OF GRANULES

AD is a very complex process that proceeds through the interactions of many biotic (microbial community) and abiotic (reactor parameters) factors. Many of these parameters are crucial and any deviation from its optimal levels can cease the whole AD process.

The parameters that have a major impact in AD reactors are pH, temperature, organic loading rate (OLR), hydraulic retention time (HRT), carbon to nitrogen (C/N) ratio, alkalinity and concentration of VFAs. Furthermore, there are also some material and conditions that can cause inhibitory effects on the process (Neshat, Mohammadi, Najafpour, & Lahijani, 2017).

PH

The pH has a direct impact on AD performance. Usually a stable pH value and a high partial pressure of hydrogen are required to maintain a good quality granulated

sludge (González, Rivera, Borja, & Sánchez, 1998). Compared to methanogens, that require a short pH range of 5.5-8.2, acidogenic bacteria are significantly less sensitive to pH variations. When pH is low, they continue producing VFAs, which results in accumulation of VFAs inside the reactor. When the concentration of VFAs exceeds the buffering capacity of the reactor content, the pH value starts to descend, and methanogens gradually die. This process can be used as a strategy to select acidogenic granules using common anaerobic biogranules from biogas production reactors.

The AD requires enzymatic reactions performed by many different microorganisms, which use diverse enzymes that requires different pH ranges to optimum performances and any significative fluctuation may stop the reaction or change the metabolic pathway (Rico, Muñoz, Fernández, & Rico, 2015). Those metabolic pathways are described further at chapter 2.4.

ALKALINITY

Alkalinity is defined as the capacity of an aqueous solution to neutralize acids. Substrates with high alkalinity can maintain the pH level when the increase of acids in solution occurs. The pH disturb results essentially from the formation of carbonic acid due to the production of carbon dioxide in the fermentation process and VFAs production.

Alkalinity together with organic load influences granulation. Alkalinity levels ranging between 5 and 19 mEq/L are considered optimal for granules formation and stability (Singh, Kumar, & Ojha, 1999).

Some chemicals can be used to maintain the pH stable between certain values, such as sodium bicarbonate ($NaHCO_3$), lime (CaO) and sodium carbonate (Na_2CO_3), however the use of these chemicals has some side effects. Ionization of these chemicals and production of certain ions, such as Na^+ at high concentrations can inhibit AD and cause the decay of the whole process.

Temperature is one of the most influential parameters in anaerobic digestion performance and stability. AD can occur in different temperature regimes, such as mesophilic condition, thermophilic condition, ambient temperature and psychrophilic condition. This last condition is less studied and normally represent temperatures between -20 °C and 10 °C.

AD process is very sensitive to temperature fluctuation, because temperature interferes directly in metabolic activity of organisms and changes properties such as gas transfer rate. Hydrolytic bacteria, which are responsible for degradation of complex materials, are also affected by temperature variations.

Thermophilic conditions (> 41 °C) can enhance the performance of AD due to higher solubility of organic compounds, higher chemical and biochemical reaction rates, lower solubility of gas in liquid and lower liquid viscosity (Buhr & Andrews, 1977). However, higher temperatures increase the inhibitory effect of ammonia and VFA by increasing and decreasing their pKa, respectively (Angelidaki & Ahring, 1992, 1993). In order to maintain such high temperature, thermophilic regimes have associated higher operational costs.

Mesophilic conditions (25-40 °C) allow satisfactory operating performance, stability and less sensitivity to inhibitors. This condition allows a more stable operation than under ambient temperature. In comparison to thermophilic regime, mesophilic condition needs less energy and can enhance process stability, though it is less efficiency in degradation of phenolic compounds, minimizing phenolic inhibition (Poirier & Chapleur, 2018; Westerholm, Hansson, & Schnürer, 2012).

HYDRAULIC RETENTION TIME

Hydraulic retention time is a major factor in both AD and waste/wastewater treatment. All bioprocesses need an appropriate HRT, this should be determined for each system/microorganism because different microorganisms have different substrate consumption and bioproducts production rates. These rates vary with the type of substrate, microorganism and operational conditions applied to the reactor. Low HRT

can result in substrate accumulation causing inhibition or wash-out of biomass when the culture suffers erosion and cannot grow as much as the quantity of biomass that is being removed from the system. Long HRT can lead to a shortage of nutrients which can result in the microorganisms death (Salminen & Rintala, 2002; Shyue Koong Chang & Schonfeld, 1991).

ORGANIC LOADING RATE

Organic loading rate (OLR) represents the amount of dry organic solids or amount of organic matter present in water (determined by chemical/biochemical oxygen demand), which is loaded per volume of system per time. Low OLR may starve the microorganisms and cause mass transfer limitation leading to disintegration of the granules. On opposite, high OLR can increase activity of hydrolysing and acidogenic bacteria compared to methanogens in AD, resulting in decrease of pH and methanogenic activity. In acidogenic reactors this condition could also lead to the accumulation of unconsumed substrate in the reactor which if too high, may cause inhibition. The optimum range of OLR depends on the strength of the microbial culture, composition of the substrate and type of process. Nutrients concentration, temperature and HRT also are parameters that had to be considered and affect the optimal OLR for a system (Neshat et al., 2017).

UPFLOW VELOCITY AND EFFECT OF GAS FORMATION

The upflow velocity (UFV) of influent and superficial velocity of biogas could have an impact on granules. UFV above 1 m/h, may disintegrate the granules due to shear stress and the eventual loss of the fragments of granules being washed-out, leading to a decrease in total volume of granules in system. The production of high amounts of gas at high OLR can shear-off bacterial cells from the granule surface, causing its erosion. Due to these factors, UASB reactors usually operate under 1 m/h of UFV (Syutsubo, Harada, Ohashi, & Suzuki, 1997).

The concentration and type of substrate suffers different consumption rates due to intra-granular diffusion and could cause impact in granular structure with easily hydrolysable noninhibitory substrates at higher concentration such as, glucose, proteins, sucrose and brewery wastes increasing the hydrolytic and acidogenic bacteria in the external layers of the granules (Guiot et al., 1992).

Low concentration of substrate results in lower production of gas which allow the intra-granular pore network to be unobstructed with gas. Consequently, these pores network are easily penetrated by substrate, increasing transfer of substrate to the core of the granule. This has an impact in large granules (up to 3 mm) at substrate limitation since it decreases their flotation due to the lower gas content inside, reducing mass-transfer of substrate (Teer, Leak, Dudeney, Narayanan, & Stuckey, 2000).

Substrates rich in fats, oil and grease are generally difficult to consume, these constituents tend to accumulate on the surface of the granules resulting in a superficial scum layer. This coating decreases the substrate transfer and can lead to a wash-out of biomass. The formation of a layer rich in lipids, can produce a hydrophobic environment which may also reduce the diffusion of water-soluble substrate into the granules (Rinzema, Boone, van Knippenberg, & Lettinga, 1994).

NITROGEN AND PHOSPHOROUS

Microorganism need carbon and nitrogen in any growth environment. Excess of nitrogen and phosphorus are reported as helpful in granule formation during the start-up of UASB reactors and can be reduced after the granules formation without negative impacts on granular structure (González et al., 1998).

The optimal ratio between C/N is described as 15-30 (Kayhanian, 1999). Deviations from this range affects AD performance. High C/N ratios cause deficiency of nitrogen which is essential for microbial growth, concentrations of nitrogen under 300 mg/L could cause massive reduction in cells growth (Singh et al., 1999). Low C/N ratios could also limit the microbial growth due to lack of carbon which leads to accumulation of ammonia nitrogen in the reactor (Kayhanian, 1999; Parkin & Owen, 1986).

Unconsumed ammonia during acidogenic step could affect the production of PHA due to C/N variations in the produced VFAs solution. Some strategies of PHA production requires specific C/N ratios and high deviations will destabilize the process (Silva et al., 2017).

MULTIVALENT CATIONS

Metals have impact on granule formation due either to its presence or absence (Fermoso, Bartacek, Manzano, van Leeuwen, & Lens, 2010). UASB granules have high porosity and large internal surface area, where metals can be adsorbed. Metal can be adsorbed at specific sites or lodged as insoluble compounds which can be increased at low pH since it allows metals to became labile.

Carboxyl and amino groups in proteins can work as binding groups on bacteria surfaces where they bind with metals. The solubility of metals increases at pH lower than 5 and a prolonged exposure of granules to low pH may influence metal retention within granules and cause catalytic or inhibitory impacts depending on the concentration of labile metals (Artola, Balaguer, & Rigola, 1997).

Calcium (Ca) is one of the essential nutrients to the growth of several microorganism and can speedup granulation. It is a constituent of extracellular polysaccharides which may help adsorption and linking processes. When Ca^{2+} precipitates, it serves as inert support during granulation, since bacterial surfaces are usually negatively charged. As shown in Figure 2.8, the presence of divalent cations can neutralize the charge when serving as a link between cells and polysaccharides (Jackson-Moss, Duncan, & Cooper, 1989). Other metals such as Iron (Fe^{2+}) and aluminium (Al^{3+}) can also be used as binding agents, linking cell surfaces and extracellular polymers (Abbasi & Abbasi, 2012).

* Multi-valence positive　　　　　　　O Negatively charged bacterium
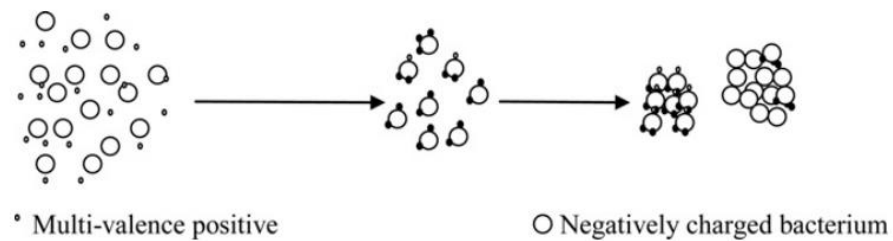
Figure 2.8 - Role of multivalent cations in granule formation (adapted from Yu Liu et al., 2003).

Iron is also required for hydrogenase, an important enzyme in fermentative hydrogen production. Iron-sulfur proteins can work as electron carriers and are involved in pyruvate oxidation to acetyl-CoA and carbon dioxide. This proteins reduce protons to form molecular hydrogen (Y. J. Lee, Miyahara, & Noike, 2001). Iron could also induce metabolic changes and is involved in the expression of Fe-S and non-Fe-S proteins related to hydrogenase (Vaňáčová et al., 2001).

2.4. METABOLIC PATHWAYS OF ACIDOGENIC BACTERIA

In acidogenic fermentation, numerous distinct pathways co-exist, carried out through a series of biochemical reactions catalysed by enzymes. As can be seen in Figure 2.9, pyruvate is a key molecule for the acidogenesis, as it can be converted into a wide range of products such as acetate, propionate, butyrate, ethanol, propanol, butanol, hydrogen and carbon dioxide (Chen, Luo, Yan, & Feng, 2013). The type of substrate has an impact in metabolic pathways and can lead to different pyruvate proportions.

The distribution of major soluble fermentation products can indicate the prevailing metabolic pathways. The most common pathways are acetate-ethanol type, propionate-type, butyrate-type, mixed-acid, lactate-type and homoacetogenic fermentation, represented in Figure 2.9.

The predominance of this pathways and the overall conversion efficiency are determined by the reactor conditions applied such as composition of substrate, inoculum, pH, temperature, system configuration, organic loading rate, hydraulic retention time and headspace hydrogen pressure (Zhou, Yan, Wong, & Zhang, 2018).
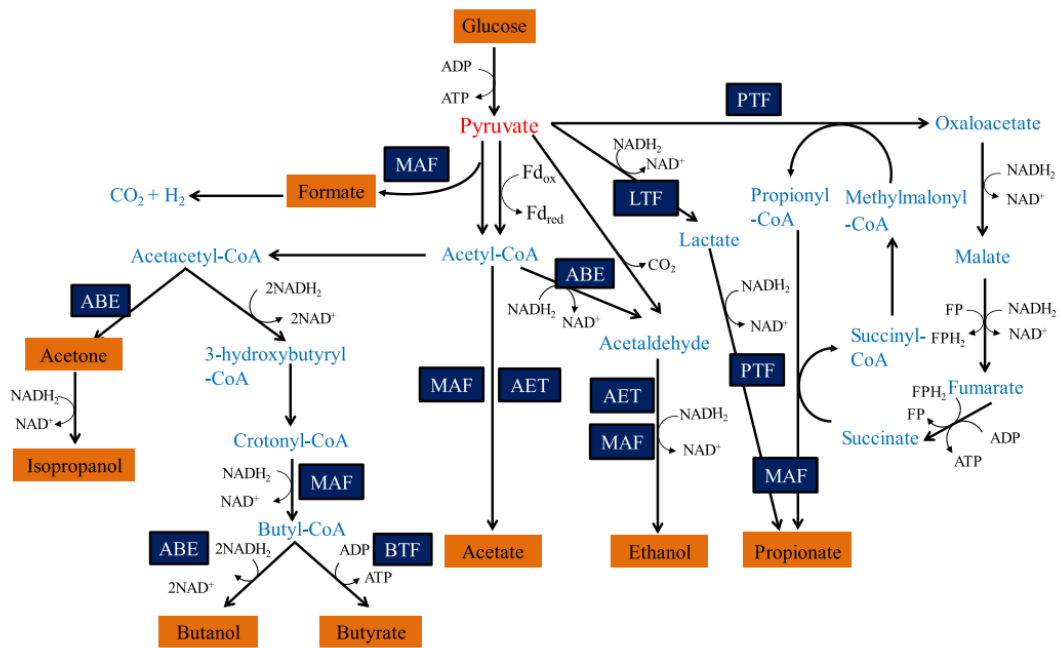
Figure 2.9 - Metabolic pathways of acidogenic fermentation. (AET, acetate-ethanol type fermentation; ABE, acetone-butanol-ethanol; PTF, propionate-type fermentation; BTF, butyrate-type fermentation; MAF, mixed-acids fermentation; LTF lactate-type fermentation) (Zhou et al., 2018).

ACETATE-ETHANOL TYPE METABOLIC PATHWAY (AET)

Acetate and ethanol are found as intermediates during acidogenic fermentation. They are commonly obtained during hydrogen production.

Acetate is an important component of carboxylic platform, being sourced from pyruvate through acetyl-CoA pathway or generated from syntrophic oxidation of ethanol or longer chain fatty acids. It is associated with functional enzymes present in each pathway (Müller, Worm, Schink, Stams, & Plugge, 2010).

Ethanol is commonly produced from glucose but it can also derived from other organic compounds like xylose and lignocellulosic materials (Govindaswamy & Vane, 2007; Sun & Cheng, 2002). The conversion of pyruvate to ethanol can be performed in two ways: a two-step process where pyruvate suffer decarboxylation to acetaldehyde and subsequent acetaldehyde reduction to ethanol (Bensaid, Ruggeri, & Saracco, 2015); or in three steps with acetyl-CoA and acetaldehyde as intermediates used by

Enterobacteriaceae (Chaganti, Kim, & Lalman, 2011). Production of ethanol could be enhanced under specific conditions like elevated hydrogen partial pressure in headspace, yet ethanol production is associated with low energy and carbon recovery rate due to loss of two carbons.

Theoretically, one mole of glucose could generate two moles of propionate, which is the main product of propionate-type pathway. However, usually microorganisms would ferment glucose to propionate together with acetate production (Zhu et al., 2009). Propionate can be produced by two different pathways, acrylate pathway and succinate or methylmalonyl-CoA pathway.

The first one comes from reduction of pyruvate with lactate as intermediate, it is converted to acryloyl-CoA, reduced to propanoyl-CoA and then to propionate. The first reaction is catalysed by lactate dehydrogenase and then lactate is reduced to propionate by propionate dehydrogenase. This reduction allows the cell to balance the anaerobic oxidation of lactate to acetate and carbon dioxide, which appears to be the primary source of ATP generation. The acrylate pathway for propionate production is applied by *Clostridium propionicum*, *Coprococcus catus*, *Clostridium homopropionicum*, *Megasphaera elsdenii* and *Prevotella ruminicola* (Dareioti, Vavouraki, & Kornaros, 2014a; H. S. Lee, Salerno, & Rittmann, 2008). The second pathway for propionate production can be done by acidogenic bacteria such as *Corynebacteria*, *Propionibacterium* and *Bifidobacterium* with transcarboxylase cycle. All the reactions of the succinate pathway are reversible. *Bacteroidetes* and several *Firmicutes* uses the succinate pathway via methylmalonyl-CoA for propionate production. While *Bacteroidetes* essentially use polysaccharides and peptides as substrates for this metabolic pathway, strains belonging to *Firmicutes* uses organic acids (Macy & Probst, 1979). *Selenomonas ruminantium* utilize carbohydrates to produce lactate, acetate and propionate. Some are also able to use lactate for growth. *Pelobacter propionicus* can consume ethanol to form propionate (Seeliger, Janssen, & Schink, 2002).

Butyrate and acetate are the main products in butyrate-type pathway followed by hydrogen production. Butyrate is synthesized by reduction and decarboxylation of pyruvate and acetate consumption. Begins with pyruvate conversion to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA is then converted to butyryl-CoA with acetoacetyl-CoA, 3-hydroxybutyryl-CoA and crotonyl-CoA as intermediates (Chaganti et al., 2011). Butyryl-CoA is catalyse by phosphotransbutyrylase or butyrate-kinase producing butyrate and by acetate CoA-transferase to produce acetate (Vital, Howe, & Tiedje, 2014).

During the process of butyrate production, there are consumption of two nicotinamide adenine dinucleotide (NADH) in order to reduce the intermediates. Compared to the process of acetate production, to produce butyrate is required extra two NADH consumption while to produce acetate NAD^+ is reduced to NADH. High production of acetate leads to surplus of NADH in the system leading to an increase of reducing equivalents that will trigger an increase of butyrate production (He et al., 2012).

MIXED-ACID METABOLIC PATHWAY (MAF)

Acidogenic microorganisms can ferment organic monomers to equal amounts of acetate, butyrate, propionate and valerate with possibility of release of carbon dioxide and molecular hydrogen. Mixed-acid metabolic pathway commonly produces acetate and butyrate as major metabolites. It is highly affected by operational conditions such as type of substrate, redox potential or pH. Reports indicates that pH lower than 4.5 enhance acetate, butyrate and ethanol production while pH higher than 6.5 leads to an increase of ethanol production and decrease in acids formation (Zhu et al., 2009).

LACTATE-TYPE METABOLIC PATHWAY

Lactate production can be divided in two types of fermentation: homolactate fermentation, characterized by a conversion of one glucose into two molecules of lactic acid; and heterolactate fermentation in which each glucose is converted to one lactic acid along with one molecule of ethanol and one of carbon dioxide (Castillo Martinez et al., 2013). At lactate-type pathway, pyruvate is produced by glycolysis, followed by a

catalysed conversion to lactate by lactate dehydrogenase (LDH), while NADH is oxidized to NAD^+.

Lactic acid can be produced from glucose or other organic compounds, converted by bacteria such as *Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus thermophilus*, etc

HOMOACETOGENIC FERMENTATION PATHWAY

Homoacetogens are a group of obligate anaerobes that can use H_2 as an electron donor to reduce CO_2 to acetate (Saady, 2013). Homoacetogenic fermentation pathway, also be called Wood-Ljungdahl pathway, is composed by two main branches, the methyl and carbonyl branches. Methyl branch begin with consumption of two reducing equivalents to produce formate by CO_2 reduction. Formate is then catalysed by successive enzymes such as formate dehydrogenase, methylene-H_4F reductase and metheyltransferase, which produce the methyl group. In this branch six reduction equivalents are consumed in order to produce the methyl group.

