Eliana da Costa Guarda

Bachelor Degree in Biochemistry

Acidogenic fermentation of brewer's spent grain towards polyhydroxyalkanoates production

Dissertation to obtain Master Degree in Biotechnology

Supervisor: Anouk F. Duque, Post-Doctoral Researcher, FCT-UNL

Júri:

Presidente: Prof. Doutor Pedro Calado Simões

Arguente: Prof. Doutora Leonor Amaral

Vogal: Doutora Anouk F. Duque

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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"(…) E aceitas as derrotas com a cabeça erguida e olhos adiante, com a graça de um adulto e não com a tristeza de uma criança. E aprendes a construir todas as tuas estradas de hoje, porque o terreno do amanhã é incerto demais para os planos, e o futuro tem o mau hábito de cair em pleno voo. (…) aprendes que paciência requer muita prática. Descobres que algumas vezes a pessoa que esperas que te empurre, quando cais, é uma das poucas que te ajuda a levantar. Aprendes que maturidade tem mais a ver com os tipos de experiência que tiveste e o que aprendeste com elas do que com quantos aniversários já comemoraste.

Portanto, planta o teu jardim e decora a tua alma, ao invés de esperares que alguém te traga flores."

William Shakespeare

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Resumo

Polihidroxialcanoatos (PHA) são poliésteres totalmente biodegradáveis, sintetizados por microrganismos a partir de recursos renováveis, que representam uma alternativa aos plásticos convencionais. A sua produção por culturas microbianas mistas, ao contrário das culturas puras, permite a utilização de subprodutos industriais na ausência de condições de esterilidade, reduzindo os custos do processo. O trabalho descrito focou-se na valorização de um resíduo da indústria cervejeira, a dreche, através da produção de ácidos orgânicos, visando a produção de PHA. O processo para produção de PHA por culturas mistas envolve três etapas: (1) fermentação acidogénica para a produção de ácidos orgânicos, (2) seleção das culturas mistas capazes de acumular PHA e (3) produção de PHA.

Por forma a extrair os açúcares necessários à produção de ácidos orgânicos (fase 1), foram estudados métodos de pré-tratamento, nomeadamente hidrólise ácida e água subcrítica. A hidrólise ácida foi o método selecionado dada a obtenção de um rendimento superior à água subcrítica (de 0.152 g açúcares totais g dreche^{-1} versus 0.008 e 0.020 g açúcares totais g dreche^{-1} a 200 °C e 230 °C, respetivamente).

Seguidamente, estudou-se o efeito do pH (5.0, 5.5 e 6.0) e da carga orgânica (10, 20 e 30 gCOD L^{-1}) na fermentação acidogénica da dreche hidrolisada em batch. Verificou-se que, independentemente da condição, os ácidos mais produzidos são o acético e butírico. Uma vez que o pH e a carga orgânica afetam o perfil dos produtos de fermentação, é possível manipular a composição do PHA.

Numa última fase, estudou-se a fermentação acidogénica da dreche hidrolisada em contínuo, usando um reator anaeróbio granular de leito expandido. Dado o período de operação demasiado curto, não foi possível atingir a estabilidade no reator. No entanto, verificou-se que os açúcares são consumidos para a produção de ácidos orgânicos, demonstrando que este reator tem potencial para a acidogénese da dreche com vista à produção de PHA.

Palavras-chave: Dreche, grânulos anaeróbios, fermentação acidogénica, ácidos orgânicos, culturas mistas microbianas, reator granular de leito expandido

Abstract

Polyhydroxyalkanoates (PHA) are fully biodegradable polyesters, synthesized by microorganisms from renewable resources, which reveal an alternative to petroleum-based plastics. Its production by mixed microbial cultures, unlike pure cultures, allows the use of industrial by-products in the absence of sterility conditions, potentially reducing process costs. The study described focused on the valorization of a waste from a brewery industry, brewer's spent grain (BSG), through organic acids production, towards PHA production. PHA production using mixed microbial cultures involves three steps: (1) acidogenic fermentation of BSG for organic acids production, (2) selection of the mixed cultures able to accumulate PHA and (3) PHA production.

In order to extract the sugars necessary to produce organic acids (phase 1), pre-treatment methods such as acid hydrolysis and subcritical water have been studied. Acid hydrolysis was selected for further experiments as the hydrolysis yield on sugars reached was higher than using subcritical water (0.152 g total sugars g BSG^{-1} versus 0.008 and 0.020 g total sugars g BSG^{-1} at 200 °C and 230 °C, respectively).

Afterwards, the pH (5.0, 5.5 and 6.0) and organic loads (10, 20 and 30 gCOD L^{-1}) effect on the acidogenic fermentation of hydrolyzed BSG was studied in batch. It was verified, regardless of the condition, that the most produced acids are acetic and butyric. As pH and organic load affects the fermentation products profile, it is possible to manipulate PHA composition.

At last, the acidogenic fermentation of hydrolyzed BSG was studied in a continuous mode, using an expanded granular sludge bed reactor (EGSB). Due to the reduced operating time, it was not possible to reach stability. However, it was observed that sugars were consumed to produce organic acids. This revealed the potential of EGSB to be used for BSG acidogenic fermentation towards PHA production.

Keywords: brewer's spent grain, anaerobic granules, acidogenic fermentation, organic acids, mixed microbial cultures, expanded granular sludge bed reactor

List of Abbreviations

BSG - Brewer's spent grain

C:N:P- Ratio of carbon:nitrogen:phosphorus (C-mol:N-mol:P-mol)

CSTR - Continuous stirred tank reactor

CoA - Coenzyme A

COD - Chemical oxygen demand (gO_2 L^{-1})

DF - Degree of fermentation (gCOD-FP $gCOD^{-1}$)

EGSB - Expanded granular sludge bed

EtOH - Ethanol

FCT - Faculdade de ciências e tecnologia

F/F - Ratio feast and famine (h h^{-1})

FP - Fermentation products (gCOD L^{-1})

GC - Gas chromatography

HAc - Acetic acid

HB - Hydroxybutyrate

HBut - Butyric acid

HIso - Isovaleric acid

HOrg - Organic acids

HPLC - High-performance liquid chromatography

HProp - Propionic acid

HRT - Hydraulic retention time (hours or days)

HV - Hydroxyvalerate

HVal - Valeric acid

K_w - Ionic product (mol^2 L^{-2})

MMC - Mixed microbial cultures

OL - Organic load (gCOD L^{-1})

OLR - Organic loading rate (gCOD L^{-1} h^{-1})

PHA - Polyhydroxyalkanoate

q_{FP} - Specific fermentation products production rate (gCOD-FP $gVSS^{-1}$ h^{-1} or gCOD-FP $gVSS^{-1}$ d^{-1})

q_S - Specific substrate uptake rate (gCOD $gVSS^{-1}$ h^{-1} or gCOD $gVSS^{-1}$ d^{-1})

r_{FP} - Volumetric fermentation products production rate (gCOD-FP L^{-1} h^{-1} or gCOD-FP L^{-1} d^{-1})

r_S - Volumetric substrate uptake rate (gCOD L^{-1} h^{-1} or gCOD L^{-1} d^{-1})

S - Substrate (gO_2 L^{-1})

SBR - Sequencing batch reactor

SCW - Subcritical water

SRT - Sludge retention time

TCA - Tricarboxylic acid cycle

TSS - Total suspended solids (g L^{-1})

V_{up} - Liquid upflow velocity (m h^{-1})

VFA - Volatile fatty acids

VSS - Volatile suspended solids (gVSS L^{-1})

$Y_{FP/S}$ - Yield of fermentation products on substrate (gCOD-FP $gCOD^{-1}$)

$Y_{X/S}$ - Yield of biomass on carbon (gVSS $gCOD^{-1}$ h^{-1})

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1. General Introduction

1.1 State of the art: how the bioplastics can reshape the plastic industry?

Currently, plastics production is continuously growing at global level (Plastics Europe, 2015), evidencing the world dependence on this synthetic plastic with an expected demand of 334.83 million tons in 2020 (Grand View Research, 2014). Plastics are polymeric molecules derived from limited fossil fuels, with a wide range of applications, due to their properties and low-cost production (Shah et al., 2008, Jain and Tiwari, 2015). However, they have been considered a serious worldwide environmental and health problem due to their non-biodegradable nature and to the lack of an appropriate treatment (Jain and Tiwari, 2015; Reddy et al., 2003). The current solutions to plastic waste, like source reduction, recycling, incineration and bio or photo-degradation appears to be insufficient and with associated disadvantages (Ben et al., 2016; Brockhaus et al., 2015; North and Halden, 2013). The increase of plastics waste makes crucial to focus on the research and development of emergent alternatives with direct association with sustainability (Alvarez-Chavez et al., 2012).