The second branch, carbonyl branch, starts with the reduction of CO_2 to carbon monoxide (CO) by acetyl-CoA synthase. This step requires the consume of two reducing equivalents. Then the methyl group and CO are converted to acetyl-CoA which is converted to acetate with ATP synthesis during catabolism or to cell carbon in anabolism (Guo, Trably, Latrille, Carrre, & Steyer, 2010; Siriwongrungson, Zeng, & Angelidaki, 2007).

2.5. BACTERIA CYTOPLASMIC MEMBRANE AND BIOENERGETICS

The cytoplasmic membrane is a barrier between the cytoplasm and the external environment which controls the movement of solutes into and out of the cell. Those movements across the membrane depends on the type of molecule. According to its characteristics may cross by passive diffusion, facilitated diffusion and active transport due to the complex structure of the membrane which is usually composed by a monolayer or a bilayer of lipids with proteins embedded. The lipids have polar heads

groups that are at the external side of the membrane in contact with water and hydrophobic hydrocarbon chains that are oriented to the interior of the membrane (Melchior, 1982).

Acidogenic bacteria have two distinct mechanisms to generate metabolic energy, the first is the use of substrate-level phosphorylation, a metabolic reaction that store chemical energy in adenosine diphosphate (ADP) by forming adenosine triphosphate (ATP) and the second mechanism occurs at the cytoplasmatic membrane with conversion of electrochemical gradients into energy using ATPases.

In the first mechanism, which begins with glycolysis, occurs the conversation of glucose to pyruvate and H^+. Those hydrogen atoms are removed from the glucose and transferred to the electron carrier molecule nicotinamide adenine dinucleotide (NAD^+), which affects its oxidation state by converting it to NADH. NADH/NAD^+ is a cofactor and exists in limited amounts inside the cell, the NAD^+ reduced into NADH during the previous step has to be regenerated, which involves the oxidation through reduction of organic intermediates, yet the process is more complex and these cofactors can suffer redox reactions by different sources (Temudo, Kleerebezem, & Van Loosdrecht, 2007).

The second mechanism that uses electrochemical gradients occurs due to membrane proteins catalysing the translocation of ions across the membrane at the expense of other forms of energy such as electron transfer systems and membrane bound ATPases. Those proteins act like pumps, generating electrochemical gradients of translocated ions. H^+ and Na^+ are the main coupling ions associated to bioenergetic processes in bacteria (Lolkema, Speelmans, & Konings, 1994).

A representation of both mechanisms of energy production are shown in Figure 2.10, with the first mechanism represented by metabolic pathways in the centre of the cell while the second mechanism is composed by all the proteins and reactions present in the external membrane.
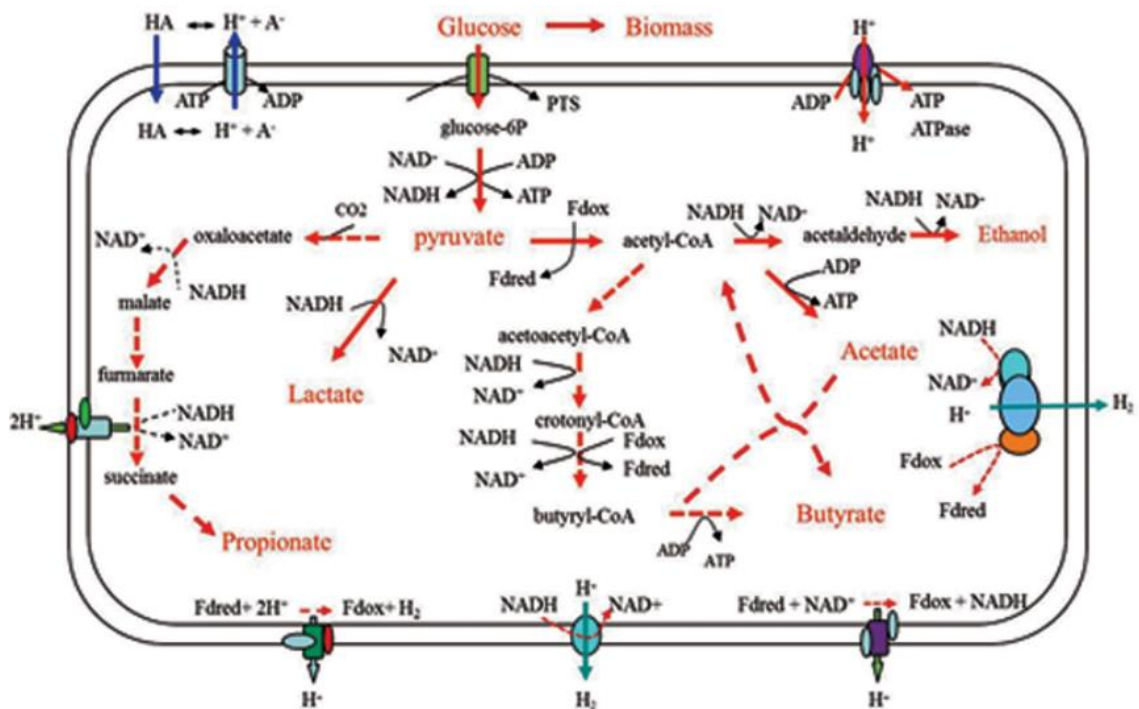
Figure 2.10 - Proposed model of bacteria mechanisms to generate metabolic energy (F. Zhang, Zhang, Chen, Van Loosdrecht, & Zeng, 2013).

Both metabolic processes of generating energy are linked and determine the overall energy status of the bacterium (Konings, Albers, Koning, & Driesen, 2002). H^+ could eventually be released as dissolved hydrogen gas, however, the higher the hydrogen partial pressure is, the more thermodynamically limited the oxidative pathways are.

Another electron carrier present in bacterial redox reactions as shown at Figure 2.10, is Ferredoxin (Fd), capable of producing hydrogen through an energy conserving electron bifurcation reaction (F. Zhang, Zhang, Chen, Van Loosdrecht, & Zeng, 2013b). Bacteria can have several types of hydrogenase enzymes which allow them to regenerate NAD^+ to NADH by production of H_2, even though a conventional reaction of this type being thermodynamically unfavourable. Bacteria could also have extrinsic membrane hydrogenase enzymes, attached to the periplasmic side of the cytoplasmic membrane that are able to catalyse the reversible oxidation of hydrogen which allow bacteria to use hydrogen as an energy source for their growth (Mendel & Robinson, 2007).

Other important factor that should be mentioned is the transport of chemical species over the cellular membranes, which can lead to generation or consumption of proton-motive energy depending on the environmental conditions. Proton-motive force (PMF) is an electrochemical gradient that origins a potential and can be interconverted into ATP equivalents (Konings, Lolkema, & Poolman, 1995).

The bacterial potential ends up being affected and affecting many parameters such as PMF, $NADH/NAD^+$ ratios and hydrogen concentration.

High hydrogen concentration increase potential, forcing the bacteria to react and compensate this variation by altering the behaviour of membrane proteins or alternating the metabolic pathways. Simultaneously to dissolved hydrogen variation, the pH variation imposed promotes changes in the PMF and transmembrane transport processes, since the bacteria respond differently to different external pH conditions.

Lower external pH leads to an increase of protonated molecules of organic acids, which can passively diffuse through the cell membrane as shown at Figure 2.11. As these molecules enter to the cytoplasm, dissociate to protons and charged derivatives which are impermeable to cell membrane (Presser et al. 1997). The accumulation of acids and alcohols may create unfavorable conditions for growth of some types of hydrolytic bacteria since some enzyme's production could stop, only bacteria that are more resistant to its harmful effects will grow and ultimately have a higher hydrolysis rate. The accumulation of protons inside bacteria may lower the intracellular pH and thus affects the transmembrane pH which contributes to the PMF, reduces the activity of acid-sensitive enzymes and damages proteins and DNA (Presser, Ross, & Ratkowsky, 1998).
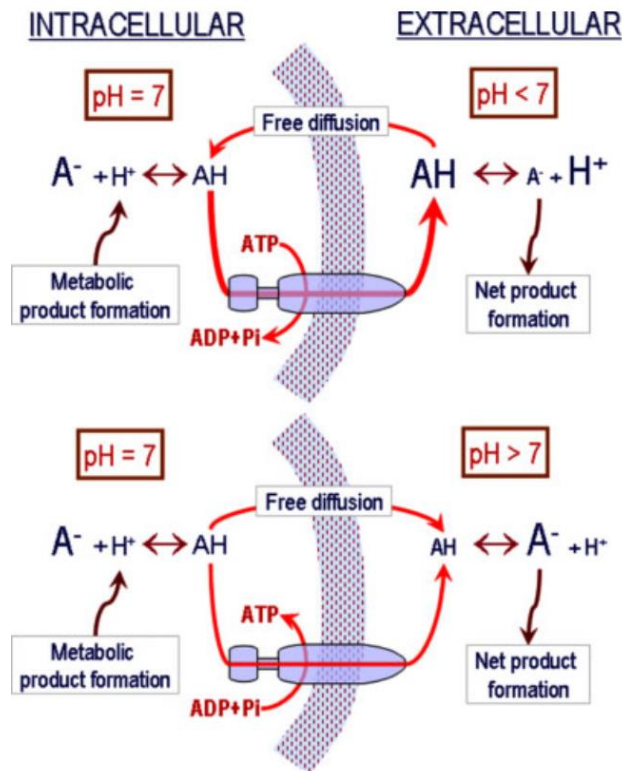
Figure 2.11 - Proposed model of transport of acidic species through the cell membrane by Rodríguez et al (Rodríguez, Kleerebezem, Lema, & Van Loosdrecht, 2006).

Acidogenic bacteria present in acidic acidogenesis have many strategies to survive in low pH (Audia, Webb, & Foster, 2001), cultures producing more acid resistant hydrolytic enzymes will also be more active and most developed defense mechanisms against environmental stress that allow them to withstand harsh conditions and sudden environmental changes due to functional conservation of several stress proteins and some of their regulators (Zverlov et al., 2010a). Defense mechanisms can usually be pumping out protons, sequestering the intracellular protons via biochemical reactions that consume protons (amino acids decarboxylation), production of ammonia, as well as modifications of the lipid content in the membrane (Lund, Tramonti, & De Biase, 2014). Even using mechanisms to maintain intracellular pH homoeostasis, many bacteria suffer some intern pH variations that end up altering the enzymatic activity and consequently the FP production and growth rates (Russell & Wilson, 1996).

3

3. Research objectives

As previously stated, PHA production is nowadays focused on using MMC and low-cost renewable feedstocks in order to lower the production costs. However, most wastes and feedstocks contain several organic compounds that are not equally suitable for PHA production ending up being used to produce glycogen instead of PHA by PHA accumulating microorganisms. It was used in the present work apple pulp, a waste produced by food industry that is composed mainly by sugars. This substrate requires a fermentation to convert those sugars into organic acids and ethanol which are PHA precursors. Apple pulp has a complex composition making it difficult to establish a static and stable profile of fermented products resulting in fluctuations on polymer composition and consequential differences in physical and mechanical properties of the final bioplastic.

Previous works stated a requirement of 15 to 20% (w/w) of HV monomers, in polymer composition to be used for food packaging. In order to be able to predict or change the fermented products' profiles to reach a certain quantity of HV precursors, two strategies were implemented in operational conditions of fermentation reactor:

— Study how a UASB reactor normally used to produce VFAs for PHA production with a ratio HB:HV of 85:15, behaviour in different pH conditions, studying performance variations such as FP profile and microbial community changes.

— Utilization of a liquid disperser inside the reactor, recirculating the reactor fluid in order to promote the transfer of dissolved hydrogen and carbon dioxide present in the liquid phase to the gas phase. This operational method was used to understand if this strategy is capable of causing variations in metabolic pathways, thus achieving variations on FP profiles, with special focus on propionate and valerate, as they are the most reported HV monomer' precursors (Chaganti et al., 2011).

4. Material and methods

4.1. EXPERIMENTAL SETUP SCHEME

The PHA production process using MMC was done in pilot scale with the process divided in three phases, acidogenic fermentation, followed by a selection phase and a final accumulation phase as shown in Figure 4.1.

The pilot plant setup was composed by: a 100 L acrylic UASB reactor used for the fermentation phase, that was performed using a working volume of 60 L; the selection phase took place in a 100 L stainless steel sequencing batch reactor (SBR); and the accumulation step was done in a 60 L stainless steel fed-batch reactor.

The experiments described in this work were all applied to the fermentation phase of the process. All the parameter variations were done under a production regime, which means all the fermented products were used in the actual PHA production.
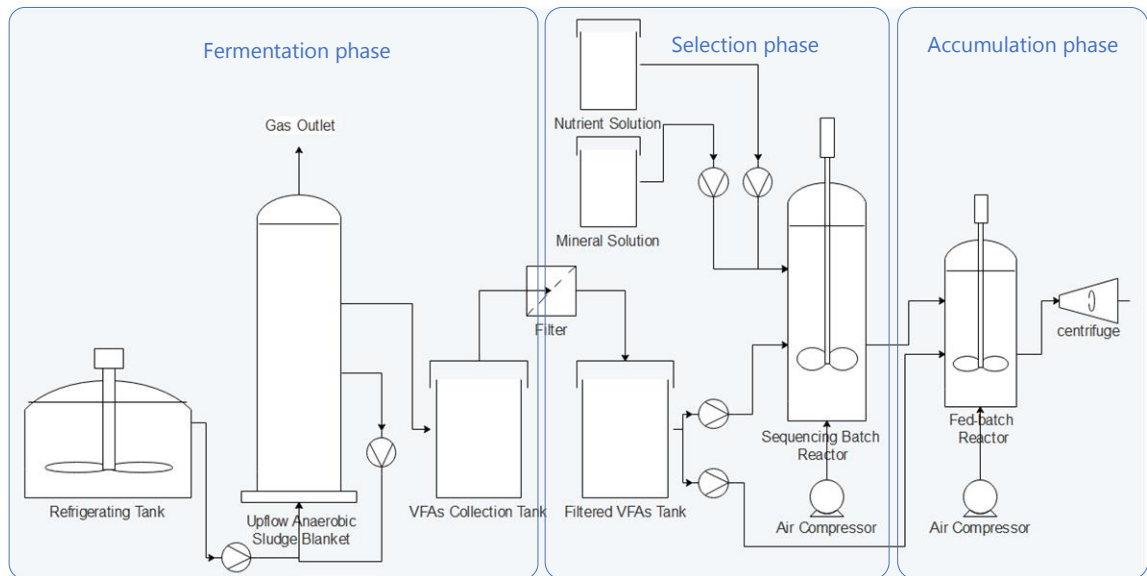
Figure 4.1 – Process diagram of PHA production at pilot scale.

4.2. SUBSTRATE

Every two weeks, it was received around 160 L of frozen apple pulp from Sumol Compal S.A. and stored in a refrigerator chest at -20 °C to maintain its characteristics. When there was a need to prepare feed solution for the reactor, the necessary amount of pulp was let at room temperature the day before to defrost.

The feed solution was prepared every three days in a 200 L refrigerating tank. The apple pulp was diluted with tap water in order to achieve a theoretical concentration of 27 g $_{COD}$/L. It was used apple pulp at low degradation state in order to minimise the variation of FP produced in reactor with the FP already present in the feed.

Since previous analysis show that this substrate have low amount of nitrogen (N) and phosphorous (P) (Carvalheira et al., 2018), the feed solution was supplemented with nitrogen, using ammonium chloride (NH_4Cl) and phosphorous, using potassium dihydrogen phosphate (KH_2PO_4) to maintain a COD:N:P ratio of 1000:5:1 (gCOD:gN:gP), thus avoiding nutrient limitation. Assuming a theoretical COD concentration in the feed solution of 27 g $_{COD}$/L, it was added 0.5158 g/L/d of NH_4Cl and 0.1185g/L/d of KH_2PO_4. To maintain the granules consistency and high productivity, the feed solution was also

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supplemented with Calcium (as CaCl$_2$) 0.5158 g/L/d, magnesium (as MgSO$_4$.7H$_2$O) 0.121235 g/L/d and Iron (as FeCl$_3$) 0.6816 g/d.

4.3. UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR

The tests were applied to a well-established 60L (operation volume) UASB reactor with 156 days of previous operation. The reactor was previously inoculated with 25 L of granular anaerobic biomass from a biobed expanded granular sludge blanket (EGSB) reactor used by Unicer bebidas SA to treat brewery wastewater.

The operational volume of granules used was 15-18 L and the suspended biomass concentration is unknow.

The reactor was operated in mesophilic condition, at 30±0.4 °C, controlled using a thermostatic bath which controls the reactors jacket temperature and with a constant HRT of 1 day.

During the start-up of the UASB reactor a gradual increase of the OLR was done reaching around 30 g $_{COD}$/L/d after 56 days of operation. That OLR was maintained for 100 days further, until the beginning of this experiment. It was established in previous work (data not shown) that the maximum OLR for the reactor work without disturbances in the granule's stability is 30 g $_{COD}$/L/d. However, due to the high variability in terms of organic load of the substrate, it was decided to work with a slightly lower OLR of 27 $_{COD}$/L/d. At the end of the experiment it was determined that the average OLR applied to the reactor was 27.7±2.3 g $_{COD}$/L/d.

The pH was online monitored using a HANNA HI8711 pH controller and manually adjusted using sodium bicarbonate that was added in the feed tank.

The recirculation flow was established at 2.4 L/min, resulting in an upflow velocity of 2.88 m/h, higher than the values normally described (Abbasi & Abbasi, 2012), yet due to the inefficient liquid dispersion at the bottom of the reactor, a higher UFV was needed to maintain a certain bouncy of the granular layer to promote higher mass transfer.

A second recirculation circuit was used in certain phases (Table 4.1) and forced to pass by a liquid disperser attached to the top of the reactor. This second recirculation

was used to cause a physical disturb which was intended to decrease the gases dissolved in the liquid phase. This alternative recirculating flow was pumped at 1 L/min from the liquid slightly above granular layer to the top of the reactor passing then by the liquid disperser (shown in Figure 4.2) and falling around 40 cm at headspace directly to the liquid inside.

Figure 4.2 - Loc-Line Swivel Nozzle used as liquid disperser.

The dissolved hydrogen was online measured using a Unisense multimeter, the headspace relative pressure was measured with an HD402T pressure transmitter from DeltaOhm and the outlet gas flow using an FMA-A2307 from OMEGA. The schematic representation of the UASB reactor and its control systems are shown in Figure 4.3.
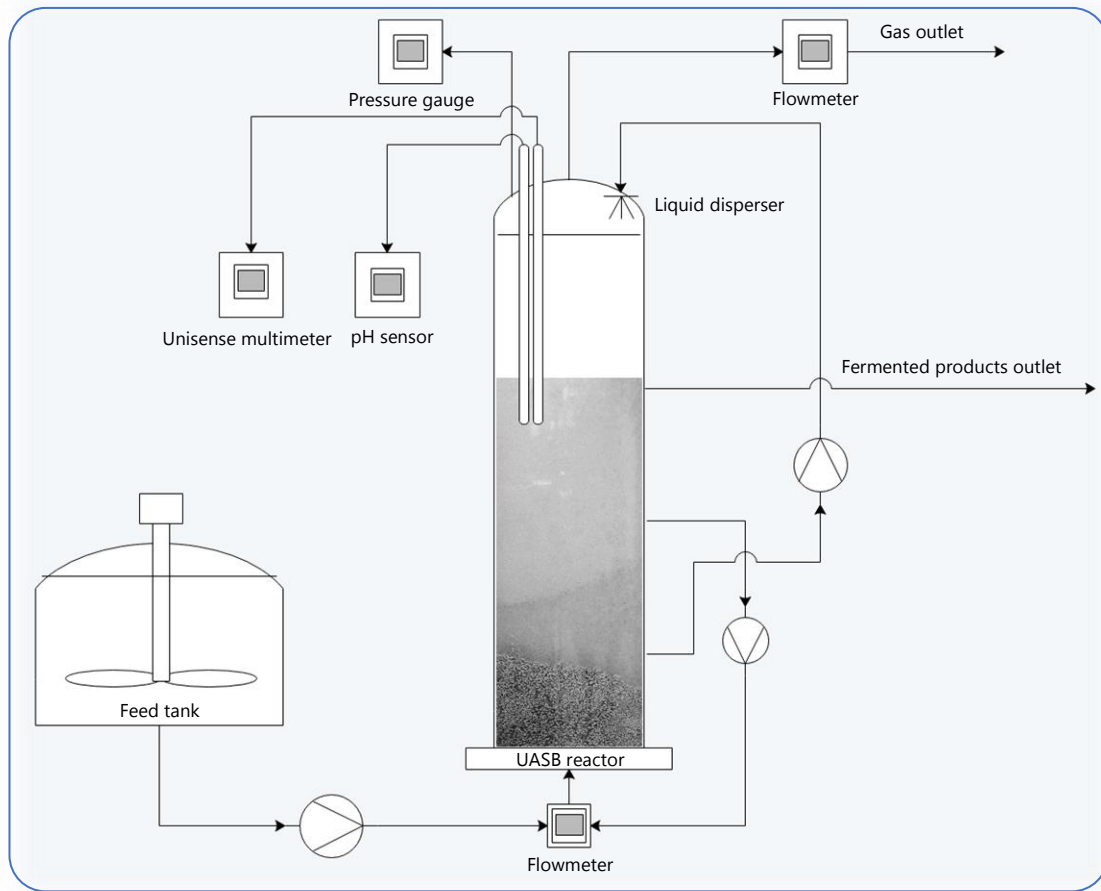
Figure 4.3 - Schematic overview of the UASB reactor.

4.4. MODE OF OPERATION

The present work intended understand which operational conditions cause variations in FP and consequently condition the polymer composition. For this propose, external variations such as different amounts and profiles of FP coming from feed solution into the UASB reactor were minimized as previously stated, by using apple pulp at low degradation state.

The reactor was used in two main experiments, the first named phase I, consisted in altering operation pH and the amount of dissolved hydrogen. Different levels of pH were proposed to operate, 4.75, 5.25, 5.75 and 6.25, however due to the way pH was controlled in this system, done manually by adding sodium bicarbonate in feed tank, pH could not be as static and specific has initially intended, yet well stablished levels were obtained.

The condition names were adapted to the average pH obtained in each stage and are shown in Table 4.1 and Table 4.2.

Table 4.1 - Operational conditions applied in UASB reactor Phase I.

Condition name	Operation period (d)	Duration (d)	Average pH
pH 4.71 Disperser Off	157-164	7	4.71 (±0.01)
pH 4.76 Disperser On	165-172	7	4.76 (±0.01)
pH 5.14 Disperser Off	173-179	6	5.14 (±0.10)
pH 5.23 Disperser On	180-188	8	5.23 (±0.04)
pH 5.89 Disperser Off	189-199	10	5.89 (±0.12)
pH 5.91 Disperser On	200-209	9	5.91 (±0.01)
pH 6.24 Disperser Off	210-220	10	6.24 (±0.06)
pH 6.36 Disperser On	221-228	7	6.36 (±0.05)

In order to study the microbial functional redundancy, the test was extended over the operation stated at Table 4.1 to a second experiment named here as phase II, to verify if MMC tends to stablish patterns over each pH during continuous operations and if FP profiles remain the same in each operational pH after being applied pH variations.

This experiment begins after the condition pH 6.36, with a slow pH descend (phase II) used to replicate two operational pH's, 5.8 and 5.1 tested before in phase I. Since the pH variation was done slowly, the data used to represent each condition incorporate only samples from a smaller period at the end of each condition in a stable period. Those periods are shown in Table 4.2.

Table 4.2 - Operational conditions applied in UASB reactor Phase II.

Condition name	Operation period (d)	Duration (d)	Average pH
pH 5.81	264-271	8	5.81 (±0.05)
pH 5.16	294-301	8	5.16 (±0.03)

The UASB reactor was operated with continuous flow with feed solution prepared every three days and continually refrigerated to reduce fermentation in the feed tank. Several parameters were monitored. In each new feed solution prepared, samples were taken to determine COD content, ammonia, phosphorous and FP produced during the degradation of the apple pulp.

The parameters analysed regularly on the reactor were temperature, pH, dissolved hydrogen in liquid phase and quantity of produced gas. Before the introduction of each new feed solution, samples were taken from the reactor to measure ammonium, phosphorous and FP. Samples from gas outlet were also taken to analyse its composition.

4.5. ANALYTICAL METHODS

4.5.1. ORGANIC ACIDS AND ETHANOL CONCENTRATION

FP were filtered (Millipore membrane of 0.45 mm) and then analysed by high performance liquid chromatography on a VWR Hitachi Chromaster with ion exchange column Aminex HPX-87H paired with an 125-0129 pre-column both from Biorad, one Diode Array Detector 5430 and a refractive index detector RI-5450 from Merck.

The eluent solution was H_2SO_4 (0.01 N) with a flow rate of 0.5 mL/min and temperature of 30 °C. The sample volume was 99 μL and a run time of 45 minutes.

The organic acids and alcohol concentrations were calculated through a standard calibration curve for every compound, with a concentration gamma of $0-1$ g/L and six different concentrations.

4.5.2. ORGANIC SUBSTRATE CONTENT

To determine the organic content in feed solution, it was measured the chemical oxygen demand, using kits LCK 914 from Hach Lange (range 5-60 g/L O_2) and read at a DR 2800 spectrophotometer from Hach Lange.

4.5.3. GAS COMPOSITION

The exhaust gas was analysed by gas chromatography on a GC 7890 B model from Agilent Technologies. The GC is equipped with three columns, PoraBOND Q PT (1), CP-Molsieve 5Å PT (2) and HayeSep Q 80/100 mesh (3). The gas carrier used was Argon at a constant flow of 10 mL/min, the run time of each sample was 7.4 min at 70 °C and a sample volume of 1 mL.

Each gas concentration is calculated through a standard calibration made with four different standard gases solutions from Air Liquid stated in Table 4.3.

Table 4.3 - Molecular percentage of each gas present in gas solution.

Gas solution	$\%CO_2$	$\%H_2$	$\%O_2$	$\%N_2$	$\%CH_4$
EK7GEF0	-	30.03	-	69.97	-
EK7GF9R	66.02	1.10	24.99	6.70	1.20
EK7GFA5	15.00	19.90	1.01	39.02	25.07
EK7GFDK	4.99	10.09	13.06	1.99	69.87

4.5.4. DISSOLVED HYDROGEN

The dissolved hydrogen on liquid phase was measured by Unisense Microsensor Multimeter using a hydrogen probe H2-500-10105. The probe calibration was made in a designated chamber where three different solutions of gases with different hydrogen concentrations were bubbling on a liquid phase composed by a liquid sample removed from the reactor. The gases solutions used are shown in Table 4.4.

Table 4.4 - Molecular percentage of each gas in hydrogen gas solutions.

Gas solution	%H$_2$	%N$_2$
UN1066	-	100.00
EF9UG1G	10.00	90.00
EK7GEF0	30.00	70.00

4.5.5. NITROGEN AND PHOSPHORUS CONCENTRATION

Phosphate and ammonia concentrations were determined by colorimetric methods implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands).

4.5.6. MICROBIAL COMMUNITY ANALYSIS THROUGH DNASENSE APS

The general microbial community analysis was done by DNASense ApS where DNA was extracted through a complex molecular process from all organisms in the samples. DNA from each microbe in the community has specific "fingerprint" genes, that can be used to identify the organisms (16S ribosomal RNA gene for Bacteria).

DNA sequencing is used to count the number of fingerprint genes from each microbe in the sample, which is then used to estimate the relative abundance of the microbes in the community. Around 10,000 fingerprints were measured from each sample to provide a high resolution of the community structure. The abundances of the species presented in the analysis represent the count of each bacterial 16S rRNA gene in the sample.

The abundance is influenced by DNA extraction, gene copy number and primer biases and does not necessarily represent the true in situ abundance, however, can be used to approximately estimate it.

The raw sequencing data was processed using the research standard UPARSE workflow and data was analysed through Rstudio using the ampvis2 package developed at Aalborg University.

4.5.7. FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Aiming the qualitative analysis of the microbial community present in UASB reactor, qualitative FISH was done according to Olson et al. (1990) in order to assess the general microbial composition, the abundance and the dynamics of the populations.

The use of fluorescent-labelled probes applied directly on the biomass samples allowed the visualization and discrimination of the microbial populations. The oligonucleotide probe used for FISH experiments was Cyanine 3 (Cy3)-labelled ARC915 probe for Archaea (Stahl et al., 1988), in order to target possible archaeal methanogens present in UASB reactor. Samples were analysed using an epifluorescence microscope Zeiss Imager D2 at 1000x.

4.6. CALCULATIONS

4.6.1. ACIDIFICATION DEGREE

The efficiency of the acidogenesis can be represented by the acidification degree which indicates how much of the organic substrate in the feed ($[COD]_{Feed\,in}$) is converted to FP ($[COD]_{FP\,out}$). Acidification degree can be calculated using the Equation 4.1 which by this method of calculation excludes the FP already present at apple pulp waste ($[COD]_{FP\,feed\,in}$) and take into consideration just the FP effectively being produced in the UASB reactor. FP represents the sum of concentrations (in terms of COD) of VFAs, lactate and ethanol.

$$Acification\ degree = \frac{[COD]_{FP\,out} - [COD]_{FP\,feed\,in}}{[COD]_{Feed\,in} - [COD]_{FP\,feed\,in}}$$

Equation 4.1

$[COD]_{FP\ out}$ is the organic load of the FP; $[COD]_{Feed\ in}$ is the organic load of the feed solution; $[COD]_{FP\ feed\ in}$ is the organic load of FP present in the feed solution due to apple pulp degradation.

4.6.2. HV% CONTENT

PHA production using MMC is affected by FP profile and it is not completely known how each acid affects PHA composition. However, in the present work it is assumed that acetic and butyric acids favour the production of the monomer 3-hydroxybutyrate (3HB) (Lemos et al., 2006), lactate is described as favouring the production of HB (Jiang et al., 2011) and ethanol, an alcohol commonly produced in fermentation, shows a trend to be used as 3HB precursor due some previous tests (data not shown).

As 3-hydroxyvalerate (3HV) precursors, the ones that generally reported are the propionic and valeric acids (Lemos et al., 2006).

HV% content results from the molar fraction between the sum of propionate and valerate molar concentrations divided by the sum of all PF molar concentrations.

$$HV\% = \frac{[Propionate] + [Valerate]}{[FP]}$$

Equation 4.2

$[Propionate]$ is the molar concentration of propionate; $[Valerate]$ is the molar concentration of valerate; $[FP]$ is the molar concentration of FP.

5. Results and discussion

5.1. IMPACT OF pH ON FERMENTATION PRODUCTS AND MICROBIOLOGY

The possibility of tailoring the polymer composition in a PHA production process by changing operational conditions makes it interesting to study these variations in FP profiles. This test mainly approach how differences in operational pH and hydrogen content will affect FP and microbial consortium during the operation of a UASB reactor previously at steady state conditions, with a well stablished and selected culture after 156 days of operation.

The granules used in the startup of this UASB reactor came from the Unicer digester that operates under alkaline conditions to produce biogas. Those are initially dark possibly because at higher pH (pH>6), sulfate-reducing bacteria present in the granules convert the sulfate into sulfide, which react with metals forming dark precipitates (Herbert H.P. Fang & Liu, 2002). After few weeks of operation under low pH they start to change to creamy white color due to reduction of sulfidogenic activity.

The difference in aspect of both types of granules can be seen in Figure 5.1.

Figure 5.1 – Unselected granules (dark) at the top, from Unicer digester in the beginning of operation and acidogenic selected granules (creamy white) on bottom.

During previous work it was stated that even though using granules, suspended cultures are present within the liquid phase and have an important role in acidogenic processes. In some small tests (data not shown) even after losing almost all granules' volume, acidogenesis was still present with an acidification degree above 50%. For this reason, all microbiology sampling and analysis present in this work were done in both granules and suspended biomass.

The granules used from Unicer are composed fundamentally by bacteria and archaea. These archaea as previously mentioned are the methane producers and should be minimized. During all experiment it was done FISH analysis and archaea were identified as "almost non-existent", with a relative abundance between 1 and 5%, for almost all samples, both for granules and suspended biomass. Only for the pH 6.25, archaea are considered "present" inside the granules, meaning a relative abundancy between 5 and 20%. These results shown that archaea can survive or reappear when favorable conditions let them prosper and grow. Three families of Archaea were identified, *Methanobacterium*, *Methanosphaera* and *Methanobrevibacter* belonging to *Methanobacteriales* order from *Euryachaeota* phylum.

The fermentation step in PHA production is intended to produce an end-stream rich in organic acids. Subsequently, the higher pH tested was 6.25 due to the increase of archaea at this condition. As previously stated, closer to neutral conditions is expected an increase in archaea since conditions are optimal for them, however archaea consume the organic acids to produce methane, reducing the content of organic acids at end-stream and for this reason, in order to avoid them, the pH was not increased to higher values.

For strong alkaline conditions like pH 10.0, which is also non favorable to the growth of archaea, was tested in previous works (data not shown), yet showed a FP profile composed mainly by lactate which is not interesting for PHA production since it significantly decrease the PHA production productivity and for this reason higher pH in strong alkaline conditions was not tested here.

5.1.1. IMPACT OF pH VARIATION IN FERMENTATION PRODUCT PROFILE

The operational conditions such as pH are crucial to stablish major FP profiles, however due to complex substrates like residues composed by apple pulp, FP production can suffer variations due to the degradation of different types of organic matter.

The results used for description of phase I, only use data from the period while the liquid disperser was off, which represent a normal operation of UASB reactors and is the same operation done at phase II. The results of each FP concentrations are shown at Table 5.1.

Table 5.1 - fermentation products concentration at different pH values (disperser off).

FP (mmol/L)	Phase I pH 4.71 (±0.02)	Phase I pH 5.14 (±0.10)	Phase I pH 5.89 (±0.12)	Phase I pH 6.24 (±0.06)	Phase II pH 5.81 (±0.05)	Phase II pH 5.16 (±0.03)
Acetate	61.63 (±1.65)	77.63 (±11.68)	78.73 (±6.80)	103.09 (±13.28)	102.31 (±1.93)	75.48 (±1.35)
Butyrate	66.87 (±3.31)	75.39 (±9.45)	50.79 (±5.07)	59.34 (±7.24)	54.82 (±2.45)	53.36 (±1.88)
Propionate	11.26 (±0.60)	11.51 (±4.39)	22.76 (±2.04)	14.41 (±1.46)	19.77 (±0.78)	20.46 (±0.92)
Valerate	8.09 (±0.35)	5.99 (±1.34)	8.79 (±0.47)	7.91 (±0.77)	13.03 (±0.73)	13.99 (±0.39)
Lactate	23.98 (±4.27)	7.08 (±1.55)	4.89 (±4.40)	3.19 (±2.09)	7.01 (±2.88)	9.64 (±2.09)
Ethanol	23.92 (±0.87)	32.70 (±2.78)	7.59 (±2.79)	7.33 (±0.88)	5.82 (±0.24)	12.39 (±2.15)
Sum FP	195.77 (±3.27)	210.30 (±21.31)	173.62 (±17.25)	195.26 (±24.69)	202.76 (±5.42)	185.31 (±0.88)

At phase I, pH 5.14 has been the best condition in terms of quantity of FP produced while pH 5.89 resulted in the condition with lowest production of FP. However, when those conditions are replicated at phase II test, the results invert with pH 5.81 having higher production than pH 5.16. The pH 4.71 and 6.24 have similar performance in terms of quantity of FP, yet the FP profile is noticeably distinct.

The major FP profiles are shown at Figure 5.2. It includes the median of molar fraction of each individual FP and respectively medians concentration values obtained at each pH tested.
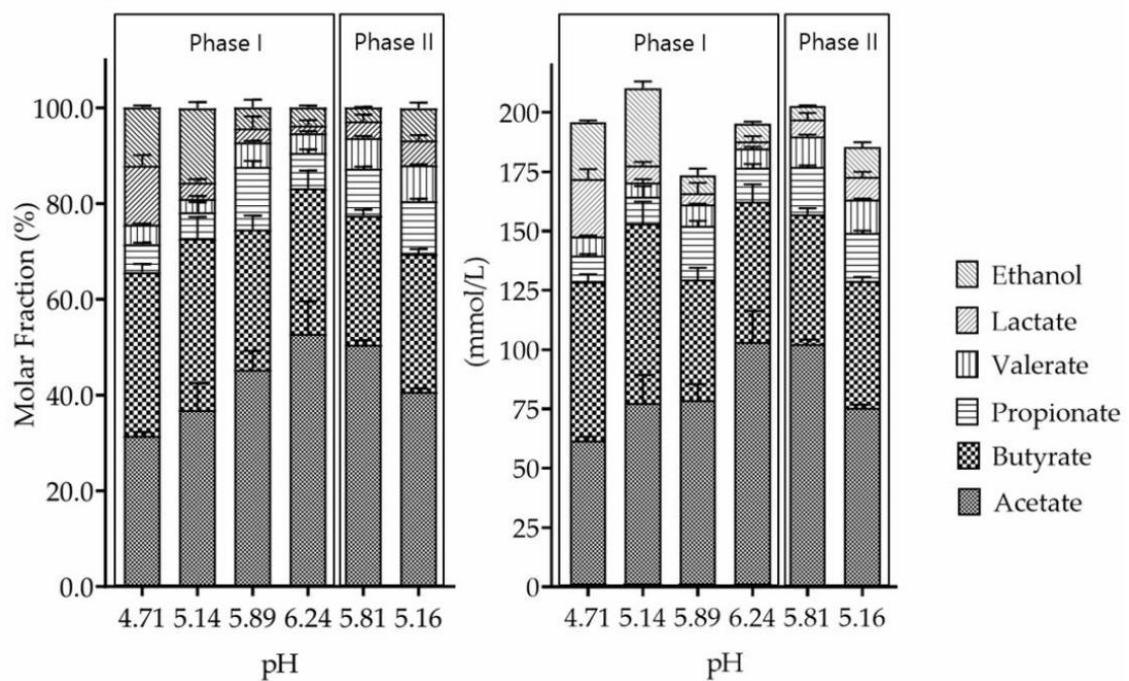
Figure 5.2 - Median of Fermentation products molar fractions (left side) and molar concentrations (right side) at different pH values.

As expected, FP profiles changed with pH variations during the tested range, with acetate and butyrate being the majors FP while ethanol, lactate, valerate and propionate produced at lower quantities.

Acetate and butyrate have almost the same molar fraction at pH 4.71 as well as at pH 5.14. At conditions approaching neutral pH leads to acetate becoming the main product and reaching in this test a molar percentage of 52.8% of all FP at pH 6.24. Otherwise, butyrate decreases at the pH 6.24 to 30.4%.