1.2 Bioplastics as a promising alternative to conventional plastics

The constant and rising demand of more sophisticated materials are responsible for the production, by bioplastics industries, of 1% of about 320 million tons of plastic produced annually, according to the European Bioplastics (Bioplastics, 2016). Bioplastics are a group of natural polymers, synthesized and catabolized by numerous organisms, considered as promising substitutes to the conventional plastics (Suriyamongkol et al., 2007). As environmental friendly, they can be produced from renewable resources (biobased) like industrial and/or agricultural wastes, reducing the dependency on limited fossil resources, decreasing greenhouse gas emissions (CO_2) and producing non-toxic by-products (Reis et al., 2011; Brockhaus et al., 2015; Gironi and Piemonte, 2011). There are three main groups of bioplastics, according to the different properties (Lackner, 2015):

1. Fully or partially biobased non-biodegradable plastics, including polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET);
2. Polylactic acid (PLA) and polyhydroxyalkanoates (PHA) are synthesized polymers from renewable sources (biobased) and biodegraded under several conditions (biodegradable);
3. Polymers such as poly(butylene adipate-co-terphthalate) (PBAT) and polycaprolactone (PLC) based on fossil resources but are biodegradable;

Despite the achievement of the criteria of a closed loop life cycle by PHA (Reis et al., 2011), its greater weakness comparatively to conventional plastics is the production price, €9/kg of P(3HB) versus €1/kg of petroleum-based plastics (Reis et al., 2003).

1.3 Polyhydroxyalkanoates (PHA)

PHA are optically active biological polyesters (Figure 1.1) synthesized by microorganisms from renewable resources and characterized by remarkable properties such as biodegradability, biocompatibility and thermoprocessibility (Amulya et al., 2016).

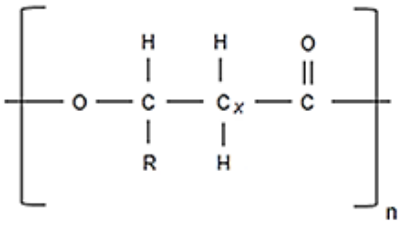

Figure 1.1: General chemical structure of PHA monomers. **R** is the variable hydrocarbon side chains, **x** refers to the size of the alkyl group and **n** represents the number of times that this monomer repeat, ranging from 100 to 30 000 (from Reddy et al., 2003).

Under aerobic or anaerobic environments, they are biodegraded into water (H_2O) and carbon dioxide (CO_2) as well as into methane (CH_4), respectively (Lee, 1996; Akaraonye et al., 2010; Khanna and Srivastava, 2005). PHA are classified as good substitutes to the non-biodegradable conventional polymers, as they have similar material properties to several conventional synthetic polymers namely PP (Lee, 1996; Ntaikou et al., 2014).

1.3.1 Chemical structure and properties

Classified as water-insoluble compounds, inert, indefinitely stable in air, non-toxic and with a very high purity within the cell (Laycock et al., 2014), PHA are composed of several hydroxyalkanoates (HA) monomers and divided into three main groups according to the number of carbon atoms: short-chain length (scl-PHA) which consists up to 5 carbon atoms, medium-chain length (mcl-PHA), which comprises 6 - 14 carbon atoms and long-chain length (lcl-PHA) if contains more than 14 carbon atoms (Zinn et al., 2001). Scl-PHA have similar thermoplastics properties to the conventional plastics, such as higher crystallinity, brittle and stiff while mcl-PHAs is an amorphous material with elastic features (Suriyamongkol et al., 2007; Reddy et al., 2003). Its molecular weights ranges from 2×10^5 to 3×10^5 Da depending on the microorganism that produces the polymer and from the growth conditions used: pH, fermentation conditions and type and concentration of the carbon source (Akaraonye et al., 2010; Sudesh et al., 2000; Anjum et al., 2016). PHA properties changes with monomer composition, microstructure and molecular weight distribution. The melting and glass transition temperatures and the level of crystallinity changes with length of monomers variations (Reis et al., 2011; Khanna and Srivastava, 2005).

The most common PHA is poly-3-hydroxybutyrate (P(3HB)), characterized by good thermoplastic properties, such as glass transition and melting temperatures of 4 °C and 180 °C, respectively (Reis et al., 2011). However, their high crystallinity (55 - 80%) is a limitation since the produced polymer is fairly stiff and brittle (Sudesh et al., 2000; Reis et al., 2011). Apart from homopolymers, containing only one type of hydroxyalkanoate as the monomer unit (e.g. P(3HB) and P(3HHx)), there are also copolymers, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), and poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-*co*-3HHx), which vary in the type and proportion of monomer (Sudesh et al., 2000). Characterized by lower melting point and glass transition temperatures than P(3HB), they have good elastic properties (Reis et al., 2011). Common PHA, defined by their respective R groups, are listed in Table 1.1.

Table 1.1: Several common PHA, defined by their respective R groups (from Lackner, 2015).

n	R chain	Short Name	PHA
1	H	poly(3-hydroxypropionate)	P(3HP)
1	CH_3	poly(3-hydroxybutyrate)	P(3HB)
1	CH_2CH_3	poly(3-hydroxyvalerate)	P(3HV)
1	C_2H_4	poly(3-hydroxyhexanoate)	P(3HHx)
2	H	poly(4-hydroxybutyrate)	P(4HB)
3	H	poly(3-hydroxyvalerate)	P(5HV)

1.3.2 Biosynthesis

PHA are formed inside bacterial cells, in aerobic and/or anaerobic environments, and stored in a granular form of 0.2 - 0.5 µm in diameter (Figure 1.2) as a reserve material during the fermentation processes (Sudesh et al., 2000).

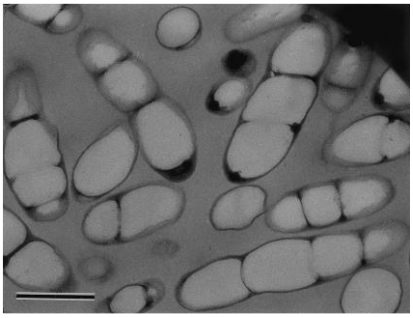

Figure 1.2: Transmission electron micrograph of thin sections of recombinant *R. eutropha* PHB^-4 cells containing large amounts (90% of the dry cell weight) of P(3HB-co-5 mol% 3HHx). Bar represents 0.5 mm (from Sudesh et al., 2000).

1.3.2.1 Pure cultures

P(3HB) was the first PHA identified in *Azotobacter chroococcum* (Sudesh et al., 2000). Actually, more than 300 species of Gram-positive, Gram-negative bacteria and some archae are capable of PHA accumulation with yields up to 80 - 90% of the cell dry weight (Laycock et al., 2014; Serafim et al., 2008; Reis et al., 2011). PHA accumulation by pure cultures occurs through nutrient limitation, usually performed by a two-stage process: (1) In the first stage the main goal is the growth of microorganisms. Under sterile conditions, they are inoculated into the medium solution containing the carbon source and nutrients and (2) in the second stage, PHA accumulation is promoted by an external limitation of essentials nutrients (i.e. N, P or O_2). Cells are unable to grow and increase in size and weight, since PHA intracellular accumulation occurs (Laycock et al., 2014; Chee et al., 2010). Industrially, PHA synthesis is mostly performed by pure cultures using wild or genetically modified strains with glucose or propionic acid as carbon source (Lemos et al., 2006, Reis et al., 2003). P(3HB-*co*-3HV) and P(3HB-*co*-3HHx), firstly commercialized by Biopol™ and Nodax™, respectively, are synthesized by genetically modified strains. Biomer™ and Biocycle™ are produced by pure cultures in their natural state, and are responsible for production of homopolymers of HB and the latter also for copolymer of HB and HV synthesis, respectively (Laycock et al., 2014, Lemos et al., 2006).