During phase II when pH is lowered from pH 6.24 to 5.81 and 5.16, acetate decreases, yet obtaining slightly higher proportions than those obtained at the correspondent pH in phase I. Butyrate ends up matching the proportions obtained during pH 5.8 in both phase I and II, however, during pH 5.16 (phase II) obtain 28.8% of FP while at pH 5.14 (phase I) had been 35.8%. This trend follows the behavior described in literature, where the increase of pH leads to an increase of acetate followed by an butyrate decrease (Herbert H.P. Fang & Liu, 2002).

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The higher lactate concentration occurs at pH 4.71 reaching 12.3% of total FP while at higher pH it reduces to less than 5.2% with a minimum value of 1.6% at pH 6.24 (phase I), indicating that pH closer to neutral conditions decreases lactate production.

Ethanol reaches its higher concentration of 15.5% at pH 5.14 (phase I), however, during pH 5.16 (phase II) only obtain 6.7%, half of the proportion at the same pH in phase I. This could be due to the system being descending from higher pH, since pH of 5.8 and 6.2 were not good for ethanol production, where ethanol was just 3 to 4% of FP.

Valerate and propionate are the main HV precursors and is important that the concentration of these FP is stabilized and replicable. Valerate is usually the lowest FP, reaching a maximum concentration of 7.5% at pH 5.16 (phase II), however, at the same pH in phase I, its concentration is just 2.8%. At pH 4.71 and pH 6.24, the concentration was 4.1% in both.

Propionate lowest concentration is at pH 4.71 and pH 5.14 (phase I) with 5.8% and 5.5% respectively of total FP, increasing to its maximum of 13.1% at pH 5.89 (phase I) and decreasing to 7.4% at pH 6.24. In phase II during pH 5.81 reached 9.8% and at pH 5.16 ends up being 2 times higher than at correspondent pH in phase I which corresponds to 11% of total FP, while in phase I it had represented just 5.5%.

These results show that microbial functional redundancy was not applicable for this process since PHA production requires very specific FP profiles or the final polymer will have different properties. As shown in Table 5.2, HV% should be similar when are applied the same operational conditions, even if the FP are the same and the variations are roughly low, the final concentrations of each product ends up changing HV precursors concentrations.

These variations have a considerable impact because in phase I the HV precursors concentration is 8.32% at pH 5.14, while in phase II pH 5.16 results in 18.59%. This variation may have impact in the final PHA HB:HV ratio, while HV precursors ratio is not the only condition to determine the final HV content in PHA, it have a major impact and if the final HV content was similar to the HV precursors ratio obtained here, it would make the polymer melting point change from around 165 °C to 145 °C and tensile strength fluctuate from 37 MPa to 32 MPa as shown in Table 2.2, which is a considerable

variation in final biopolymer physical properties. However, at pH 5.8 the results in both phases have similar FP profiles and the final polymer should be comparable.

Table 5.2 - HV% precursors at different pH values in operation with disperser off.

	Phase I				Phase II	
Operational conditions	pH 4.71 (±0.02)	pH 5.14 (±0.10)	pH 5.89 (±0.12)	pH 6.24 (±0.06)	pH 5.81 (±0.05)	pH 5.16 (±0.03)
HV% precursors	9.89 (±1.99)	8.32 (±6.58)	18.17 (±4.09)	11.43 (±4.45)	16.18 (±1.59)	18.59 (±1.51)

5.1.2. IMPACT OF pH VARIATION IN MIXED MICROBIAL CONSORTIA

DNA sequencing was used to count the number of fingerprint genes from each microbe in the sample and used as an estimate of the relative abundance of the microbe in the community. Those fingerprint genes were matched with a database of known references and thereby most of the microbes in the community were identified. High throughput amplicon sequencing of the bacterial 16S rRNA gene allows to identify a total of 1062 operational taxonomic units (OTUs) on granules and suspension biomass combined.

The OTUs were classified till the level of species yet much of those lack identifications at this level, only being possible to be identified at higher levels such us genus or family. As shown in Figure 5.3, all metadata is rearranged in phylum, class and order. The data shown represents more than 95.5% of all identified bacterial OTUs.

Note that OTUs from *Saccharibacteria* lack more information in DNA reports and literature about classes or lower levels, so this group of bacteria will be discussed here always as being from a single group in class or order and will be referenced by phylum name.
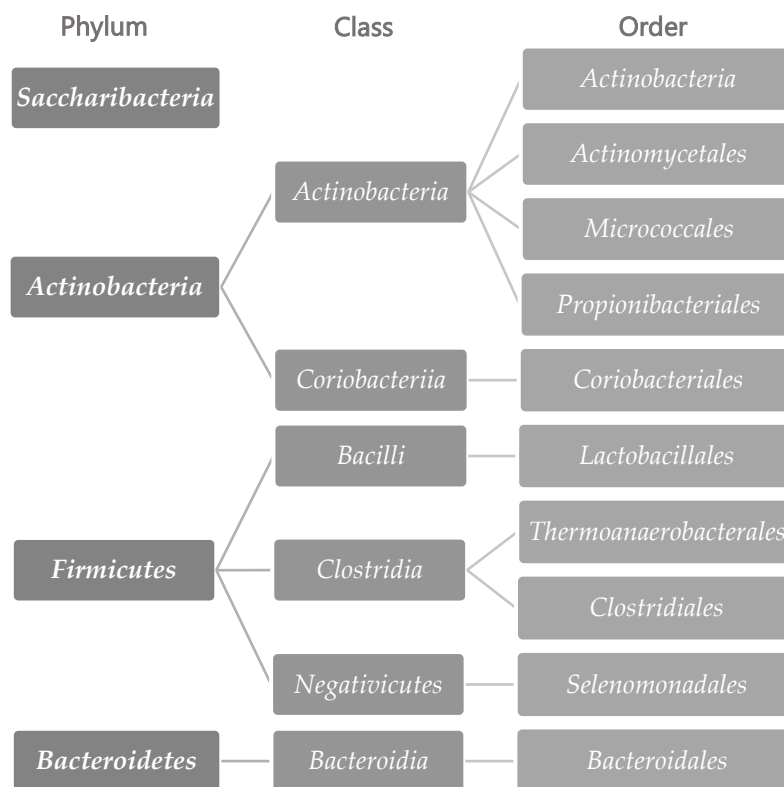
Figure 5.3 - Taxonomic diagram of the most common identified OTUs distributed by phylum, class and order.

IMPACTS IN MMC AT PHYLUM LEVEL

In Figures 5.4 and 5.5, it is shown the difference between MMC at phylum level in granules and suspension. As previously mentioned, granules act as a protective environment to microbial cells which end up minimizing the effect of pH variations, specially between lower pH changes. At pH 4.7 to pH 5.1 the variations in granules (at phylum level) are almost neglectable, however, in suspension is possible to see a notorious difference between different pH values which are also distinct in proportion in relation to granular MMC.

Hydrolytic bacteria are phylogenetically diverse but mostly fall into two phylum, *Bacteroidetes* and *Firmicutes* (Venkiteshwaran et al., 2015). Previous works did state *Firmicutes* and *Bacteroidetes* as the most predominant phylum in fermentation of waste activated sludge (Cheng, Chen, Yan, & Su, 2014; Cirne, Bond, Pratt, Lant, & Batstone,

2012), similar results were observed in this work, with both phylum representing more than 70% of MMC for both granules and suspension.
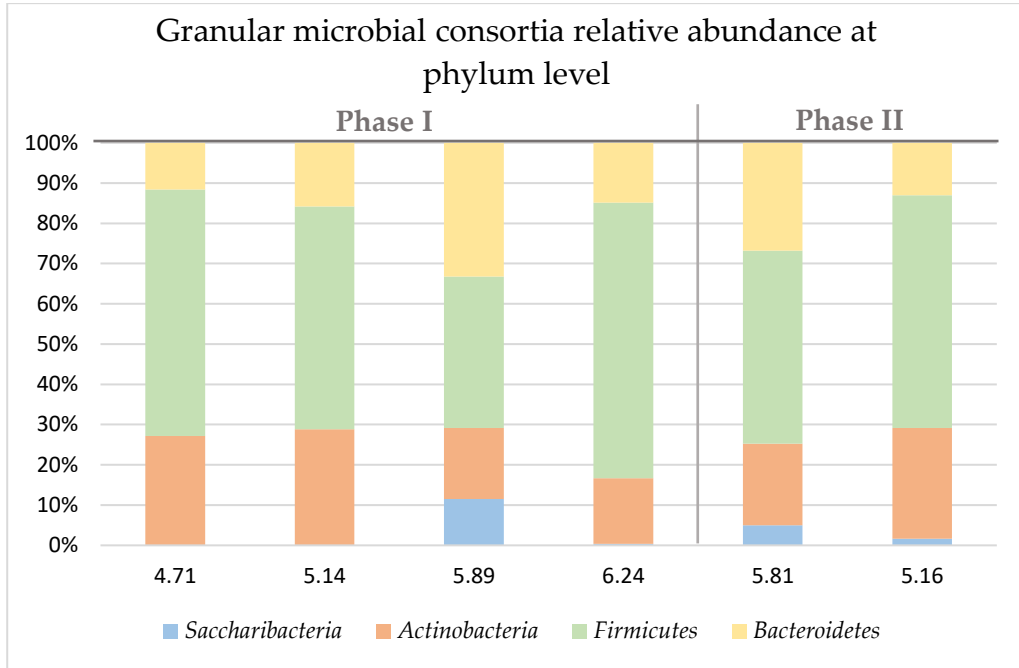
Figure 5.4 - Relative abundance of bacteria phylum inside the granules.
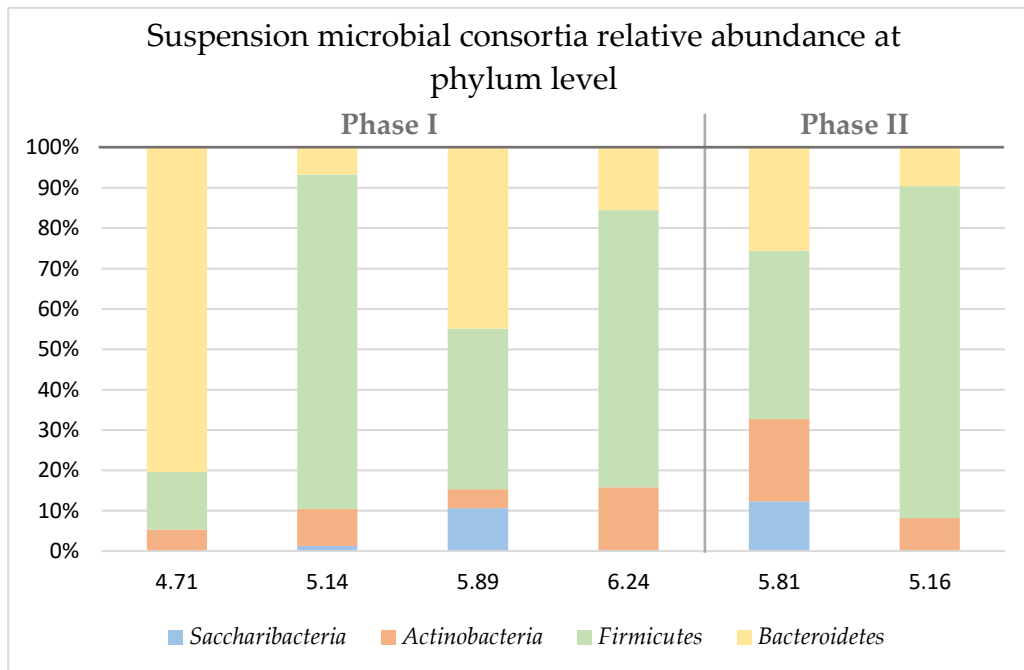
Figure 5.5 - Relative abundance of bacteria phylum in suspension.

UASB suspension at the lower pH has a major amount of *Bacteroidetes*, which represent 80.3% of MMC in suspension, yet at pH 5.1 the MMC completely changes with *Bacteroidetes* decreasing to just 6.7% and *Firmicutes* increasing from 14.3% to 81.7%. This major difference reiterates the importance of sampling both granules and suspension microbial consortia, allowing the perception that even slight pH variations can drastically change the microbial consortia composition.

This can be related to *Bacteroidetes* possibly being more acid tolerant than most of *Firmicutes*, which allow them to have less decrease in growth rates than *Firmicutes*. Since suspension is an environment more harmful than inside the granules this occurrence can only be seen at the suspension. When pH increase *Firmicutes* should have higher growth rates than *Bacteroidetes* and rapidly increase their proportion inside the reactor in suspension.

The pH 5.8 in both phases is the condition which promotes the biggest variation, resulting in a very distinct profile with a considerable amount of the *Saccharibacteria*. This phylum reaches around 5 to 11% in granules and 10 to 12% in suspension. Those bacteria appear to prosper only at pH range of 5.8 to 5.9, since it is barely present (1.2%) in suspension biomass at pH 5.14 (phase I) and increases to 10% at pH 5.8, followed by an almost total inexistence (0.3%) at pH 6.2.

The phase II was important to establish an optimum growth range for this phylum, is possible to point that at pH 5.14 the condition is not favourable to *Saccharibacteria* growth because if these bacteria had a proper environment to prosper, at pH 5.18 (phase II) the amount of them remaining should at least maintain, yet the results reveal a very accentuate decrease (complete loss in suspension), clearly demonstrating that for the tested conditions, *Saccharibacteria* only have acceptable growth and surviving conditions at pH around 5.8.

Actinobacteria is the phylum that suffers lower variations in relative abundance, remaining in granules between 26% to 29% at low pH (4.7 to 5.1) and decreases to 16% and 20% at pH of 5.8 to 6.2, respectively. *Actinobacteria* might be important bacteria for holding granule structural integrity since some species present in this phylum are known for encoding various cell surface or surface-exposed proteins that may exhibit cell-

adherent properties (Comfort & Clubb, 2004). Its abundance is generally lower in suspension, except for the pH 6.2 at phase I and pH 5.8 at phase II, which is very similar between both granules and suspension.

IMPACT IN MMC AT CLASS LEVEL

Regarding the classification at taxonomic class, the rearrangement of the microbiology data is shown in Figures 5.6 and 5.7.
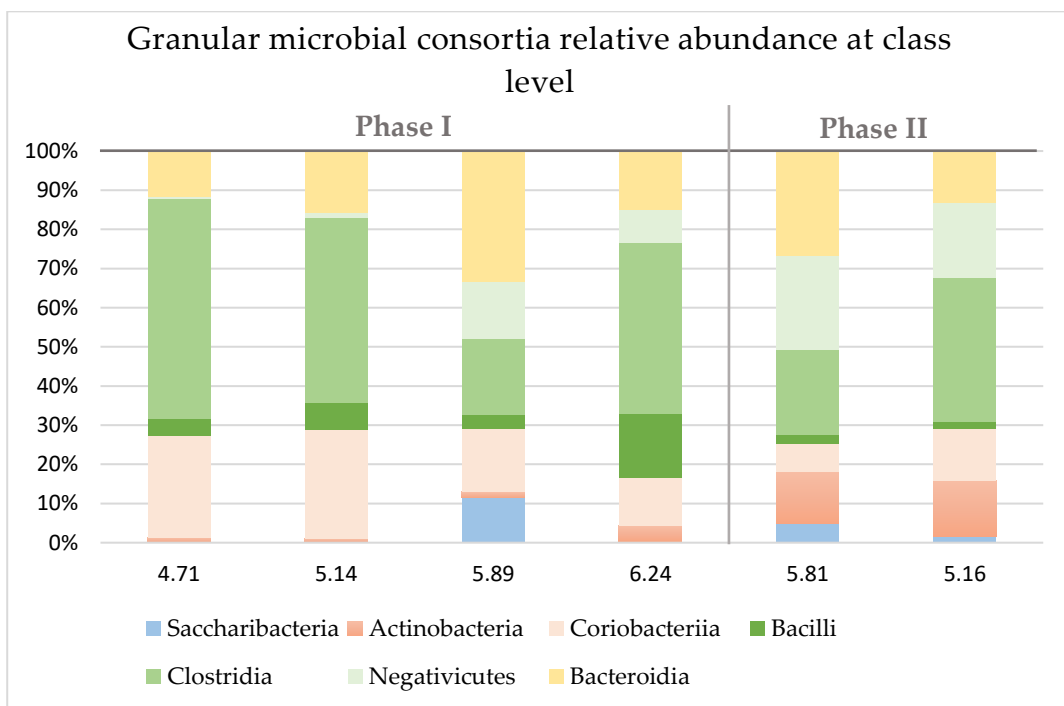
Figure 5.6 - Relative abundance of bacteria class inside the granules.
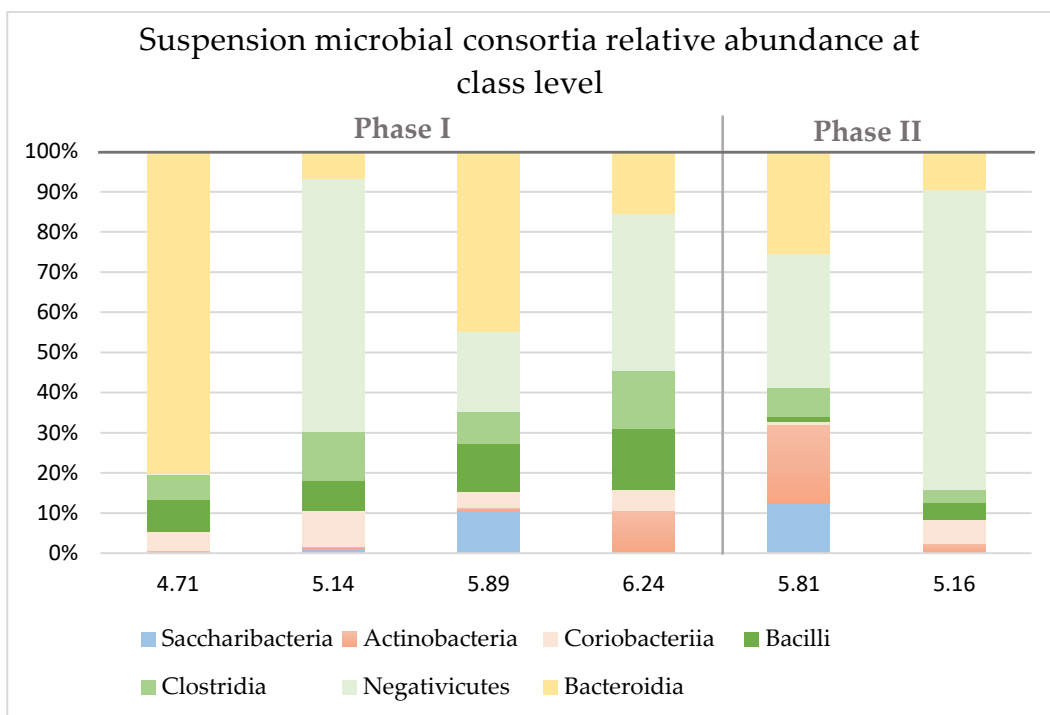
Figure 5.7 - Relative abundance of bacteria class in suspension.

Bacteroidia is the most abundant class of bacteria at low pH in suspension, indicating that this is probably de most adapted class of bacteria for this pH. *Prevotella*, a gram-negative bacterium, is the species that mostly represent this class at such low pH and represents 80% of MMC in suspension, yet only 12% in granules.

Bacteroidia altogether decrease at pH 5.1, probably due to the increase of *Firmicutes* that should have high growth rates in these conditions. At pH 5.8 *Bacteroidia* increase again both in granules and suspension, matching the abundance of *Firmicutes* yet slightly decrease at pH 6.2.

Clostridia, a class of gram-negative bacteria of phylum *Firmicutes*, is one of the most common classes of bacteria present in anaerobic digesters. In this study, it represents between 21% and 56% of MMC in granules and some studies report this class of bacteria as dominant microorganisms in alkaline treatment fermentation (P. Zhang et al., 2010). However, it is also stated as very sensitive to the environmental conditions (Bensaid et al., 2015), as is possible to see in Figure 5.7, *Clostridia* only represents between 3 and 14% of MMC in suspension, indicating that even if this class of bacteria

can survive in acidic environments, its probable optimal growth conditions should be at neutral or alkaline conditions and use the granules as a protection to prosper in acidic environments.

Bacilli includes species gram-positive, capable of surviving at low pH of 3.5 since their high-affinity transport system have binding proteins that are able to function at very acidic conditions (Hulsmann, Lurz, Scheffel, & Schneider, 2000). Here these bacteria remain slightly constant during the whole course experiment in both granules and suspension with a maximum abundance of 15% at pH 6.2 in both granules and suspension.

Negativicutes are similar in their cell membrane to *Clostridia*, being gram-negative in contradiction with general *Firmicutes* bacteria. It starts to appear at pH 5.1 in suspension, rapidly achieving 62% of MMC. The dynamics of MMC variation inside granules is slower and at pH 5.1 in phase I the *Negativicutes* only represent 1% of the culture. However, this class of bacteria is common in this type of processes (Joyce et al., 2018) and gradually reach a maximum of one quarter of all MMC.

Actinobacteria and *Coriobacteria* are both classes of gram-positive bacteria from the phylum *Actinobacteria*. These classes represent 20 to 30% of MMC in granules excepting for pH 6.2, where only reached 15%. In suspension, except for pH 6.2 and 5.8 (phase II), the abundance was usually below 10% and at those higher pH values is possible to see a change from *Coriobacteria* to A*ctinobacteria,* which during the beginning of the test represented almost the totality of phylum *Actinobacteria* and end up achieving 19% of MMC in granules at pH 5.16 (phase II).

The abundance of *Saccharibacteria* is the same discussed in its phylum and is described in previous chapter, since there is scarcity of knowledge about the detailed phylogeny and physiology of *Saccharibacteria*. Nevertheless, they are described as gram-positive bacteria and include filamentous morphotype that could play a pivotal role in granule formation and granule stability (Nielsen, Kragelund, Seviour, & Nielsen, 2009).

Some research works, namely the study of Cheng et al. (2014), have done a statistical analysis comparing microbial communities and FP to study if there are any correlations. Those studies are merely to indicate possible new points of focus for new research and usually require the inclusion of more external data, since the number of samples should be as much as possible and with lower variables.

Cheng used the Pearson correlation to point a positive correlation between the orders *Bacteroidales* and *Clostridiales* with the distribution of the FP, whereas bacteria from *Lactobacillales* and *Bacillales* had a significant negative correlation. However, Pearson correlation should be used for linear correlations and the results obtained here as shown in chapter 5.3 do not follow linear trends. Taking this into account, it was used Spearman's Rs correlation since this method can be used to analyze monotonic trends with much better performance (Puth, Neuhäuser, & Ruxton, 2015).

The distribution of the bacterial communities and FP production in different pH values was analyzed with PAST 3.22 to compare the relationships between the MMC using the data grouped in their taxonomic unit "order" and the FP concentrations using Spearman's Rs correlation. The results are shown in Table 5.3, strong positive correlations are shown in blue and represent values close to 1.00 while strong negative correlations are shown in red and represent values close to -1.00.

The results showed that *Actinomycetales* had a significant positive correlation with the increase of propionate and valerate, whereas the correlation with butyrate is strongly negative, meaning a possible correlation with the decrease of butyrate. This finding matches the results obtained by Horiuchi, Shimizu, Tada, Kanno, & Kobayashi (2002) where the effect of changes in operating pH (ranging from 5.0 to 8.0) on organic acid production in continuous reactors was studied. Their reactors were inoculated with anaerobic digester sludge and fed with a glucose–yeast extract medium where occurred a switch from butyric acid to propionic acid as the pH increased. At that time, it was attributed to a change in the dominant microbial population during the transition period of around 120 h, rather than a metabolic pathway change within the same bacterial

population, which would be expected to occur more quickly (Dareioti, Vavouraki, & Kornaros, 2014b).

Propionibacteriales are described as propionate producers (Cheung, Walsh, & Fung, 1975), here the results show a slightly positive correlation with propionate and valerate production and a significative negative correlation with butyrate and ethanol.

Clostridiales have a significant negative correlation with propionate production yet it was not found any study demonstrating this trend.

Coriobacteriales, which are known producers of ethanol, acetate and lactate (König, 2015) are here positively correlated with ethanol production.

Valerate production by sugars is not well perceived, yet it can be associated with the production of propionate (Lens, O'Flaherty, Dijkema, Colleran, & Stams, 1996) and this statistical analysis shows a similar mathematical trend. Positive correlations detected with some Orders and propionate, could in a very rough way point a production trend. The same correlations are obtained for valerate except for *Saccharibactera*, nevertheless this bacteria order does not have any strong correlation with the rest of FP.

Table 5.3 - Spearman's Rs analysis.

Order	Acetate	butyrate	Lactate	Propionate	Valerate	Ethanol	Sum VFAs	pH
Actinobacteria	0.56	0.21	-0.56	0.05	0.10	-0.82	0.10	0.56
Actinomycetales	0.10	**-1.00**	-0.10	**0.90**	**0.90**	-0.70	-0.70	0.50
Bacteroidales	-0.10	0.30	0.10	-0.60	-0.10	-0.20	0.30	-0.20
Clostridiales	-0.50	0.80	0.50	**-0.90**	-0.60	0.80	0.20	-0.70
Coriobacteriales	-0.60	0.70	0.60	-0.60	-0.50	**1.00**	0.30	-0.80
Lactobacillales	0.30	0.60	-0.30	-0.50	-0.80	0.40	0.00	0.10
Micrococcales	0.30	-0.50	-0.30	0.70	0.20	-0.10	-0.60	0.50
Propionibacteriales	0.30	**-0.90**	-0.30	0.80	0.80	**-0.90**	-0.40	0.60
Saccharibacteria	0.30	-0.60	-0.30	0.80	0.50	-0.50	0.10	0.40
Selenomonadales	0.00	-0.40	0.00	0.70	0.30	0.10	-0.10	0.10
Thermoanaerobacterales	-0.11	-0.11	0.11	-0.11	0.34	-0.45	0.34	-0.11

5.2. IMPACT OF DISSOLVED HYDROGEN CONCENTRATION IN FERMENTATION PRODUCTS

During the phase I of the test two different variables were studied in UASB operation, accompanying the pH variations that were already discussed during each pH stage was applied a mechanical method to reduce dissolved hydrogen in liquid phase, which is assumed that will relieving hydrogen inhibition by improving mass transfer of hydrogen from the cell into the medium (Lovitt, Shen, & Zeikus, 1988).

The measurements of hydrogen inside UASB reactor pointed, contrarily to the general assumption, a difference in hydrogen concentration in liquid phase (dissolved hydrogen) and the hydrogen in gas phase (hydrogen present in headspace). This concentrations in some previous measurements were three times higher in liquid than in gas phase, indicating that in this system the production of hydrogen was higher than the normal mass transfer rate between both phases, probably due to the low surface area. Regarding this, all the concentrations used to discuss results will be the ones measured in liquid phase since it is there that occurs the direct contact with bacterial cells.

This mechanism causes a dilution effect on the hydrogen partial pressure whose value depends not only on the hydrogen content but also on the carbon dioxide which is also affected by the operational pH since it affects the gas/liquid phase distribution of the carbon dioxide produced. The average concentration of dissolved hydrogen in each pH stage and corresponding FP obtained during the test are shown in Table 5.4.

The concentration of dissolved hydrogen in the stages with disperser off have a much higher variation (higher standard deviation) due to the time that the reactor needs to recover to high hydrogen concentrations while simultaneously occurs the increase of the pH. To minimize the errors due to these variations, the studied data did not include samples from the first three days between each operational condition.

The use of liquid dispenser inside the UASB reactor was effective to decrease the hydrogen concentration in liquid phase in all pH stages tested here matching the hydrogen concentration in gas phase. However, with the increase of pH the overall amount of hydrogen measured in the phases where the disperser was set off are lower.

This could happen due to a better equilibrium between hydrogen in liquid and gas phase or a lower production by bacteria.

The variation of dissolved hydrogen concentrations did not cause major differences in FP, however, at some specific stages it is possible to notice some important shifts. Those occur almost exclusively at pH 5.1 which could be attributed to two factors, the variation in dissolved hydrogen only is enough to impact in metabolic pathways at this specific pH value or only at this pH the dissolved hydrogen can make an noticeable impact in metabolic pathways shift surpassing higher major impact factor such as pH and substrate variations.

As shown in Figure 5.8, the global impact that hydrogen variation had in the efficiency of the acidogenesis process both at acidification degree, as HV%. This last parameter as previously mentioned is a key factor in final PHA polymer characteristics. During the European project were this system is being used, it was stated an optimum HV% content of between 15% and 20% (w/w) in PHA.

Impact of pH in acidification degree and HB:HV ratio

Maximum standard deviations

Acidification degree < 6.3%

pH   HV %
< 0.1   <2.5%

	pH 4,7 Disp Off	pH 4,7 Disp On	pH 5,1 Disp Off	pH 5,1 Disp On	pH 5,8 Disp Off	pH 5,8 Disp On	pH 6,2 Disp Off	pH 6,2 Disp On
Average acidification degree	69.64%	69.00%	82.58%	78.26%	70.62%	66.92%	79.06%	72.48%
Average pH	4.71	4.76	5.14	5.23	5.89	5.91	6.24	6.36
Average HV %	9.88%	7.67%	8.26%	13.97%	18.37%	15.01%	11.48%	11.17%

Figure 5.8 - Global impact of dissolved hydrogen test in acidification degree and HV%.

Conditions	pH 4.71 (±0.02) Disp off	pH 4.76 (±0.01) Disp on	pH 5.14 (±0.10) Disp off	pH 5.23 (±0.04) Disp on	pH 5.89 (±0.12) Disp off	pH 5.91 (±0.01) Disp on	pH 6.24 (±0.06) Disp off	pH 6.36 (±0.05) Disp on
Dissolved Hydrogen ($\mu mol/L$)	318.70 (±136.02)	53.14 (±8.80)	184.99 (±69.47)	51.46 (±22.26)	156.11 (±132.13)	79.77 (±45.85)	120.24 (±32.20)	26.10 (±25.40)
FP (mmol/L)								
Acetate	61.63 (±1.65)	62.64 (±1.65)	77.63 (±11.68)	85.90 (±4.62)	78.73 (±6.80)	77.63 (±2.47)	103.09 (±13.28)	92.12 (±6.88)
Butyrate	66.87 (±3.31)	59.81 (±2.79)	75.39 (±9.45)	59.21 (±3.61)	50.79 (±5.07)	49.01 (±0.76)	59.34 (±7.24)	58.04 (±2.35)
Propionate	11.26 (±0.60)	8.88 (±0.93)	11.51 (±4.39)	19.91 (±0.87)	22.76 (±2.04)	16.28 (±2.95)	14.41 (±1.46)	12.22 (±1.13)
Valerate	8.09 (±0.35)	5.39 (±0.92)	5.99 (±1.34)	7.66 (±0.65)	8.79 (±0.47)	7.64 (±0.68)	7.91 (±0.77)	8.10 (±0.30)
Lactate	23.98 (±4.27)	25.63 (±1.24)	7.08 (±1.55)	7.65 (±4.07)	4.89 (±4.40)	1.79 (±0.88)	3.19 (±2.09)	3.46 (±1.03)
Ethanol	23.92 (±0.87)	24.20 (±2.63)	32.70 (±2.78)	17.66 (±4.28)	7.59 (±2.79)	6.94 (±0.61)	7.33 (±0.88)	7.81 (±1.19)
Sum FP	195.77 (±3.27)	186.56 (±3.70)	210.30 (±21.31)	198.00 (±11.27)	173.62 (±17.25)	159.29 (±0.90)	195.26 (±24.69)	181.47 (±5.80)

The results present in Table 5.4 and Figure 5.8, shows that the major advantage of using the liquid disperser can be seen at pH 5.1, while disperser was off the production of HV precursors only reach 8.26%, yet when disperser is used it increases the HV content to 13.97%, which turns the FP profile closely correct to produce PHA for food packaging. This pH condition is followed by the higher acidification degree of all pH stages tested here.

The stage at pH 5.8 shows the maximum content of HV precursors with the disperser off with FP profile reaching 18.37% of HV precursors that is suitable to be used

for food packing, yet the HV content can be lowered using the disperser, since at this pH its use decreases HV precursors to 15.01%. However, at this pH is observed a reduction of around 10% in acidification degree, resulting in overall less efficient process especially considering that this process has as side objective of being a residue treatment.

The operational pH of 4.71 did result in the lower acidification degree and lower HV precursors as well. This condition apparently is the least affected by the dissolved hydrogen in terms of acidification degree variations while HV% precursors change from 9.9% with disperser off to 7.7% while on.

For the last condition, operation at pH 6.2, the acidification degree is comparable to the obtained at pH 5.1 which was the most efficient, yet the FP profile has a HV precursors content of only 11% for both disperser on and off.

The pH between 5.1 and 5.8 is the best pH range to produce the correct FP profile, however, at some pH values the use of the disperser seems to be a good strategy to comply with the correct HV% precursors.

5.3. FERMENTATION PRODUCTS AT DIFFERENT OPERATING CONDITIONS

In Figures 5.9 to 5.14, the data represented as black dots are the concentration of each FP in each sample at the corresponding pH. In all studied pH conditions, it can be observed a larger variability in the data obtained when the disperser is off, since it is at this stage that the changes of the operational pH are made, and this control is done manually. Furthermore, the maximum dissolved hydrogen content also takes some time to be attained, contributing to the data dispersion.

The data was analysed in PAST 3.22 and was applied a polynomial model to trace the equation that shows the trends in FP production. The model represented as a red line was adjusted to fit the dots up to a reasonable lower error (R^2 closer to 1) using orders of 3 up to 5 depending on how much the data was dispersed. The mathematical expression of this polynomial model is shown on top of all charts.

ACETATE PRODUCTION

Generally, the Embden-Meyerhof pathway provides the main route for the conversion of glucose. Bacteria use this pathway to obtain energy from the oxidation of glucose which mainly produces acetate. Figure 5.9 shows that low pH is unfavourable for acetate production, even being the most abundant FP at this pH, acetate production was the lowest of all test. The production increases at higher pH, however, at pH around 5.8 there is a decrease in the absolute concentration, yet in terms of relative concentration, the acetate concentration did not decrease compared to the others FP. This behaviour is, as further showed, due to an overall descend in acidification efficiency followed by an increase in propionate and valerate production.

The main trends observed can be related to the energetics of the transport processes and the free diffusion of the undissociated acids. At neutral pH values, acetate is predicted as the main product since it yields one extra ATP per pyruvate. However, the acetate production decreases at lower pH values since the concentration of the protonated forms of acids increases, resulting in higher energy requirements for outwards transport of acetic acid. This energy required to export molecules outwards of the cell is describe at Figure 2.10. Butyrate replaces a considerable amount of the acetate at lower pH values since the production of one butyrate prevents the production of one acetate and consequently fewer acid molecules need to be transported outside of the bacteria per glucose converted.
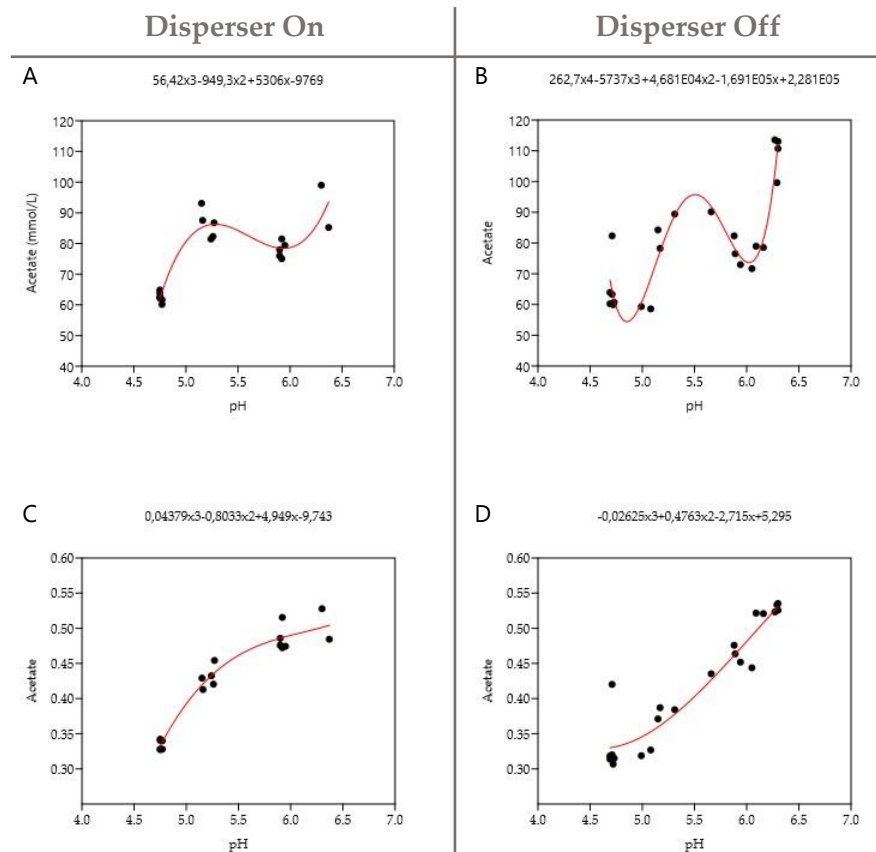
Figure 5.9 – Acetate concentration at different pH: A-disperser on and B-disperser off; Acetate relative concentration at different pH: C-disperser on and D-disperser off.

Whereas during acetate production net NADH is formed, as it derives from pyruvate, it is generally assumed that the NADH that is left cannot be oxidized into H_2, unless the hydrogen partial pressure is very low. Thus, this NADH produced during glycolysis must be oxidized to NAD^+, by reducing other organic compounds and therefore producing more reduced compounds.

Acetate production was not considerably affected by using the disperser, however, at pH 6.3 with high hydrogen concentration, its production is about 10% higher.

It should be expected that lower hydrogen concentrations increase the activity of hydrogenases in the membrane cell, where NADH is oxidized to NAD^+ releasing H_2 outside of the cell. NAD^+ would be used further to oxidize glucose and increase the acetate production however this is not what was observed.

It was confirmed that the feed COD in both conditions was similar, so discarding the variation in substrate content, it is pointed that a global increase can be related to the overall better performance of acidification since all other FP have similar results between the high and low hydrogen content at this pH, which does not indicate a shift in metabolic pathways to a specific product.