The entire process of PHA production depends essentially on the substrate cost, the PHA yield on substrate and the downstream processing. Despite the high cellular density (e.g. 100 g L^{-1}) and volumetric productivities (up to 5 g-PHA L^{-1} h^{-1}) achieved by pure cultures (Reis et al., 2011), its high substrate and downstream process costs are its main weaknesses (Chen et al., 2015). To reduce PHA production costs, substrate behaves as a critical factor directly related to the overall fermentation stage. Thus, substrate should be renewable, inexpensive and readily available as it is the case of waste feedstock's (Chee et al., 2010). However, the use of waste feedstock's by pure cultures, characterized by defined metabolic pathways, is impossible due to their complex and unsterile nature (Tamis et al., 2015).

1.3.2.1 Mixed microbial cultures (MMCs)

Mixed microbial cultures (MMCs) are microbial populations with undefined composition, whose selection depends on the operational conditions applied into the biological system (Albuquerque et al., 2007). MMCs are able to consume a large range of cheap substrates, containing diverse organic chemical compounds, such as waste feedstock's (e.g. industrial and/or agricultural wastes and by-products). Therefore, MMCs have demonstrated to be an excellent alternative to pure cultures potentially reducing PHA production costs (Gurieff and Lant, 2007). The absence of aseptic conditions, the low-cost substrate instead of a defined substrate and the lower control requirements are the main positive approaches to use MMCs (Lemos et al., 2006; Serafim et al., 2004). Several substrates can be used by MMCs for PHA production, namely sugar cane molasses (Albuquerque et al., 2007), cheese whey, (Duque et al., 2014; Bengtsson et al., 2008a), cellulose, sucrose and starch (Reddy et al., 2003) or effluents and wastewaters such as olive oil (Dionisi et al., 2005; Ntaikou et al., 2014), paper mill (Bengtsson et al., 2008b) and brewery wastewater (Ben et al., 2016).

However, lower cellular concentrations with PHA storage capacity are obtained by MMCs, rounding 55% of the dry weight using synthetic feedstocks, resulting in lower volumetric productivities instead of those obtained by pure cultures (Albuquerque et al., 2011; Jiang et al., 2012).

1.3.3 PHA production by MMCs

PHA synthesis by MMCs was firstly observed in aerobic wastewater treatment plants (WWTP) (Reis et al., 2011). Through the "Feast and Famine (FF)" strategy or aerobic dynamic feeding (ADF), culture selection occurs by transient conditions of substrate feed (Reis et al., 2003; Gurieff and Lant, 2007). Feast period comprises the culture supplementation, in a short period of time, of excess carbon. PHA storage is the dominant process, as the enzymes required to reach the maximum growth are not sufficient while enzymes needed for PHA storage are present. During the famine period, stored PHA is used for cell growth and cell maintenance (Reis et al., 2011, Dias et al., 2006, Serafim et al., 2008). The FF cycles repetition will favor the cell growth on storage products, creating a selection for organisms with high PHA-storing capacity (Dias et al., 2006; Albuquerque et al., 2011). Bacteria selection step carried out under aerobic conditions is shown in Figure 1.3:

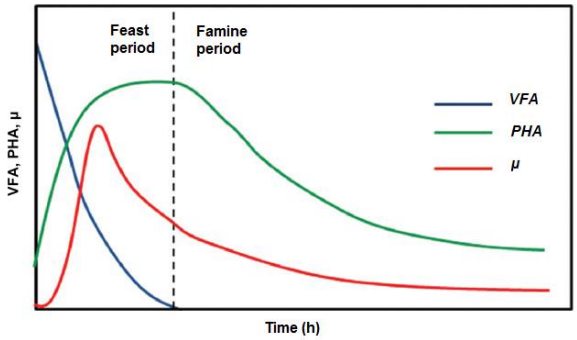

Figure 1.3: Common behaviour of MMC for PHA storage under fully aerobic feast and famine conditions. Blue line represents volatile fatty acids (VFA), green line corresponds to PHA and red line to the specific growth rate (μ) (from Reis et al., 2011).

Physical adaptation of the microorganisms to the FF strategy and selection of enriched culture with high and stable capacity of PHA accumulation results from alternate substrate availability (Albuquerque et al., 2010). Serafim et al. (2004) showed that, using a pulse substrate feeding strategy, 65% of intracellular PHA content was achieved. This simpler strategy requiring less investment and operation costs, is economically competitive with PHA production by pure cultures (M.L. Dias et al., 2006).

Depending on the substrate used as feedstock, PHA production can be operated in two or three-step process (Albuquerque et al., 2007; Serafim et al., 2008). Both processes involve selection of PHA-storage organisms followed by PHA accumulation (Step 2 and 3 in Figure 1.4) (Serafim et al., 2008). However, using waste feedstock's, many being carbohydrate-rich, PHA production by MMCs is operated in three separate stage (Figure 1.4), with different optimal conditions (Lemos et al., 2006; Serafim et al., 2008): (1) acidogenic fermentation where organic content in the waste is biologically converted to organic acids and other fermentation products (FP), (2) culture selection (under FF strategy), using the fermented feedstock from stage 1 and (3) PHA accumulation using the selected culture (stage 2) and the organic acids produced in stage 1. PHA produced is then extracted and purified (Albuquerque et al., 2007, Gouveia et al., 2016).

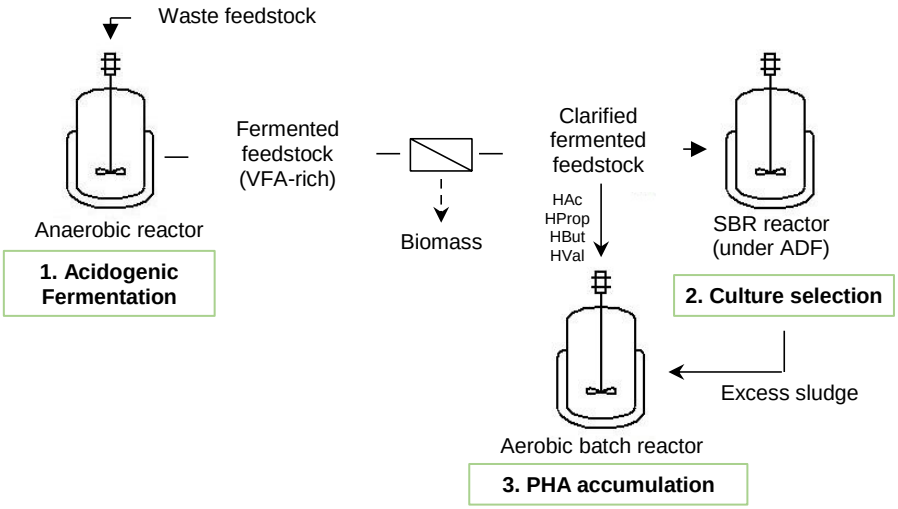

Figure 1.4: Three-step process for PHA production by mixed microbial cultures from waste streams (from Albuquerque et al., 2007).

1.3.3.1 Acidogenic Fermentation

PHA production by MMCs is mostly performed using organic acids (HOrg) as substrate. As MMCs allow the utilization of wastes aiming at organic acids production, a previous anaerobic step called acidogenic fermentation (stage 1, Figure 1.4) is required, where sugars in the wastes are converted into fermentation products including organic acids (e.g. acetic (HAc), propionic (HProp), butyric (HBut) and valeric (HVal) acids), hydrogen (H_2), carbon dioxide (CO_2) and ethanol (EtOH) (Albuquerque et al., 2007, Lier et al., 2008). During this stage, CH_4 production is not desired. As such, the use of low HRT, temperature and pH or a combination of the different approaches are strategies to prevent methanogenic activity (Reis et al., 2011), resulting in PHA precursors accumulation, namely organic acids (Tamis et al., 2015).