BUTYRATE PRODUCTION

Butyrate as previously stated replaces part of the acetate when pH is 5.2 because at more acidic conditions the production of one butyrate reduce the production of one acetate and consequently fewer acid molecules need to be transported outside of the bacteria, yet at an even lower pH such as pH 4.7 butyrate starts to decrease as well leading to an increase of lactate and ethanol. At these low pH values, any acid transport outwards becomes thermodynamically very expensive, so bacteria end up producing high amounts of ethanol reducing the amount of acids produced (Rodríguez et al., 2006). Simultaneously with the increase in ethanol production, the increase on lactic acid which have the lowest pK_a (3.85) of all FP reported here, allows that when this acid is exported to outside of the bacteria cell most of them will be deprotonated which prevents it from re-entering the bacterial cell.
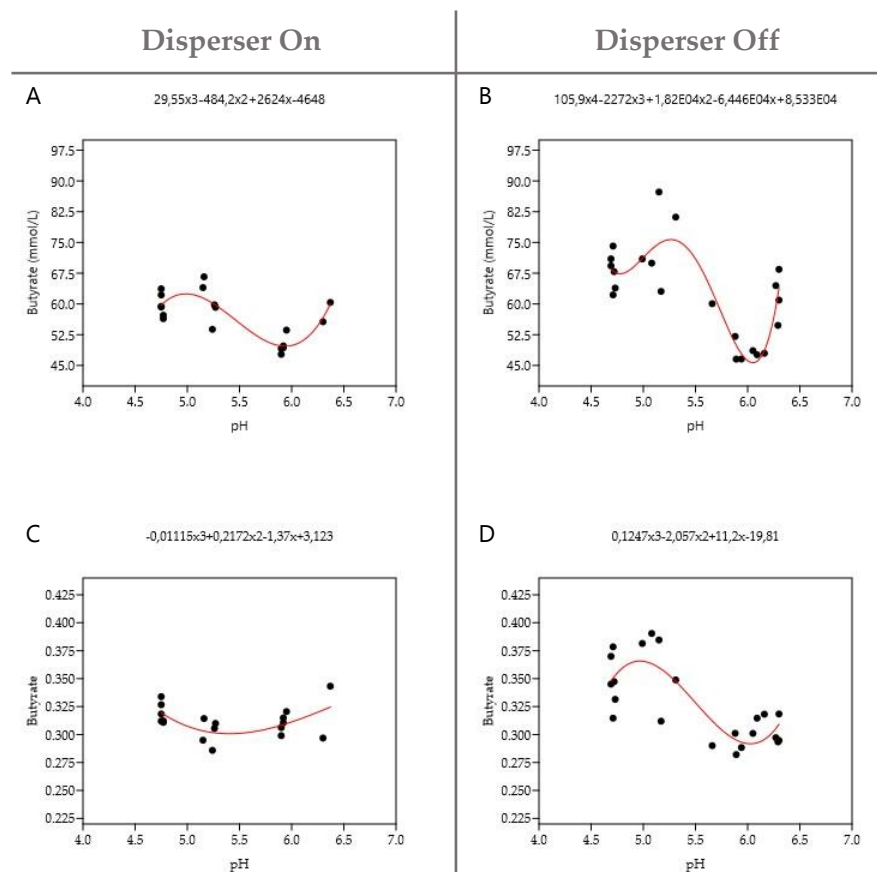
Figure 5.10 - Butyrate concentration at different pH: A-disperser on and B-disperser off; Butyrate relative concentration at different pH: C-disperser on and D-disperser off.

Butyrate and acetate were the two most abundant species in the effluent. These results are consistent with those described by Fang and Liu (Herbert H.P. Fang & Liu, 2002) in some works using glucose as substrate, that consisted in varying pH from 4.0 to 7.0. They observed an increase of acetate followed by a decrease in H_2 and butyrate production as pH increased.

Except at pH 6.0, the high dissolved hydrogen content did increase the butyrate production as shown in Figure 5.10. The increase in the hydrogen pressure should cause a thermodynamic limitation in the acetate production reaction forcing a decrease in its intracellular concentration and making its transport outwards the cell more energetically expensive. This causes a shift to produce more butyrate since hydrogen and acetate are incorporated per butyrate produced.

At pH of 6.3 in both low and high dissolved hydrogen content, the amount of butyrate slightly increases contradicting the general trend where was expected a decrease of butyrate since the amount of acetate also increases, however, as shown in Figure 5.8 and Table 5.4, the acidification degree and the sum of FP also increased. The higher efficiency in acidification increased both the quantity of butyrate and acetate as well, however, as shown in Table 5.4, the difference between both these FP increments, shows higher increase in the production of acetate than the one seen in butyrate, confirming the change of butyrate towards acetate production even at pH close to neutrality.

LACTATE PRODUCTION

The production of lactate was mainly reported as a response of bacteria at low pH as shown in Figure 5.11, in addition to H-ATPase activity which expels protons to outside of the cell. Bacteria have other stress protection mechanisms; many display an inducible acid tolerance response that protects them from being killed in acidic conditions.

Some acid resistant bacteria species did not use this H-ATPase when pH outside of the cell drops to low, using it mainly at low pH gradients, closer to neutral conditions. They maintain a relatively constant pH gradient as the pH outside falls by allowing the pH intern to decrease. This capability is thought to reduce the energy demand for proton translocation through the H-ATPase and has been shown as helping in prevention of intracellular accumulation of organic acid anions formed by the increase of protonated acids being dissociated inside the cell (Zverlov et al., 2010b). Probably due to this reason, when present in acidic environment bacteria increases the synthesis of lactate dehydrogenase since this enzyme have high activity in acidic conditions which greatly increase the lactate production, minimizing the production of acetate and butyrate as previously explained.
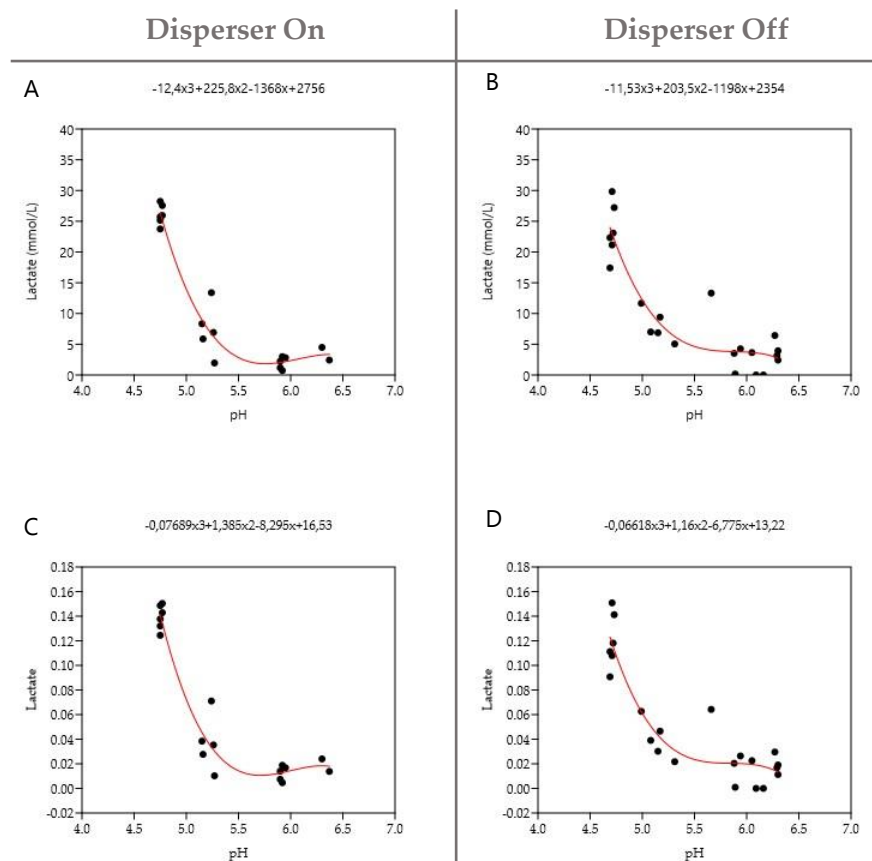
Figure 5.11 - Lactate concentration at different pH: A-disperser on and B-disperser off; Lactate relative concentration at different pH: C-disperser on and D-disperser off.

There is no perceptible difference in lactate production between both low and high hydrogen content, yet in both cases, the lactate production sharply decrease from pH 4.7 to pH 5.1, which may indicate that is not just lactate dehydrogenase losing activity but also that could be coupled with the consume of lactate to other FP such as acetate and propionate.

ETHANOL PRODUCTION

Some authors refer a maximum ethanol concentration at pH 5.0-6.0 (Herbert H.P. Fang & Liu, 2002), while there are different reported results using also fruit and vegetable waste, where the optimal pH for ethanol production was at 4.0 and decreasing while pH increases to 6.0 (Wu et al., 2017). This experiment shows a mix of both results got by

those authors and the concentration of dissolved hydrogen may be an answer for the differences in results.

Ethanol was the third most abundant FP produced with two different trends in production as shown in Figure 5.12. When operating with low hydrogen content, the production follows a trend like results obtained by Wu, where the major production occurs at pH of 4.7 and decreases as pH change to more neutral conditions. However, at high hydrogen content ethanol production increase at pH 5.1 and then rapidly decreases until pH 6.2.

Production of ethanol could be enhanced under specific conditions such as elevated hydrogen concentration, yet ethanol production is associated with low energy and carbon recovery rate due to loss of two carbons.
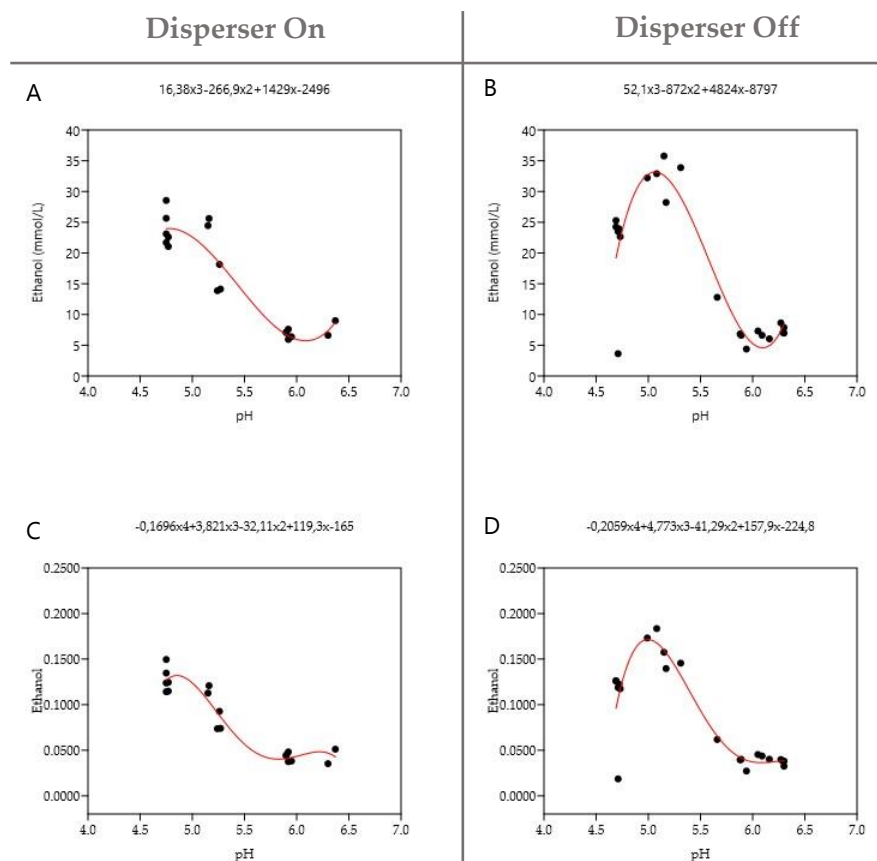

Figure 5.12 - Ethanol concentration at different pH: A-disperser on and B-disperser off; Ethanol relative concentration at different pH: C-disperser on and D-disperser off.

As mentioned before, bacteria increase lactic acid production at low pH since it requires less energy to be exported, yet the production of one mole of lactate can consume the equivalent of one quarter of ATP while its production only generates 1 ATP. As a result, some bacteria may prefer include ethanol production since it does not require transporting ATP for exportation (Maris, Konings, Dijken, & Pronk, 2004).

The increment of ethanol at acidic conditions may causes changes in membrane fluidity and inactivation of H^+/K^+-ATPase activity (Mazzeo, Nandi, & Levine, 1988), which could take an important role in the acid tolerance response. Since many bacteria in low pH change structurally the membrane, the increase of ethanol may take a role in slowing down H-ATPase activity to reduce the consumption of ATP while other strategies are being used to overcome acidic conditions.

PROPIONATE PRODUCTION

Propionate, as the major acid used for HV production, is especially important to be able to change the characteristics of the final PHA. The production trends are described in Figure 5.13 which shows a very different pattern between low and high hydrogen content.

As previously mentioned, the propionate can be produced by two different pathways, acrylate pathway, the most common, where lactate is reduced to propionate when catalysed by propionate dehydrogenase (H. S. Lee et al., 2008) or produced in methylmalonyl-CoA pathway which involves the production of intermediates such as malate and succinate (Vidra et al., 2018). Formation of propionate is usually accompanied by the production of acetate for stoichiometric reasons and to maintain hydrogen and redox balances (Boyaval & Corre, 1995).

The abrupt decrease in lactate production at pH 5.1, could not be due only to LDH loss of activity, but also to the increase of lactate consumption which lead to the formation of acetate and propionate. At low hydrogen concentration, probably due to the activation of the acrylate pathway, lactate concentration decreases, and propionate increases, which can indicate that lactate is being used as intermediate to produce propionate. At pH 5.1 and high hydrogen concentrations, the propionate is the lowest

during all test, a similar behaviour can be seen in the acetate trend at this pH, yet the concentration of lactate in both low and high hydrogen remains similar. This might be explained by three proposed hypotheses.

First hypothesis using acrylate pathway, is that at high hydrogen concentration the activity of propionate and lactate dehydrogenases are reduced at similar rates. The decrease in lactate production at high hydrogen content is comparable to lactate consumption when hydrogen content is low, which ends up showing a similar trend in lactate concentration at both low and high concentrations, while propionate production is much different.

At high hydrogen concentration, pyruvate instead of being used in propionate production, could be converted by Fd to acetyl-CoA, further reduced to acetaldehyde and reduced again to ethanol. This option oxidizes two NADH to NAD^+, the same as in succinate pathway yet with less intermediates, which could explain the increase of ethanol.

Second hypothesis is that acrylate pathway is not the major pathway in propionate production at pH above 5 where propionate production does not interfere with lactate concentration. Here propionate is being produced by methylmanolyl-CoA pathway, which is supported by an increase in *Firmicutes* at this pH as shown in chapter 5.1.2, that are described as being capable of using this pathway, yet this pathway is not very common and the energy balance is not particularly favourable (Dareioti et al., 2014a).

The third hypothesis for the decrease of propionate could be more complex and can be used for both acrylate and methylmanolyl-CoA pathways. This decrease in propionate could be due to the increase in ethanol concentration that reach its maximum where propionate reach its minimum. Both pathways use decarboxylation while producing propionate, the decarboxylation leads to the extrusion of sodium ions, in this way a sodium gradient across the cytoplasmic membrane is built up which is the driving force for ATP synthesis and use it to produce energy (Hilpert, Schink, & Dimroth, 1984). Methylmanolyl-CoA pathway involves a membrane-bound methylmalonyl-CoA decarboxylase which does decarboxylation with Na^+ transport across the membrane while a membrane-bound ATPase specifically activated by Na ions catalyse the transport

of Na$^+$ ions into bacterial vesicles. As previously explained part of the produced ATP is done by the creation of an Na ion gradient which can be generated when decarboxylation of methylmalonyl-CoA occurs (Hilpert et al., 1984). If more species of bacteria use a similar process, this probably could be suffering inhibition by high concentrations of ethanol because this could be interfering with Na$^+$ ATPases and other proteins in membrane. This last hypothesis could be supported by studies that have shown inhibition of the Na$^+$/K$^+$-ATPase by ethanol exposure using brain homogenates, synaptosomes, microsomes and cultured neurons. The precise mechanism responsible for this inhibitory effect of ethanol is unknown, yet it was pointed as a consequence of stabilization of the ATP-bound state and impairing the formation of some of the enzyme's conformational states (Israel, Kalant, & Laufer, 1965).
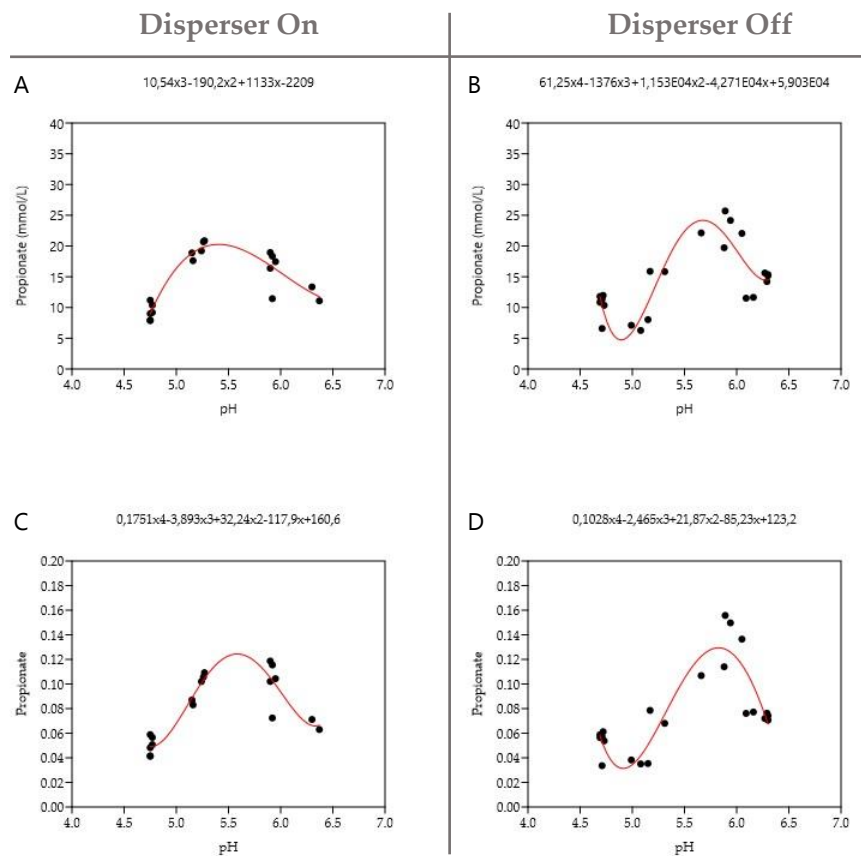

Figure 5.13 - Propionate concentration at different pH: A-disperser on and B-disperser off; Propionate relative concentration at different pH: C-disperser on and D-disperser off.