Acidogenic fermentation can be operated using suspended biomass (e.g. cells, flocs) or immobilized biomass (e.g. biofilm, granules). Considering immobilized biomass, the microorganisms can be attached to a support (biofilm) or can be aggregated (granules) (Guiot et al., 1992). Anaerobic granules (0.5 - 3 mm) are aggregated microorganisms formed in high-rate upflow anaerobic reactors (Hickey et al., 1991). Its internal architecture promotes species to substrate transfers and increase resistance to high OLR and to toxic shocks (Amani et al., 2010; Guiot et al., 1992).

1.3.3.2 Culture selection

Culture selection is the second stage of the three-step process for PHA production by MMCs (stage 2, Figure 1.4). The main goal is to achieve an enriched culture with high storage capacity (Reis et al., 2011). The common configuration used are sequencing batch reactors (SBR), compact systems, easily controlled and highly flexible, where selection of microorganisms with high ability for PHA storage is ideal and biomass grows under transient (unsteady) conditions (Serafim et al., 2008; Reis et al., 2011). Two sequentially disposed continuous reactors are followed by a settler is an alternative configuration (Serafim et al., 2008). Different reactor operating conditions (SRT, HRT, pH, T, cycle length, OLR, influent substrate and nutrient concentrations) and feedstocks act as the main regulatory factors on this step, providing different responses and directly influence the selective pressure in favour of PHA-storing organisms (Reis et al., 2011; Albuquerque et al., 2010).

1.3.3.3 PHA accumulation

PHA accumulation stage is the last of the three-stage process for PHA production (step 3 in Figure 1.4). It is performed in batch or fed-batch mode in an open system where the reactor is inoculated with the selected culture (step 2) and fed in the form of a pulse of substrate with a high organic acids concentration (from step 1) (Ben et al., 2016; Chen et al., 2015). Operated under growth limiting conditions (e.g. ammonia and/or phosphate limitation) or by nutrients and carbon sources continuous exposition, higher PHA cell content is achieved in the first case. The highest PHA content from waste or by-products feedstocks was obtained using fermented molasses, 74.6% (gPHA $gVSS^{-1}$), under N limitation (Reis et al., 2011), whereas using pure HAc these value increased to 89% (Johnson et al., 2009; Albuquerque et al., 2011). The overall PHA production

performance is evaluated according to the PHA cell content, yield on substrate, specific storage rate and cell volumetric productivity (Reis et al., 2011).

1.3.4 Metabolic pathways

PHA production occurs under stress conditions caused by the external nutrient limitation (oxygen, nitrogen and phosphorus) or by an internal limitation in anabolic enzyme levels or activity. As a consequence, cells are unable to grow (Reis et al., 2011). Pathway I (Figure 1.5), where carbohydrate catabolic degradation occurs, is used for pure cultures resulting in pyruvate, energy and reducing equivalents synthesis (Reis et al., 2011). Without growth-limitation conditions, pyruvate conversion into acetyl-CoA is enhanced (Zinn et al., 2001). In the tricarboxylic acid cycle (TCA), this product is oxidized into CO_2 generating anabolic precursors, energy and reducing equivalents. Under growth-limitation conditions, the acetyl-CoA level is reduced due to its conversion into P(3HB) by three enzymes: 3-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) (Zinn et al., 2001; Sudesh et al., 2000; Reis et al., 2011). Pathway III, involves the activation of short-chain organic acids to the corresponding acyl-CoA molecules, which are different HA monomers precursors (Figure 1.5) (Braunegg et al., 1998; Reis et al., 2011; Loo and Sudesh, 2007). When the carbon source is HAc, the process follow Pathway I. HProp is converted to propionyl-Co-A with 3-hydroxy-2-methylvalerate (3H-2-MV) synthesis, or to acetyl-Co-A with or 3HV or 3-hydroxy-2-methylbutyrate (3H-2-MB) production. HBut and HVal are converted directly to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, with 3HB and 3HV synthesis, respectively (Reis et al., 2011; Serafim et al., 2008; Dias et al., 2006). Fatty acid β-oxidation pathway (Pathway II, not shown) is related to fatty acids uptake by microorganisms to produce mcl-PHA. Subsequent pathways (not shown) involve the synthesis of alternative copolymers such as P(4HB) (Laycock et al., 2014).

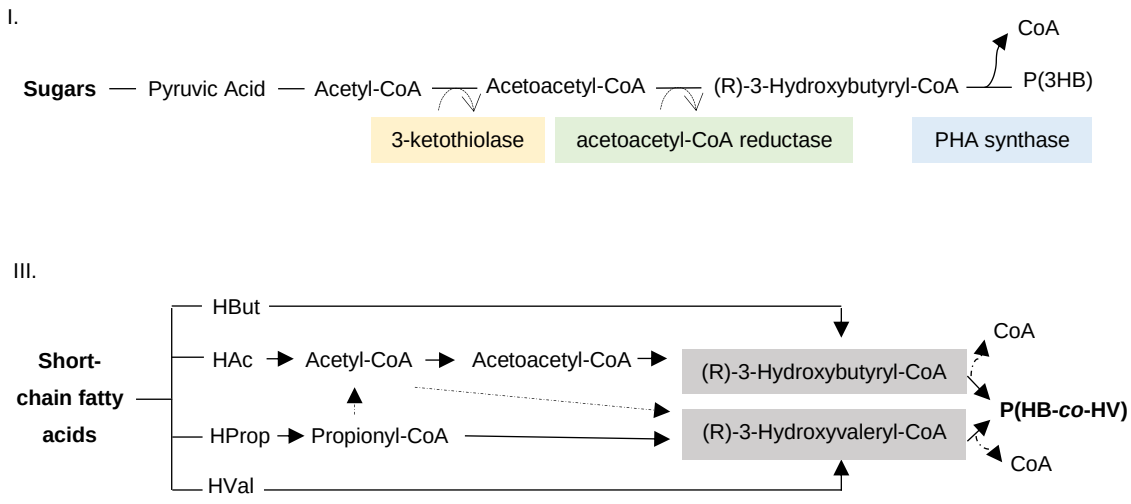

Figure 1.5: Metabolic pathways involved in polyhydroxyalkanoate (PHA) synthesis from sugars (I) and from short-chain fatty acids (III) (from Reis et al., 2011).

Polymer composition reflects the combination of different mixtures of substrates used by the culture (Serafim et al., 2008). PHA production can be promoted through inhibition of metabolic pathways or activation of specific enzymes that are directly or indirectly involved in PHA synthesis (Laycock et al., 2014, Gouveia et al., 2016). PHA synthesis by MMCs involves similar metabolic pathways described by pure cultures using organic acids as carbon source (Reis et al., 2011).

1.3.5 Extraction and Purification

Downstream processing is a decisive cost-determining factor in biopolymer production, regardless of the raw material costs and the fermentation process itself. To achieve a high yield in PHA extraction it is fundamental to concentrate PHA-containing cells by centrifugation, cross-flow filtration and flocculation (Zinn et al., 2001, Muhammadi et al., 2015). PHA extraction was performed using solvents, such as chloroform, methylene chloride, propylene carbonate and dichloromethane due to their simplicity and rapidity. Although the recovered PHA has a higher degree of purity, they are non-environmental friendly methods, requiring large amounts of toxic and volatile solvents, negatively affecting the total production costs (Kunasundari and Sudesh, 2011; Reis et al., 2011). Chemical and enzymatic digestion, through solubilization of cellular materials surrounding the PHA granules, can also be applied. Chemical digestion with sodium hypochlorite or surfactants to solubilize the non-PHA cellular mass (Muhammadi et al., 2015) are not a good choice due to the low purity degree and/or molecular weight (Zinn et al., 2001; Kunasundari and Sudesh, 2011). Despite of the good quality of PHA resultant from enzymatic digestion, the high cost of very specific enzymes and the complexity of the recovery process are not favourable parameters (Kunasundari and Sudesh, 2011). PHA downstream process is a constant challenge, being cost the key factor in the selection of the method, generally focusing on the minimization of using strong chemicals and solvents. Moreover, it depends on the final application, since for medical applications it must be free of bacterial endotoxins, contaminating chemicals and solvents (Kunasundari and Sudesh, 2011). If it is to be used in garbage bags production, it is not necessary such a high purity degree (Kunasundari and Sudesh, 2011).

1.3.6 Applications

As a conceivable substitute to the conventional plastics, PHA can be applied in an extensive range of applications, some of which being listed in Table 1.2.

Table 1.2: Applications of polyhydroxyalkanoates (from Muhammadi et al., 2015).

Field	Applications
Packaging, molding and coating (food packaging)	Bags, containers, paper coatings, pens, razors, utensils, diapers, feminine hygiene products, cosmetic containers, bottles and cups
Chemical area	Optically active compounds synthesis Biodegradable career or drug delivery systems for controlled release of medicine, hormones, insecticides and herbicides
Medical area	Surgical sutures, staples, swabs, bone replacements and plates, blood vessel replacements, stimulation of bone growth by piezoelectric properties

Several applications are still emerging, such as a new source of small molecules or acting as tissue adhesives or sealants (Akaraonye et al., 2010).

2. Motivation and Thesis Outline

2.1 Motivation

2.1.1 PHA production by mixed microbial cultures (MMCs) using waste as feedstock

Over the years, plastic production has been causing a huge negative impact in the environment due to its harmful effects. Bioplastics, such as polyhydroxyalkanoates (PHA), are considered as potential substitute of conventional plastics due to its biobased and biodegradable nature (Reis et al., 2011). Its industrial production, performed mostly by pure cultures, depends on expensive carbon sources and maintenance process costs (Albuquerque et al., 2011). Alternatively, mixed microbial cultures (MMCs) allow the substitution of refined carbon sources by low cost complex feedstock (e.g. agricultural/industrial residues or by-products), removing the requirement of sterile conditions, therefore potentially decreasing PHA production costs (Reis et al., 2011). This work was developed in order to simultaneous treat and valorize a raw material, using a three-stage PHA production process, firstly producing organic acids as PHA precursors.

2.1.2 Valorization of brewer's spent grain (BSG)

Brewer's spent grain (BSG) is the main low-value solid waste and by-product of the brewery industry, counting with about 3.4 million metric tons of spent grains produced per year in the European Union (Fărcaş et al., 2014). Being mainly composed by fibers like cellulose, hemicellulose and lignin, as well as some proteins, minerals and lipids, it is currently being used for animal feed, due to its high nutritive value (Mussatto, 2014). Additionally, BSG can be composted, incinerated, dumped or anaerobically fermented (Fărcaş et al., 2014). Hence, BSG can be used to produce an added value compound such as PHA, contributing to circular economy.

2.1.3 Objetives

The present thesis aims to investigate, at laboratory scale, a strategy for the valorization of brewery waste, brewer's spent grain (BSG), through organic acids production, using anaerobic granular sludge. Lab scale reactors were operated and the impact of operating parameters on organic acids production, namely pH and organic load, were evaluated.

2.2 Thesis Outline

This thesis comprises six chapters - **Chapter 1** is a general introduction focused on the PHA development: their importance as well as PHA properties, synthesis and applications. The current chapter (**Chapter 2**) describes the motivation and the outline of the work developed during the master project. **Chapter 3** is dedicated to the BSG pre-treatment by acid hydrolysis and subcritical water. **Chapter 4** focus on the effect of pH and organic load on the acidogenic fermentation of hydrolyzed brewer's spent grain in batch. **Chapter 5** reports brewer's spent grain acidogenic fermentation in an expanded granular sludge bed reactor. In **Chapter 6** general conclusions are summarized and future work suggestions are presented.

3. Brewer’s spent grain (BSG) pre-treatment strategies

3.1 Introduction

In the brewery industry, barley malt is partially liquefied resulting in a liquor (wort), used for beer production, and a solid residue named as brewer's spent grain (BSG) (Forssell et al., 2008). As the main by-product of brewery industry, BSG is a lignocellulosic material, recalcitrant and with chemical composition dependent on various factors, such as barley variety, conditions of malting and mashing, and the quality and type of adjuncts added (Mussatto et al., 2006). Despite of the fluctuations in the amount of each constituent reported, its main composition consists on fibers like cellulose, hemicellulose and lignin. Some proteins, minerals and lipids can also be found in BSG's composition (Mussatto et al., 2006). Comprising 50% (w/w) of BSG's composition, cellulose and hemicellulose fractions are mainly composed by sugars such as glucose, xylose and arabinose (Mussatto, 2014; Fărcaş et al., 2014). Due to its high water content (80%) it is essential a long-term storage, in order to prevent rapid microbial spoilage (Macheiner et al., 2003). In Table 3.1 several studies using different BSG with different compositions are listed.

Table 3.1: BSG's chemical composition according to several studies.

Study	Cellulose (% w/w)	Hemicellulose (% w/w)	Protein (% w/w)	Ash (% w/w)	Lipid (% w/w)	Lignin (% w/w)
Beldman et al., 1987	15.1	24.8	23.8	3.5	25.0	
Carvalheiro et al., 2004	21.9	29.6	24.6	1.2	n.a	21.7
Mussatto and Roberto, 2005	16.8	28.4	15.2	4.6	n.a	27.8
Kopsahelis et al., 2007	9.0	19.0	23.0	4.0	8.0 - 9.0	16.0

*n.a – not available; % in dry weight matter

Currently, BSG is used for animal feed due to its high nutritive value (Mussatto and Roberto, 2005). However, other several applications can be found such as biogas production, where BSG act as a substrate with high biotechnological value. Some interesting compounds can be extracted and used as hydrocolloid, such as arabinoxylan or corresponding oligosaccharides, as antioxidant from extracted phenolic compounds and as emulsifying agents in the case of lignin and proteins extraction (Mussatto, 2014; Forssell et al., 2008; Treimo et al., 2009).

These lignocellulosic materials are currently pre-treated to convert polysaccharides present in BSG into fermentable sugars (Macheiner et al., 2003). The main pre-treatment procedures are classified as physical (crushing, grinding and microwave radiation), chemical (alkaline and organic acids solvents, oxidizing agents and ionic liquid as well), physico-chemical (auto-hydrolysis, hot water, steam and supercritical fluids) and biological (enzymes, fungi and bacteria) (Macheiner et al., 2003; Partida-Sedas et al., 2017). Low sugar yields, optimal reaction conditions, high capital investment and processing costs are some disadvantages of these methods (Partida-Sedas et al., 2017). Chemical processes are chosen as more favourable, usually involving sulphuric acid or hydrochloric acid at concentrations of 1 - 10% (v/v) and temperatures ranging from 100 to 150 °C, in order to hydrolyse hemicelluloses into xylose and arabinose and cellulose into glucose (Lenihan et al., 2010). Mussatto and Roberto (2005), who have studied acid hydrolysis of BSG under liquid/solid ratio of 8 g g^{-1}, 100 mg g^{-1} of H_2SO_4 and a reaction time of 17 minutes, reported an efficiency of extracted hemicellulosic fraction of 92.7% (w/w) in dry matter.

However, from degradation of glucose and monosaccharides, some undesirable compounds can be produced such as furfural, hydroxymethylfurfural (5-HMF), HAc and phenolic compounds (Lenihan et al., 2010). The concentrations of these inhibitory compounds increases with temperature and acid concentration (Lenihan et al., 2010; Mussatto, 2014). Furthermore, acid hydrolysis results in environmental concerns and corrosion problems (Haghighi Mood et al., 2013).

Hot-compressed water (HCW) or subcritical water (SCW) is a potential greener alternative to hydrolyse lignocellulosic materials, constituted by polysaccharides, into oligomers and monomers (Pourali et al., 2009, Brunner, 2009). SCW is an environmentally friendly solvent process, being non-explosive, non-flammable, non-toxic and cheap. This process uses hot water at temperatures ranging from 100 to 374 °C, maintained in liquid state by high pressure application (Rogalinski et al., 2008, Pourali et al., 2009). The different behaviour of the water at ambient conditions is directly related to the dielectric strength and ionic product, both monitored by temperature and pressure changes (Rogalinski et al., 2008). Above the triple point (point 1 in Figure 3.1) and below the critical point (point 3 in Figure 3.1 where T = 374 °C and ρ = 22.1 MPa), water is either liquid or gaseous (Möller et al., 2011).