VALERATE PRODUCTION

Valerate as shown in Figure 5.14 is the lowest FP obtained here and the total variation drifts between 4 and 10 mmol/L, which is difficult to determine effective production trends in so low variation range.
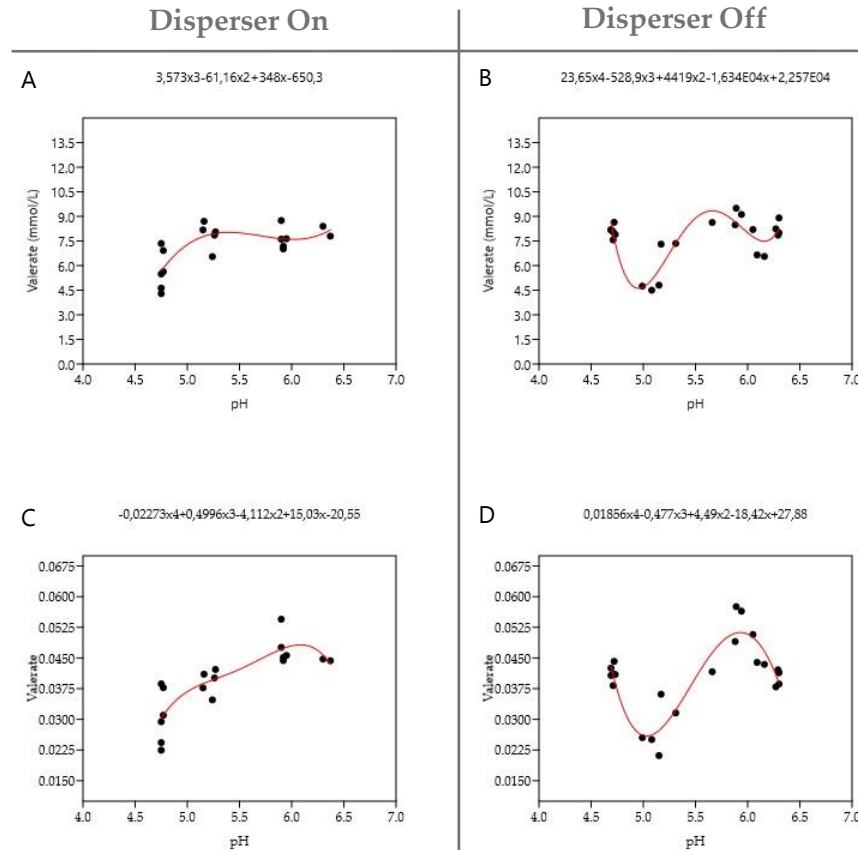

Figure 5.14 - Valerate concentration at different pH: A-disperser on and B-disperser off; Valerate relative concentration at different pH: C-disperser on and D-disperser off.

There was not found any reported mechanisms for valerate production and is not much mentioned in fermentations using sugars as substrate, being more described as being produced by substrates rich in proteins. However, the formation of higher fatty acids like valerate and methylbutyrate by mixed microbial communities has been observed by using 13C-labelled propionate (Lens et al., 1996).

Propionate oxidation is a relatively slow process and it is easily negatively affected by changes in the environmental conditions. For this reason, side reactions in its

biodegradation route are possible as well yet propionate oxidation is reported to occur under methanogenic conditions and is thermodynamically difficult. It can only be degraded by obligatory syntrophic consortia of microorganisms (Stams, Dijkema, Plugge, & Lens, 1998).

Since here there is no active methanogenic bacteria that can degrade propionate it is proposed that valerate might be produced by chain elongations using propionate. If this assumption is correct and since the valerate production follows closely the trend of propionate production, the rate of propionate elongation to valerate should be stable and is not noticeably affected by pH and dissolved hydrogen variations.

IMPACT OF OPERATING CONDITIONS IN HV PRECURSORS CONTENT

The variation of HV precursors as shown in Figure 5.15, follows the same trend as shown in propionate production since this acid is the main HV precursor. The valerate has a lower concentration which does not have major impact in the HV precursors production trend, yet valerate follows a trend mainly like propionate.
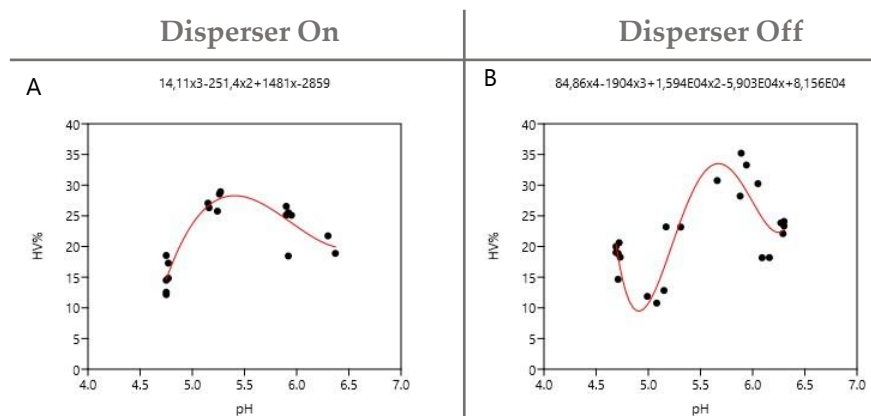
Figure 5.15 - HV% precursors at different pH: A-disperser on and B-disperser off.

Depending on the hydrogen content, it is possible to modify the content of HV precursors. While the disperser was on, the maximum HV content occurs between 5.1 and 5.8. Out of this range, the HV% decreases. During the operation with disperser off,

at pH 5.1 the HV% abruptly decrease since propionate and valerate decreases as described before. For any other tested pH with the same type of operation, it is not perceptible any notable difference between the use or not of the disperser.

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6. Conclusions

Bioplastics have captured the attention recently with many technologies and bioprocesses reaching the market. PHA is one of the biopolymers that could be an important substitute to conventional fossil-based plastic. This work mainly focused in future strategies to control the biopolymer production in fermentation step using MMC, which is an important factor to reduce production costs.

Controlling acidogenic fermentation is crucial to be able to stipulate certain PHA characteristics by changing key points in operational conditions. During this experiment, variations in two different operational parameters of a UASB reactor were made. It was applied a pH increase, named phase I, beginning in pH 4.7 up to 6.2 and were reached four stable pH stages (4.7, 5.1, 5.8 and 6.2). Simultaneous was used at each pH tested a period with a liquid disperser turned on and off to promote two different dissolved hydrogen concentrations. Followed by this test was applied a pH descend to pH 5.8 and 5.2, named phase II, in order to study if the reactor can recover from pH variations and if microbial functional redundancy is verified in this system.

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During the phase I, the maximum FP concentration was reached at pH 5.1, achieving 210 mmol/L, while at pH 5.8 results in the minimum concentration of 173 mmol/L, yet this lower concentration at pH 5.8 is not described by any similar results on literature. In phase II, pH 5.8 reached an FP concentration of 202 mmol/L while at pH 5.1 the concentration is 185 mmol/L. These results are considerably different from the results obtained during the phase I and some variations in fermentation products profiles are also noticed. For this reason, it is stated that microbial functional redundancy was not applied or at least for the exactitude needed to be able to control PHA characteristics has intended. During phase II, pH 5.8 was the best condition, being the only pH stage that complied with the requirement percentage of HV precursors that is between 15% and 20%. However, the strategy of using the liquid disperser to lower the dissolved hydrogen content in the liquid phase of the UASB reactor was effective at pH 5.1, increasing HV precursors from 8.26 to 13.97%, while acidification degree was higher than that at pH 5.8. This strategy allows that at conditions closer to those tested here, the UASB reactor can operate at higher productivity and at the same time, complying with the requirements defined to obtain the desired final polymer composition.

This work shows that this innovative strategy of modifying hydrogen content in the liquid phase using a liquid disperser could be a new strategy to eventually control the final PHA physical characteristics, by altering the fermented product profile produced in the acidogenic step, which is the first step in PHA production using mixed microbial consortia.

7

7. References

Abbasi, T., & Abbasi, S. A. (2012). Formation and impact of granules in fostering clean energy production and wastewater treatment in upflow anaerobic sludge blanket (UASB) reactors. *Renewable and Sustainable Energy Reviews*, *16*(3), 1696–1708. https://doi.org/10.1016/j.rser.2011.11.017

Angelidaki, I., & Ahring, B. K. (1992). Effects of free long-chain fatty acids on thermophilic anaerobic digestion. *Applied Microbiology and Biotechnology*, *37*(6), 808–812. https://doi.org/10.1007/BF00174850

Angelidaki, I., & Ahring, B. K. (1993). Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. *Applied Microbiology and Biotechnology*, *38*(4), 560–564. https://doi.org/10.1007/BF00242955

Artola, A., Balaguer, M. D., & Rigola, M. (1997). Heavy metal binding to anaerobic sludge. *Water Research*, *31*(5), 997–1004. https://doi.org/10.1016/S0043-1354(96)00345-4

Audia, J. P., Webb, C. C., & Foster, J. W. (2001). Breaking through the acid barrier: An

orchestrated response to proton stress by enteric bacteria. *International Journal of Medical Microbiology*, *291*(2), 97–106. https://doi.org/10.1078/1438-4221-00106

Bensaid, S., Ruggeri, B., & Saracco, G. (2015). Development of a photosynthetic microbial electrochemical cell (PMEC) reactor coupled with dark fermentation of organic wastes: Medium term perspectives. *Energies*, *8*(1), 399–429. https://doi.org/10.3390/en8010399

Blakeney, M. (2019). *Food loss and food waste: Causes and solutions. Food Loss and Food Waste: Causes and Solutions*. https://doi.org/10.4337/9781788975391

Bonkowski, M. (2004). Protozoa and plant growth: The microbial loop in soil revisited. *New Phytologist*, *162*(3), 617–631. https://doi.org/10.1111/j.1469-8137.2004.01066.x

Buhr, H. O., & Andrews, J. F. (1977). The thermophilic anaerobic digestion process. *Water Research*. https://doi.org/10.1016/0043-1354(77)90118-X

Byrom, D. (1987). Polymer synthesis by microorganisms: technology and economics. *Trends in Biotechnology*. https://doi.org/10.1016/0167-7799(87)90100-4

Cai, J., Liu, Y., Li, S., Gao, M., & Qin, G. (2016). Photoelectrochemical Behavior of Sn-Doped α-Fe 2 O 3 Photoanode with Different Reducer. *Chinese Journal of Chemistry*, *34*(8), 778–782. https://doi.org/10.1002/cjoc.201600204

Carvalheira, M., Cassidy, J., Ribeiro, J. M., Oliveira, B. A., Freitas, E. B., Roca, C., … Reis, M. A. M. (2018). Performance of a two-stage anaerobic digestion system treating fruit pulp waste: The impact of substrate shift and operational conditions. *Waste Management*, *78*, 434–445. https://doi.org/10.1016/j.wasman.2018.06.013

Castillo Martinez, F. A., Balciunas, E. M., Salgado, J. M., Domínguez González, J. M., Converti, A., & Oliveira, R. P. de S. (2013). Lactic acid properties, applications and production: A review. *Trends in Food Science and Technology*. https://doi.org/10.1016/j.tifs.2012.11.007

Chaganti, S. R., Kim, D. H., & Lalman, J. A. (2011). Flux balance analysis of mixed anaerobic microbial communities: Effects of linoleic acid (LA) and pH on biohydrogen production. In *International Journal of Hydrogen Energy* (Vol. 36, pp. 14141–14152).

https://doi.org/10.1016/j.ijhydene.2011.04.161

Chee, J., Yoga, S., Lau, N., Ling, S., & Abed, R. M. M. (2010). Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, (April 2016), 1395–1404.

Chen, Y., Luo, J., Yan, Y., & Feng, L. (2013). Enhanced production of short-chain fatty acid by co-fermentation of waste activated sludge and kitchen waste under alkaline conditions and its application to microbial fuel cells. *Applied Energy*, *102*, 1197–1204. https://doi.org/10.1016/j.apenergy.2012.06.056

Cheng, W., Chen, H., Yan, S. H., & Su, J. (2014). Illumina sequencing-based analyses of bacterial communities during short-chain fatty-acid production from food waste and sewage sludge fermentation at different pH values. *World Journal of Microbiology and Biotechnology*, *30*(9), 2387–2395. https://doi.org/10.1007/s11274-014-1664-6

Cheung, Y. F., Walsh, C., & Fung, C. H. (1975). Stereochemistry of Propionyl-Coenzyme A and Pyruvate Carboxylations Catalyzed by Transcarboxylase. *Biochemistry*, *14*(13), 2981–2986. https://doi.org/10.1021/bi00684a029

Cirne, D. G., Bond, P., Pratt, S., Lant, P., & Batstone, D. J. (2012). Microbial community analysis during continuous fermentation of thermally hydrolysed waste activated sludge. *Water Science and Technology*, *65*(1), 7–14. https://doi.org/10.2166/wst.2011.705

Colombo, B., Favini, F., Scaglia, B., Sciarria, T. P., D'Imporzano, G., Pognani, M., … Adani, F. (2017). Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. *Biotechnology for Biofuels*, *10*(1), 1–15. https://doi.org/10.1186/s13068-017-0888-8

Comfort, D., & Clubb, R. T. (2004). A Comparative Genome Analysis Identifies Distinct Sorting Pathways in Gram-Positive Bacteria. *Infection and Immunity*, *72*(5), 2710–2722. https://doi.org/10.1128/IAI.72.5.2710-2722.2004

Dareioti, M. A., Vavouraki, A. I., & Kornaros, M. (2014a). Effect of pH on the anaerobic

acidogenesis of agroindustrial wastewaters for maximization of bio-hydrogen production: A lab-scale evaluation using batch tests. *Bioresource Technology*, *162*(3), 218–227. https://doi.org/10.1016/j.biortech.2014.03.149

Dareioti, M. A., Vavouraki, A. I., & Kornaros, M. (2014b). Effect of pH on the anaerobic acidogenesis of agroindustrial wastewaters for maximization of bio-hydrogen production: A lab-scale evaluation using batch tests. *Bioresource Technology*, *162*, 218–227. https://doi.org/10.1016/j.biortech.2014.03.149

Elbeshbishy, E., Hafez, H., Dhar, B. R., & Nakhla, G. (2011). Single and combined effect of various pretreatment methods for biohydrogen production from food waste. *International Journal of Hydrogen Energy*, *36*(17), 11379–11387. https://doi.org/10.1016/j.ijhydene.2011.02.067

European Commission, & Report, T. (2010). *Preparatory Study on Food Waste Across Eu 27. October* (Vol. 33). https://doi.org/10.2779/85947

Fang, H. H.P. (2000). Microbial distribution in UASB granules and its resulting effects. *Water Science and Technology*, *42*(12), 201–208. https://doi.org/10.2166/wst.2000.0272

Fang, H. H P, Liang, D. W., Zhang, T., & Liu, Y. (2006). Anaerobic treatment of phenol in wastewater under thermophilic condition. *Water Research*, *40*(3), 427–434. https://doi.org/10.1016/j.watres.2005.11.025

Fang, Herbert H.P., & Chui, H. K. (1993). Maximum COD loading capacity in UASB reactors at 37°C. *Journal of Environmental Engineering (United States)*, *119*(1), 103–119. https://doi.org/10.1061/(ASCE)0733-9372(1993)119:1(103)

Fang, Herbert H.P., & Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology*, *82*(1), 87–93. https://doi.org/10.1016/S0960-8524(01)00110-9

Fermoso, F. G., Bartacek, J., Manzano, R., van Leeuwen, H. P., & Lens, P. N. L. (2010). Dosing of anaerobic granular sludge bioreactors with cobalt: Impact of cobalt retention on methanogenic activity. *Bioresource Technology*, *101*(24), 9429–9437. https://doi.org/10.1016/j.biortech.2010.07.053

FUSIONS. (2016). *Estimates of European food waste levels. Available in https://www.eu-fusions.org/index.php/publications. IVL-report C 186.* Retrieved from www.eu-fusions.org

Girotto, F., Alibardi, L., & Cossu, R. (2015). Food waste generation and industrial uses: A review. *Waste Management*, *45*, 32–41. https://doi.org/10.1016/j.wasman.2015.06.008

González, J. S., Rivera, A., Borja, R., & Sánchez, E. (1998). Influence of organic volumetric loading rate, nutrient balance and alkalinity: COD ratio on the anaerobic sludge granulation of an UASB reactor treating sugar cane molasses. *International Biodeterioration and Biodegradation*, *41*(2), 127–131. https://doi.org/10.1016/S0964-8305(98)00003-1

Govindaswamy, S., & Vane, L. M. (2007). Kinetics of growth and ethanol production on different carbon substrates using genetically engineered xylose-fermenting yeast. *Bioresource Technology*, *98*(3), 677–685. https://doi.org/10.1016/j.biortech.2006.02.012

Guiot, S. R., Arcand, Y., & Chavarie, C. (1992). Advantages of fluidization on granule size and activity development in upflow anaerobic sludge bed reactors. In *Water Science and Technology* (Vol. 26, pp. 897–906). https://doi.org/10.2166/wst.1992.0470

Guo, X. M., Trably, E., Latrille, E., Carrre, H., & Steyer, J. P. (2010). Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy*, *35*(19), 10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008

Han, W., Fang, J., Liu, Z., & Tang, J. (2016). Techno-economic evaluation of a combined bioprocess for fermentative hydrogen production from food waste. *Bioresource Technology*, *202*, 107–112. https://doi.org/10.1016/j.biortech.2015.11.072

He, M., Sun, Y., Zou, D., Yuan, H., Zhu, B., Li, X., & Pang, Y. (2012). Influence of Temperature on Hydrolysis Acidification of Food Waste. *Procedia Environmental Sciences*, *16*, 85–94. https://doi.org/10.1016/j.proenv.2012.10.012

Hilpert, W., Schink, B., & Dimroth, P. (1984). Life by a new decarboxylation-dependent

energy conservation mechanism with Na + as coupling ion . *The EMBO Journal*, *3*(8), 1665–1670. https://doi.org/10.1002/j.1460-2075.1984.tb02030.x

Horiuchi, J. I., Shimizu, T., Tada, K., Kanno, T., & Kobayashi, M. (2002). Selective production of organic acids in anaerobic acid reactor by pH control. *Bioresource Technology*, *82*(3), 209–213. https://doi.org/10.1016/S0960-8524(01)00195-X

Hulsmann, A., Lurz, R., Scheffel, F., & Schneider, E. (2000). Maltose and maltodextrin transport in the thermoacidophilic gram-positive bacterium Alicyclobacillus acidocaldarius is mediated by a high-affinity transport system that includes a maltose binding protein tolerant to low pH. *Journal of Bacteriology*, *182*(22), 6292–6301. https://doi.org/10.1128/JB.182.22.6292-6301.2000

Israel, Y., Kalant, H., & Laufer, I. (1965). Effects of ethanol on Na, K, Mg-stimulated microsomal atpase activity. *Biochemical Pharmacology*, *14*(12), 1803–1814. https://doi.org/10.1016/0006-2952(65)90270-4

Jackson-Moss, C. A., Duncan, J. R., & Cooper, D. R. (1989). The effect of calcium on anaerobic digestion. *Biotechnology Letters*, *11*(3), 219–224. https://doi.org/10.1007/BF01026064

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G., & van Loosdrecht, M. C. M. (2011). Polyhydroxybutyrate production from lactate using a mixed microbial culture. *Biotechnology and Bioengineering*, *108*(9), 2022–2035. https://doi.org/10.1002/bit.23148

Joyce, A., Ijaz, U. Z., Nzeteu, C., Vaughan, A., Shirran, S. L., Botting, C. H., … Abram, F. (2018). Linking microbial community structure and function during the acidified anaerobic digestion of grass. *Frontiers in Microbiology*, *9*(MAR), 1–13. https://doi.org/10.3389/fmicb.2018.00540

Kayhanian, M. (1999). Ammonia inhibition in high-solids biogasification: An overview and practical solutions. *Environmental Technology (United Kingdom)*, *20*(4), 355–365. https://doi.org/10.1080/09593332008616828

Kim, D. Y., Hyung, W. K., Moon, G. C., & Young, H. R. (2007). Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates.