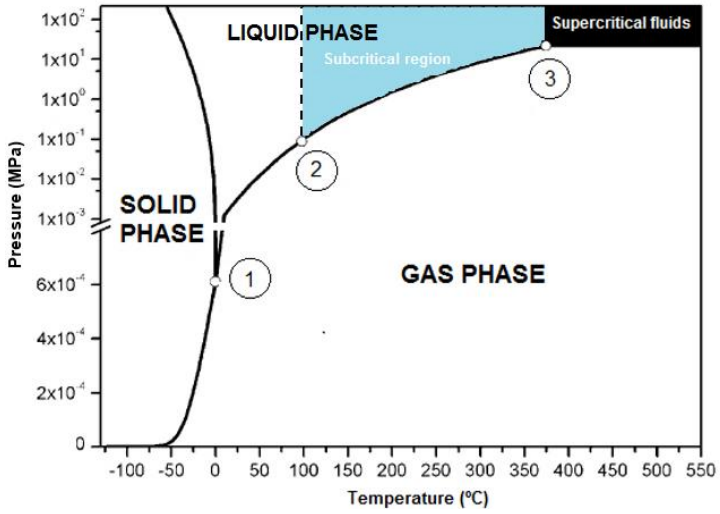

Figure 3.1: Phase diagram of water presenting subcritical region (in blue colour), the triple point (1), the boiling point at ambient pressure (2) and critical point (3) (adapted from Möller et al., 2011).

As mentioned above, temperature and pressure changes affect water properties. Increasing the temperature, the ionic product (K_w) is up to three orders of magnitude higher, from 10^{-14} mol^2 L^{-2} at 25 °C to 10^{-11} mol^2 L^{-2} at 300 °C but above the critical point it decreases drastically to 10^{-20} at 380 °C (Möller et al., 2011). This increase in ionic product, consequence of higher H_3O^+ and OH^- concentrations, enhance the reactivity of water, acting as an acid/base catalyst precursor (Kruse and Dinjus, 2007). On the other hand, dielectric constant of water decreases with temperature increase, resulting in a decrease of the number of hydrogen bonds per molecule of water due to its dissociation (Möller et al., 2011).

Due to its several properties, SCW is currently applied on extraction processes (Möller et al., 2011). Several studies using SCW have been performed for the hydrolysis of different agro-

industrial residues such as sugar cane bagasse (Prado et al., 2014), bean dregs waste (Zhu et al., 2011) and rice bran (Pourali et al., 2009). SCW was tested as a potential alternative to the acid hydrolysis in order to extract sugars from BSG, due to its environmentally friendly nature, quickness and to the necessity of relatively simple equipment (Pedras et al., 2017). In order to compare two possible BSG pre-treatment strategies, namely acid hydrolysis and SCW, BSG hydrolysis using SCW was investigated, at four temperatures (T = 150, 180, 200 e 230 °C) with a constant water flow rate of 10 mL min^{-1}.

3.2 Materials and Methods

3.2.1 Brewer's spent grain preparation

BSG was supplied by a Portuguese brewery industry, dried in the oven at 70 °C for one week and grinded using a hammer mill to obtain smaller particles. BSG was stored at -18 ºC.

3.2.2 Experimental set-up

Sugar extraction of BSG was performed by acid hydrolysis with sulfuric acid (95 - 98% (w/v) H_2SO_4, Sigma-Aldrich) under conditions previously optimized by Carvalheiro et al. (2004). To maintain the liquid/solid ratio of 8 g g^{-1}, 50 g of BSG was added to 388 mL deionized water and 12 mL of H_2SO_4. The hydrolysis was performed at 121 °C for 20 minutes. Subsequently, the hydrolysates were centrifuged (4-16 KS, Sigma) for 15 minutes (10 000 × g, 4 °C), and the resulting supernatant was clarified through filtration. In order to improve hydrolysis yield, a second hydrolysis was performed to the pellets resultant from the first hydrolysis. Deionized water and H_2SO_4 were added to the pellets (ca. 388 mL and 12 mL, respectively) and the second hydrolysis was performed using the same conditions as in the first hydrolysis. The first and second hydrolyzed BSG were mixed and stored at 4 °C. The hydrolysed BSG was neutralized (pH 7) using $Ca(OH)_2$. After neutralization, hydrolysed BSG was centrifuged for 15 minutes (10 000 × g, 4 °C) and the supernatant was characterized.

The experimental set-up used for the hydrolysis reactions using SCW is shown in Figure 3.2. Deionized water was pumped through a heating section before entering the tubular reactor (stainless steel tube with 51 cm long, 5 cm of external diameter and 2.6 cm of internal diameter) placed in an electric oven with temperature control. BSG was placed inside the reactor and kept between porous discs to avoid biomass to pass to the pipes. A heating cable connected to a temperature controller was used. Pressure was controlled by a back-pressure regulator valve placed at the outlet of the reactor after a cooling section. Temperature and pressure of the outlet water were monitored. The outlet stream, hydrolyzed BSG, was collected for analysis.

Figure 3.2: SCW apparatus used for BSG hydrolysis.

The water flow used, 10 mL min^{-1}, was the same for all the assays. The pressure was kept constant, at 65 bar and four maximum temperatures were tested: 150, 180, 200 and 230 °C. Several samples were collected during the warm-up period, at different outlet stream temperatures, namely at 50 °C, 50 °C to 100 °C, 100 °C to 150 °C and 150 °C up to the maximum temperature, selected for each assay. The collected samples were stored at -18 °C for analysis.

3.2.3 Analytical Procedures

The characterization of acid hydrolysate for ions, sugars, furfural and 5-HMF, was performed by the analytical laboratory, LAQV-REQUIMTE. Briefly, Ca^{2+}, Mg^{2+}, Na^{+} and S^{2-} quantification was conducted by inductively coupled plasma atomic emission spectroscopy (ICP-AES) through ICP spectrophotometer (Horiba Jobin-Yvon Ultima) equipped with 40.68 MHz RF generator, 1.00m Czerny-Turner monochromator, AS500 automatic sampler and CMA device (Concomitant Metals Analyzer). Sugars were analyzed by high performance liquid chromatography (HPLC Dionex ICS-3000) using an amperometric detector (PAD-Pulse amperemetric detection), CarboPAC-PA10 250 x 4mm column and an Amino-trap pre-column. Sodium hydroxide (NaOH) 18 mM was used as eluent with 1 mL min^{-1} flow rate and T = 30 °C. SO_4^{2-} was also quantified by HPLC (Dionex ICS-3000) using a conductivity detector, ionPAC AS16 250 x 4mm column. NaOH 22 mM was used as eluent at a flow rate of 1.5 mL min^{-1} at T = 30 °C. Furan compounds were analyzed by HPLC (Thermo Finnigan Surveyor) with a UV/Vis detector (Accela λ = 280 nm) through a Biorad Aminex 87H 300 x 7.8 mm column. The analysis was performed using H_2SO_4 10 mN as eluent with a 0.6 mL min^{-1} flow rate at T = 30 °C. Organic acids of filtered samples (VWR, spin filter 0.2 μm) were measured by HPLC using a Biorad Aminex HPX-87H column (300 x 7.8 mm) and a Biorad pre-column (125-0129 30 x 4.6 mm) coupled to an IR detector. The analysis was conducted at 30 °C with sulphuric acid (H_2SO_4 0.01 N) as eluent at a 0.5 mL min^{-1} flow rate (Duque et al., 2014). The organic acids concentrations were calculated through a standard calibration curve (31 - 1000 mg L^{-1} of each organic acid).

The characterization of hydrolyzed BSG from SCW in terms of sugars and organic acids were performed as described above.

3.3 Calculations

The hydrolysis yield on sugars was calculated according to Eq 3.1 by the dividing the amount of sugars extracted (g L^{-1}) by the initial weight of BSG (g) used for hydrolysis:

$$\text{Hydrolysis yield on sugars } (g \text{ sugars } g\ BSG^{-1}) = \frac{\text{Amount of sugars extracted}}{\text{Initial weight of BSG}} \quad \text{Eq 3.1}$$

The extent of extraction/hydrolysis of BSG by SCW was determined by dividing the amount of BSG hydrolyzed by the initial amount of BSG placed in the reactor (Eq 3.2):

$$\text{Extent of hydrolysis } (\%) = \frac{\text{Initial weight of BSG - Final weight of BSG}}{\text{Initial weight of BSG}} \times 100 \quad \text{Eq 3.2}$$

The yield of BSG extract (Eq 3.3) using SCW strategy was calculated by dividing the amount of extract obtained by lyophilizing the samples collected throughout the assay by the initial amount of BSG placed in the reactor:

$$\text{Yield of BSG extract } (\%) = \frac{\text{Weight of BSG extract}}{\text{Initial weight of BSG}} \times 100 \quad \text{Eq 3.3}$$

3.4 Results and Discussion

Brewer's spent grain, as mentioned above, is a lignocellulosic material constituted mainly by hemicellulose and cellulose. As a strategy for BSG pre-treatment, acid hydrolysis and subcritical water were performed in order to produce an extract rich in sugars, but with low inhibitory compounds concentrations (Mussatto, 2014). Both pre-treatment methods tested were compared.

3.4.1 Acid hydrolysate characterization

Acid hydrolysis was performed under optimized conditions with a liquid/solid ratio of 8 g g^{-1}, as described in the Materials and Methods section. The analysis of acid hydrolyzed BSG, namely first and second hydrolysis and the mix of both hydrolysis, is presented in Table 3.2.

Table 3.2: Initial characterization of acid hydrolyzed BSG.

	First hydrolysis	Second hydrolysis	Mix*
Calcium (g L^{-1})	1.42	1.07	1.12 ± 0.04
Magnesium (g L^{-1})	2.36	1.10	1.76 ± 0.03
Sodium (mg L^{-1})	5.29	4.65	5.05 ± 0.07
Sulfur (g L^{-1})	19.7	27.8	23.50 ± 1.36
Sulfate (g L^{-1})	52.92	80.12	66.65 ± 2.93
Furfural (g L^{-1})	0.66	0.57	0.71 ± 0.07
5-HMF (g L^{-1})	0.07	0.04	0.07 ± 0.03
Acetic acid (g L^{-1})	1.42	0.58	1.13 ± 0.14
Glucose (g L^{-1})	5.37	2.65	3.55 ± 0.10
Xylose (g L^{-1})	13.17	6.26	9.98 ± 0.65
Arabinose (g L^{-1})	7.75	3.41	5.56 ± 0.04

* The values listed are averages ± standard deviations

The main constituents identified were calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), sulfur (S^{2-}), sulfate (SO_4^{2-}), acetic acid (HAc), furfural, 5-HMF as well as glucose, xylose and arabinose. According to the literature, the presence of mineral elements such as Ca^{2+}, Mg^{2+}, Na^{2+} and S^{2-} was expected, although in concentrations lower than 0.5%, which was also observed in the present study (Mussatto et al., 2006). From acid hydrolysis, the extracted and identified sugars were xylose, arabinose and glucose (Table 3.2). Xylose was the main extracted sugar, followed by arabinose and glucose. These results are in accordance with Mussatto (2014), which also found that the most abundant sugars in BSG's fractions corresponds xylose, arabinose and glucose. Furthermore, pentose sugars are the main compounds formed providing a high presence of monosaccharides such as xylose and arabinose (Mussatto and Roberto, 2005).

Additionally, sugars can be degraded during the hydrolysis to some inhibitory compounds, such as HAc, furfural and 5-HMF. In this work, low concentrations of the last two compounds, furfural and 5-HMF, were detected (0.71 ± 0.07 and 0.07 ± 0.03 g L^{-1}, respectively). These results were similar to the reported by Mussatto and Roberto (2005) that studied acid hydrolysis of BSG at 120 °C under the same liquid/solid ratio, sulfuric acid concentration of 100 mg g^{-1} and at two reaction times (0.63 and 0.09 g L^{-1} after 17 minutes and 0.87 and 0.07 g L^{-1} after 37 minutes, of furfural and 5-HMF, respectively). Furfural is always higher than 5-HMF concentrations, providing that pentose sugars (xylose and arabinose) were more susceptible to degradation than hexose sugars

(glucose), under the applied conditions (Mussatto and Roberto, 2005). Roberto et al. (1991), that studied acid hydrolysis of sugar cane bagasse to produce ethanol, reported that furfural only represents a problem at levels above 1 g L^{-1}. HAc was also detected in a concentration of 1.13 ± 0.14 g L^{-1}. Mussatto and Roberto (2005), under the same conditions described for furfural and 5-HMF, reported HAc concentrations of 1.20 and 1.34 g L^{-1} for 17 and 37 minutes of reaction time, respectively. From a previous study, HAc concentrations above 5 g L^{-1}, in their undissociated form, totally inhibit growth of *Saccharomyces cerevisiae* (Taherzadeh et al., 1997).

As it is possible to verify in Table 3.2, most of the compounds were extracted during the first hydrolysis. At high concentrations of extracted sugars, higher concentrations of inhibitory compounds such as HAc, furfural and 5-HMF were achieved, as more extracted sugars can be degraded. In the second hydrolysis, lower concentrations of sugars and mineral elements were obtained, since much of it was already hydrolysed. The increase on S^{2-} and SO_4^{2-} was expected as sulfuric acid was used to carry out the hydrolysis. Concerning BSG mix, slight variations were detected, since the volume obtained after each hydrolysis is quite different, due to some losses during the extraction process.

The highly acidic nature of hydrolysates can affect fermentation stage (Chandel et al., 2011). To adjust the pH, a process of neutralization in the acid hydrolysates was used, resulting in salts formation (Lenihan et al., 2010). The overall characterization before and after this step is presented in Table 3.3.

Table 3.3: Hydrolyzed BSG characterization before (first column) and after $Ca(OH)_2$ addition (second column).

	Before $Ca(OH)_2$ addition*	**After $Ca(OH)_2$ addition***
Calcium (g L^{-1})	1.20 ± 0.04	4.60 ± 0.33
Magnesium (g L^{-1})	1.76 ± 0.03	3.90 ± 0.04
Sodium (mg L^{-1})	5.05 ± 0.07	4.90 ± 0.01
Sulphur (g L^{-1})	23.50 ± 1.36	3.14 ± 0.72
Sulphate (g L^{-1})	66.65 ± 2.93	4.61 ± 1.81
Furfural (g L^{-1})	0.71 ± 0.07	0.26 ± 0.05
5-HMF (g L^{-1})	0.07 ± 0.03	0.03 ± 0.005
Acetic acid (g L^{-1})	1.13 ± 0.14	1.52 ± 0.000
Glucose (g L^{-1})	3.55 ± 0.10	3.35 ± 0.31
Xylose (g L^{-1})	9.98 ± 0.65	9.20 ± 0.23
Arabinose (g L^{-1})	5.56 ± 0.04	4.94 ± 0.06
Total protein (g L^{-1})	-	19.7 ± 2.10
$NH4^+$ (mmol N L^{-1})	-	45.8 ± 0.10
PO_4^{3-} (mg P L^{-1})	-	2.33 ± 0.79
Total nitrogen (mmol N L^{-1})	-	188.7 ± 33.6
C:N:P ratio (C-mol:N-NH_4^+-mol:P-mol)	-	100:33:2

*Average values ± standard deviations are presented.

As expected, addition of $Ca(OH)_2$ increase the calcium content of hydrolyzed BSG. Sulfur and sulfate content decreases due to calcium sulphate precipitate formation. Furfural and 5-HMF concentrations decrease with $Ca(OH)_2$ addition (Table 3.3). This might be due to its conversion to other less toxic compounds (Purwadi et al., 2004). Furthermore, although a small decrease of

sugars concentration with $Ca(OH)_2$ addition was observed, it was not significant. To evaluate the overall acid hydrolysis process, the hydrolysis yield on sugars from BSG was calculated and summarized in Table 3.4.

Table 3.4: Hydrolysis yield on sugars from BSG by acid hydrolysis.

	First hydrolysis	Second hydrolysis	Mix* (First and second hydrolysis)
Glucose (g glucose g BSG^{-1})	0.025	0.009	0.028 ± 0.00
Xylose (g xylose g BSG^{-1})	0.061	0.021	0.080 ± 0.01
Arabinose (g arabinose g BSG^{-1})	0.036	0.011	0.044 ± 0.00
Total sugars (g total sugars g BSG^{-1})	0.123	0.041	0.152 ± 0.01

*Average ± standard deviations are presented.