Journal of Microbiology, *45*(2), 87–97. https://doi.org/1

Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology*, *37*, 24–38. https://doi.org/10.1016/j.nbt.2016.05.001

König, H. (2015). Coriobacterium . *Bergey's Manual of Systematics of Archaea and Bacteria*, 1–3. https://doi.org/10.1002/9781118960608.gbm00216

Konings, W. N., Albers, S. V., Koning, S., & Driesen, A. J. M. (2002). The Cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*(1–4), 61–72. https://doi.org/10.1023/A:1020573408652

Konings, W. N., Lolkema, J. S., & Poolman, B. (1995). The generation of metabolic energy by solute transport. *Archives of Microbiology*. https://doi.org/10.1007/BF02529957

Kunasundari, B., & Sudesh, K. (2011). Isolation and recovery of microbial polyhydroxyalkanoates. *Express Polymer Letters*. https://doi.org/10.3144/expresspolymlett.2011.60

Lee, H. S., Salerno, M. B., & Rittmann, B. E. (2008). Thermodynamic evaluation on H2 production in glucose fermentation. *Environmental Science and Technology*, *42*(7), 2401–2407. https://doi.org/10.1021/es702610v

Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering*. https://doi.org/10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.3.CO;2-1

Lee, Y. J., Miyahara, T., & Noike, T. (2001). Effect of iron concentration on hydrogen fermentation. *Bioresource Technology*, *80*(3), 227–231. https://doi.org/10.1016/S0960-8524(01)00067-0

Lemos, P. C., Serafim, L. S., & Reis, M. A. M. (2006). Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. *Journal of Biotechnology*, *122*(2), 226–238. https://doi.org/10.1016/j.jbiotec.2005.09.006

Leng, L., Yang, P., Singh, S., Zhuang, H., Xu, L., Chen, W. H., … Lee, P. H. (2018). A review on the bioenergetics of anaerobic microbial metabolism close to the

thermodynamic limits and its implications for digestion applications. *Bioresource Technology*, *247*(September), 1095–1106. https://doi.org/10.1016/j.biortech.2017.09.103

Lens, P. N. L., O'Flaherty, V., Dijkema, C., Colleran, E., & Stams, A. J. M. (1996). Propionate degradation by mesophilic anaerobic sludge: Degradation pathways and effects of other volatile fatty acids. *Journal of Fermentation and Bioengineering*, *82*(4), 387–391. https://doi.org/10.1016/0922-338X(96)89156-2

Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W., & Klapwijk, A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*, *22*(4), 699–734. https://doi.org/10.1002/bit.260220402

Li, Y.-Y., P. Fang, H. H., Chen, T., & Chui, H.-K. (1995). UASB Treatment of Wastewater Containing Concentrated Benzoate. *Journal of Environmental Engineering*, *121*(10), 748–751. https://doi.org/10.1061/(asce)0733-9372(1995)121:10(748)

Liu, Yu, Xu, H. Lou, Yang, S. F., & Tay, J. H. (2003). Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. *Water Research*, *37*(3), 661–673. https://doi.org/10.1016/S0043-1354(02)00351-2

Liu, Yuchen, & Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. In *Annals of the New York Academy of Sciences* (Vol. 1125, pp. 171–189). https://doi.org/10.1196/annals.1419.019

Lolkema, J. S., Speelmans, G., & Konings, W. N. (1994). Na+-coupled versus H+-coupled energy transduction in bacteria. *BBA - Bioenergetics*, *1187*(2), 211–215. https://doi.org/10.1016/0005-2728(94)90113-9

Lovitt, R. W., Shen, G. J., & Zeikus, J. G. (1988). Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in Clostridium thermohydrosulfuricum. *Journal of Bacteriology*, *170*(6), 2809–2815. https://doi.org/10.1128/jb.170.6.2809-2815.1988

Lund, P., Tramonti, A., & De Biase, D. (2014). Coping with low pH: Molecular strategies in neutralophilic bacteria. *FEMS Microbiology Reviews*, *38*(6), 1091–1125.

https://doi.org/10.1111/1574-6976.12076

Macy, J. M., & Probst, I. (1979). The Biology of Gastrointestinal Bacteroides. *Annual Review of Microbiology*, *33*(1), 561–594. https://doi.org/10.1146/annurev.mi.33.100179.003021

Maris, A. J. A. V., Konings, W. N., Dijken, J. P. V., & Pronk, J. T. (2004). Microbial export of lactic and 3-hydroxypropanoic acid: Implications for industrial fermentation processes. *Metabolic Engineering*. https://doi.org/10.1016/j.ymben.2004.05.001

Mazzeo, A. R., Nandi, J., & Levine, R. A. (1988). Effects of ethanol on parietal cell membrane phospholipids and proton pump function. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *254*(1), G57–G64. https://doi.org/10.1152/ajpgi.1988.254.1.g57

McHugh, S., Carton, M., Mahony, T., & O'Flaherty, V. (2003). Methanogenic population structure in a variety of anaerobic bioreactors. *FEMS Microbiology Letters*, *219*(2), 297–304. https://doi.org/10.1016/S0378-1097(03)00055-7

Melchior, D. L. (1982). Lipid Phase Transitions and Regulation of Membrane Fluidity in Prokaryotes. In F. Bronner & A. Kleinteller (Eds.), *Current Topics in Membranes and Transport* (Vol. 17, pp. 263–316). Academic Press. https://doi.org/10.1016/S0070-2161(08)60314-7

Mendel, S., & Robinson, C. (2007). Targeting of Proteins by the Twin-Arginine Translocation System in Bacteria and Chloroplasts. In R. E. Dalbey, C. M. Koehler, & F. Tamanoi (Eds.), *Enzymes* (Vol. 25, pp. 69–91). Academic Press. https://doi.org/10.1016/S1874-6047(07)25003-6

Muhammadi, Shabina, Afzal, M., & Hameed, S. (2015). Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications. *Green Chemistry Letters and Reviews*, *8*(3–4), 56–77. https://doi.org/10.1080/17518253.2015.1109715

Müller, N., Worm, P., Schink, B., Stams, A. J. M., & Plugge, C. M. (2010). Syntrophic butyrate and propionate oxidation processes: From genomes to reaction mechanisms. *Environmental Microbiology Reports*. https://doi.org/10.1111/j.1758-

2229.2010.00147.x

Neshat, S. A., Mohammadi, M., Najafpour, G. D., & Lahijani, P. (2017). Anaerobic co-digestion of animal manures and lignocellulosic residues as a potent approach for sustainable biogas production. *Renewable and Sustainable Energy Reviews*, *79*(July 2016), 308–322. https://doi.org/10.1016/j.rser.2017.05.137

Nielsen, P. H., Kragelund, C., Seviour, R. J., & Nielsen, J. L. (2009). Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS Microbiology Reviews*. https://doi.org/10.1111/j.1574-6976.2009.00186.x

Ojumu, T. V., Yu, J., & Solomon, B. O. (2004). Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. *African Journal of Biotechnology*, *3*(1), 18–24. https://doi.org/10.5897/AJB2004.000-2004

Pakalapati, H., Chang, C. K., Show, P. L., Arumugasamy, S. K., & Lan, J. C. W. (2018). Development of polyhydroxyalkanoates production from waste feedstocks and applications. *Journal of Bioscience and Bioengineering*, *126*(3), 282–292. https://doi.org/10.1016/j.jbiosc.2018.03.016

Parawira, W., Murto, M., Zvauya, R., & Mattiasson, B. (2006). Comparative performance of a UASB reactor and an anaerobic packed-bed reactor when treating potato waste leachate. *Renewable Energy*, *31*(6), 893–903. https://doi.org/10.1016/j.renene.2005.05.013

Parkin, G. F., & Owen, W. F. (1986). Fundamentals of anaerobic digestion of wastewater sludges. *Journal of Environmental Engineering (United States)*, *112*(5), 867–920. https://doi.org/10.1061/(ASCE)0733-9372(1986)112:5(867)

Pfaltzgraff, L. A., De Bruyn, M., Cooper, E. C., Budarin, V., & Clark, J. H. (2013). Food waste biomass: A resource for high-value chemicals. *Green Chemistry*, *15*(2), 307–314. https://doi.org/10.1039/c2gc36978h

Poirier, S., & Chapleur, O. (2018). Inhibition of anaerobic digestion by phenol and ammonia: Effect on degradation performances and microbial dynamics. *Data in Brief*, *19*, 2235–2239. https://doi.org/10.1016/j.dib.2018.06.119

Presser, K. A., Ross, T., & Ratkowsky, D. A. (1998). Modelling the growth limits (growth/no

growth interface) of Escherichia coli as a function of temperature, pH, lactic acid concentration, and water activity. *Applied and Environmental Microbiology*, *64*(5), 1773–1779.

Puth, M. T., Neuhäuser, M., & Ruxton, G. D. (2015). Effective use of Spearman's and Kendall's correlation coefficients forassociation between two measured traits. *Animal Behaviour*. https://doi.org/10.1016/j.anbehav.2015.01.010

Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration and Biodegradation*, *126*(January 2017), 45–56. https://doi.org/10.1016/j.ibiod.2017.10.001

Reis, M., Albuquerque, M., Villano, M., & Majone, M. (2011). *Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks. Comprehensive Biotechnology, Second Edition* (Second Edi, Vol. 6). Elsevier B.V. https://doi.org/10.1016/B978-0-08-088504-9.00464-5

Rhu, D. H., Lee, W. H., Kim, J. Y., & Choi, E. (2003). Polyhydroxyalkanoate (PHA) production from waste. In *Water Science and Technology* (Vol. 48, pp. 221–228). https://doi.org/10.2166/wst.2003.0472

Rico, C., Muñoz, N., Fernández, J., & Rico, J. L. (2015). High-load anaerobic co-digestion of cheese whey and liquid fraction of dairy manure in a one-stage UASB process: Limits in co-substrates ratio and organic loading rate. *Chemical Engineering Journal*, *262*, 794–802. https://doi.org/10.1016/j.cej.2014.10.050

Rinzema, A., Boone, M., van Knippenberg, K., & Lettinga, G. (1994). Bactericidal effect of long chain fatty acids in anaerobic digestion. *Water Environment Research*, *66*(1), 40–49. https://doi.org/10.2175/wer.66.1.7

Rodríguez, J., Kleerebezem, R., Lema, J. M., & Van Loosdrecht, M. C. M. (2006). Modeling product formation in anaerobic mixed culture fermentations. *Biotechnology and Bioengineering*, *93*(3), 592–606. https://doi.org/10.1002/bit.20765

Russell, J. B., & Wilson, D. B. (1996). Why Are Ruminal Cellulolytic Bacteria Unable to Digest Cellulose at Low pH? *Journal of Dairy Science*, *79*(8), 1503–1509.

https://doi.org/10.3168/jds.S0022-0302(96)76510-4

Saady, N. M. C. (2013). Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *International Journal of Hydrogen Energy*. https://doi.org/10.1016/j.ijhydene.2013.07.122

Salminen, E. A., & Rintala, J. A. (2002). Semi-continuous anaerobic digestion of solid poultry slaughterhouse waste: Effect of hydraulic retention time and loading. *Water Research*, *36*(13), 3175–3182. https://doi.org/10.1016/S0043-1354(02)00010-6

Schmidt, J. E., & Ahring, B. K. (1996). Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering*. https://doi.org/10.1002/(SICI)1097-0290(19960205)49:3<229::AID-BIT1>3.0.CO;2-M

Seeliger, S., Janssen, P. H., & Schink, B. (2002). Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiology Letters*, *211*(1), 65–70. https://doi.org/10.1016/S0378-1097(02)00651-1

Shen, F., Yuan, H., Pang, Y., Chen, S., Zhu, B., Zou, D., … Li, X. (2013). Performances of anaerobic co-digestion of fruit & vegetable waste (FVW) and food waste (FW): Single-phase vs. two-phase. *Bioresource Technology*, *144*, 80–85. https://doi.org/10.1016/j.biortech.2013.06.099

Show, K. Y., Wang, Y., Foong, S. F., & Tay, J. H. (2004). Accelerated start-up and enhanced granulation in upflow anaerobic sludge blanket reactors. *Water Research*, *38*(9), 2293–2304. https://doi.org/10.1016/j.watres.2004.01.039

Shyue Koong Chang, & Schonfeld, P. M. (1991). Multiple period optimization of bus transit systems. *Transportation Research Part B*, *25*(6), 453–478. https://doi.org/10.1016/0191-2615(91)90038-K

Silva, F., Campanari, S., Matteo, S., Valentino, F., Majone, M., & Villano, M. (2017). Impact of nitrogen feeding regulation on polyhydroxyalkanoates production by mixed microbial cultures. *New Biotechnology*, *37*, 90–98. https://doi.org/10.1016/j.nbt.2016.07.013

Singh, R. P., Kumar, S., & Ojha, C. S. P. (1999). Nutrient requirement for UASB process: A review. *Biochemical Engineering Journal*, *3*(1), 35–54. https://doi.org/10.1016/S1369-703X(98)00043-6

Siriwongrungson, V., Zeng, R. J., & Angelidaki, I. (2007). Homoacetogenesis as the alternative pathway for H2 sink during thermophilic anaerobic degradation of butyrate under suppressed methanogenesis. *Water Research*, *41*(18), 4204–4210. https://doi.org/10.1016/j.watres.2007.05.037

Stams, A. J. M., & Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology*. https://doi.org/10.1038/nrmicro2166

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology*, *83*(1), 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Syutsubo, K., Harada, H., Ohashi, A., & Suzuki, H. (1997). An effective start-up of thermophilic UASB reactor by seeding mesophilically-grown granular sludge. In *Water Science and Technology* (Vol. 36, pp. 391–398). https://doi.org/10.1016/S0273-1223(97)00547-7

Teer, J. E., Leak, D. J., Dudeney, A. W. L., Narayanan, A., & Stuckey, D. C. (2000). Changes in pre-formed granule composition and structure in a sulphidogenic uasb treating a synthetic ferric oxalate wastewater. *Environmental Technology (United Kingdom)*, *21*(12), 1325–1335. https://doi.org/10.1080/09593332208618165

Temudo, M. F., Kleerebezem, R., & Van Loosdrecht, M. (2007). Influence of the pH on (Open) mixed culture fermentation of glucose: A chemostat study. *Biotechnology and Bioengineering*, *98*(1), 69–79. https://doi.org/10.1002/bit.21412

Tuck, C. O., Pérez, E., Horváth, I. T., Sheldon, R. A., & Poliakoff, M. (2012). Valorization of biomass: Deriving more value from waste. *Science*, *337*(6095), 695–699. https://doi.org/10.1126/science.1218930

Uçkun Kiran, E., Trzcinski, A. P., Ng, W. J., & Liu, Y. (2014). Enzyme Production from Food Wastes Using a Biorefinery Concept. *Waste and Biomass Valorization*, *5*(6), 903–917.

https://doi.org/10.1007/s12649-014-9311-x

Vaňáčová, Š., Rasoloson, D., Rázga, J., Hrdý, I., Kulda, J., & Tachezy, J. (2001). Iron-induced changes in pyruvate metabolism of Tritrichomonas foetus and involvement of iron in expression of hydrogenosomal proteins. *Microbiology*, *147*(1), 53–62. https://doi.org/10.1099/00221287-147-1-53

Venkiteshwaran, K., Bocher, B., Maki, J., & Zitomer, D. (2015). Relating Anaerobic Digestion Microbial Community and Process Function : Supplementary Issue: Water Microbiology. *Microbiology Insights*, *8s2*, MBI.S33593. https://doi.org/10.4137/mbi.s33593

Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *MBio*, *5*(2). https://doi.org/10.1128/mBio.00889-14

Westerholm, M., Hansson, M., & Schnürer, A. (2012). Improved biogas production from whole stillage by co-digestion with cattle manure. *Bioresource Technology*, *114*, 314–319. https://doi.org/10.1016/j.biortech.2012.03.005

Witholt, B., & Kessler, B. (1999). Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics. *Current Opinion in Biotechnology*. https://doi.org/10.1016/S0958-1669(99)80049-4

Wu, Y., Wang, C., Zheng, M., Zuo, J., Wu, J., Wang, K., & Yang, B. (2017). Effect of pH on ethanol-type acidogenic fermentation of fruit and vegetable waste. *Waste Management*, *60*, 158–163. https://doi.org/10.1016/j.wasman.2016.09.033

Yan, B. H., Selvam, A., & Wong, J. W. C. (2014). Application of rumen microbes to enhance food waste hydrolysis in acidogenic leach-bed reactors. *Bioresource Technology*, *168*, 64–71. https://doi.org/10.1016/j.biortech.2014.03.085

Yu, J. (2007). Microbial Production of Bioplastics from Renewable Resources. In *Bioprocessing for Value-Added Products from Renewable Resources* (pp. 585–610). https://doi.org/10.1016/B978-044452114-9/50024-4

Zhang, B., Carlson, R., & Srienc, F. (2006). Engineering the monomer composition of polyhydroxyalkanoates synthesized in Saccharomyces cerevisiae. *Applied and*

Environmental Microbiology, *72*(1), 536–543. https://doi.org/10.1128/AEM.72.1.536-543.2006

Zhang, F., Zhang, Y., Chen, M., Van Loosdrecht, M. C. M., & Zeng, R. J. (2013a). A modified metabolic model for mixed culture fermentation with energy conserving electron bifurcation reaction and metabolite transport energy. *Biotechnology and Bioengineering*, *110*(7), 1884–1894. https://doi.org/10.1002/bit.24855

Zhang, F., Zhang, Y., Chen, M., Van Loosdrecht, M. C. M., & Zeng, R. J. (2013b). A modified metabolic model for mixed culture fermentation with energy conserving electron bifurcation reaction and metabolite transport energy. *Biotechnology and Bioengineering*, *110*(7), 1884–1894. https://doi.org/10.1002/bit.24855

Zhang, P., Chen, Y., Zhou, Q., Zheng, X., Zhu, X., & Zhao, Y. (2010). Understanding short-chain fatty acids accumulation enhanced in waste activated sludge alkaline fermentation: Kinetics and microbiology. *Environmental Science and Technology*, *44*(24), 9343–9348. https://doi.org/10.1021/es102878m

Zheng, D., Angenent, L. T., & Raskin, L. (2006). Monitoring granule formation in anaerobic upflow bioreactors using oligonucleotide hybridization probes. *Biotechnology and Bioengineering*, *94*(3), 458–472. https://doi.org/10.1002/bit.20870

Zhou, M., Yan, B., Wong, J. W. C., & Zhang, Y. (2018). Enhanced volatile fatty acids production from anaerobic fermentation of food waste: A mini-review focusing on acidogenic metabolic pathways. *Bioresource Technology*, *248*, 68–78. https://doi.org/10.1016/j.biortech.2017.06.121

Zhu, H., Parker, W., Basnar, R., Proracki, A., Falletta, P., Béland, M., & Seto, P. (2009). Buffer requirements for enhanced hydrogen production in acidogenic digestion of food wastes. *Bioresource Technology*, *100*(21), 5097–5102. https://doi.org/10.1016/j.biortech.2009.02.066

Zverlov, V. V., Hiegl, W., Köck, D. E., Kellermann, J., Köllmeier, T., & Schwarz, W. H. (2010a). Hydrolytic bacteria in mesophilic and thermophilic degradation of plant biomass. *Engineering in Life Sciences*, *10*(6), 528–536. https://doi.org/10.1002/elsc.201000059

Zverlov, V. V., Hiegl, W., Köck, D. E., Kellermann, J., Köllmeier, T., & Schwarz, W. H. (2010b). Hydrolytic bacteria in mesophilic and thermophilic degradation of plant biomass. *Engineering in Life Sciences*, *10*(6), 528–536. https://doi.org/10.1002/elsc.201000059