Comparing the hydrolysis yield on sugars from BSG using acid hydrolysis (Table 3.4) it was observed that the hydrolysis yields of each sugar were higher in the first hydrolysis than in the second hydrolysis. Consequently, higher hydrolysis yield on total sugars was obtained in the first hydrolysis (0.123 g total sugars g BSG^{-1} in the first hydrolysis versus 0.041 g total sugars g BSG^{-1} in the second hydrolysis). As the most extracted sugars were xylose and arabinose, regardless of the hydrolysis, higher yields were obtained. Second hydrolysis was performed in order to extract sugars still present, improving hydrolysis yield. Since acid hydrolysis was performed twice, hydrolysis yield was achieved by mixing the first and second hydrolyzed BSG, resulting in a 0.152 ± 0.01 g total sugars g BSG^{-1}.

3.4.2 Characterization of BSG hydrolysate using SCW

To evaluate a greener BSG pre-treatment strategy, SCW was selected and the effect of temperature on extraction/hydrolysis efficiency of BSG was studied. Four temperatures (T = 150, 180, 200 and 230 °C) were tested and water flow rate was maintained at 10 mL min^{-1} for all assays. During the hydrolysis process, it was observed a change in the color of the samples recovered. In the beginning, samples were clear and slightly yellow but along the hydrolysis, they became darker and turbid until maximum temperature was reached, where samples became brown (Figure 3.3). Pourali et al. (2009), that used SCW to treat rice bran to produce valuable materials, observed a similar colour change to brown at moderate temperatures (i.e. 160 - 280 °C) and with 5 minutes of reaction time. Hata et al. (2008) studied extraction of defatted rice bran using SCW and reported that brown color could be a result from Maillard reaction or from carbohydrates caramelization.

Figure 3.3: Samples collected during hydrolysis reactions using SCW at P = 65 bar. From left to right are presented the first (lower temperature) to the last sample (higher temperature).

The influence of temperature on the extraction/hydrolysis efficiency using SCW in the range 150 - 230 °C is shown in Table 3.5.

Table 3.5: Influence of temperature on the BSG extraction/hydrolysis using SCW. P = 65 bar.

Temperature (°C)	**Flow rate** (mL min^{-1})	**Extent of hydrolysis** (%)	**Yield** (%)
150	10.0	21.2	-
180	10.0	35.3	-
200	10.0	42.4	15.3
230	10.0	69.3	24.6

As expected, increasing the temperature in the range tested led to an increase in the extent of hydrolysis, from ca. 21% at 150 °C to ca. 69% at 230 °C (Table 3.5). This shows that temperature promotes an increase of ionic product of water, which becomes a stronger catalyst for the hydrolysis of biomass (Kruse and Dinjus, 2007). Using SCW at 150 °C and 180 °C, the extent of hydrolysis was very low (21.2 and 35.3% at 150 and 180 °C, respectively). Thus, the yield of soluble compounds was not calculated for these two temperatures. For 200 °C and 230 °C, the yield was 15.3% and 24.6%, respectively. It was possible to verify a higher yield with temperature increase, because of higher extraction of soluble compounds. Comparing the extent of hydrolysis and the yield for both temperatures (T = 200 and 230 °C), the second one is lower than the first one, with 42.4% and 15.3% at T = 200 °C and 69.3% and 24.6% for T = 230 °C, respectively. Pedras et al. (2017), that used SCW to valorize white wine grape pomace, reported that this difference can be related to the release of volatile compounds produced during the process. As temperature of the assay increases, greater is the duration of the cooling phase, where volatile compounds can also be extracted and are not taken into account in the quantification of the yield of water soluble compounds. Furthermore, there is always mass loss in the process, which also can reflect the difference between the extent of hydrolysis and the yield of soluble compounds. BSG hydrolysates obtained at 200 °C and 230 °C were characterized in terms of sugars and organic acids content (Table 3.6).

Table 3.6: Total recovered sugars and HOrg at different temperatures (200 and 230 °C) with water flow rate at 10 mL min^{-1} and P = 65 bar.

Extracted compounds	T = 200 (°C)	T = 230 (°C)
Arabinose (mg L^{-1})	121.1	284.3
Glucose (mg L^{-1})	37.7	74.1
Xylose (mg L^{-1})	86.1	183.8
Total Sugars (mg L^{-1})	244.9	542.2
HAc (mg L^{-1})	590.0	1810.0
HIso (mg L^{-1})	40.00	80.00
Total acids (mg L^{-1})	630.0	1890.0

The results presented in Table 3.6 show the extracted compounds at two different temperatures (200 and 230 °C). Similarly to acid hydrolysis, the identified sugars were xylose, arabinose and glucose, being the first two the main sugars extracted. Some organic acids (HOrg) were also detected, namely acetic and isovaleric acids (HAc and HIso, respectively). Their presence can be explained by the fact that some degradation of fermentable sugars can occur, resulting in organic acids formation (Prado et al., 2014). Both, sugars and acids, were extracted in higher concentrations at 230 °C than at 200 °C, probably due to the increase in water reactivity. In order to prevent sugars degradation and, consequently, decrease the acids concentration, the water residence time should be decreased at higher temperatures (Prado et al., 2014). However, this was not tested in the present study and should be deeper investigated.

3.4.3 Pre-treatment strategies comparison

To evaluate the BSG pre-treatment strategies studied, acid hydrolysis and SCW, a comparison between them is necessary. Table 3.7 present sugars and HOrg identified by acid hydrolysis and by SCW, at T = 200 °C and T = 230 °C.

Table 3.7: Extracted sugars and HOrg from acid hydrolysis and SCW (T = 200 °C and T = 230 °C, respectively).

	Acid hydrolysis*	SCW (T = 200 °C)	SCW (T = 230 °C)
Arabinose (g L^{-1})	4.94 ± 0.06	0.121	0.284
Glucose (g L^{-1})	3.35 ± 0.31	0.038	0.074
Xylose (g L^{-1})	9.20 ± 0.23	0.086	0.184
Total sugars (g L^{-1})	17.49	0.245	0.542
Acetic acid (g L^{-1})	1.52 ± 0.000	0.590	1.81
Isovaleric acid (g L^{-1})	-	0.040	0.080
Total acids (g L^{-1})	1.520	0.630	1.890

*Average values ± standard deviations

It was observed that more sugars were extracted in acid hydrolysis than SCW (17.49 g L^{-1} versus 0.245 and 0.542 g L^{-1} at T = 200 °C and 230 °C, respectively) (Table 3.7). In acid hydrolysis, it was possible to detect HAc. In SCW, HAc and HIso were detected. With respect to acid hydrolysis, the HAc concentration was higher than the obtained in SCW at T = 200 °C but lower than T = 230 °C. The hydrolysis yield on sugars for the studied strategies is presented in Table 3.8.

Table 3.8: Hydrolysis yield on sugars from BSG for the two pre-treatment strategies, namely acid hydrolysis and SCW (T = 200 and 230 °C).

	Acid hydrolysis*	**Subcritical water**	
		T = 200 °C	**T = 230 °C**
Hydrolysis yield on sugars (g total sugars g BSG^{-1})	0.152 ± 0.01	0.008	0.020

*Average values ± standard deviations

Although SCW is considered an environmental friendly method, when compared to acid hydrolysis, lower yields were obtained for the tested conditions (0.152 g total sugars g BSG^{-1} versus 0.008 at 200 °C and 0.020 g total sugars g BSG^{-1} at 230 °C, respectively). From SCW, the main sugars extracted were solubilized with oligosaccharides, resulting in low yield. Schacht et al. (2008), who studied the production of ethanol from plant materials, stated that sugars can be protected from decomposition by keeping them in oligomeric form.

Despite of its disadvantages, acid has the potential to penetrate lignin, breaking down cellulose and hemicellulose structure, forming individual sugar molecules (Verardi et al., 2012). The combination of both methods could probably increase the hydrolysis yield on sugars. However, it does not bring any advantage to the pre-treatment process as it continues to involve the use of non-environmental friendly solvents. Regardless of their non-environmental friendly nature, acid hydrolysis has been currently applied since it does not need to be conjugated with another preliminary method (K. Chandel et al., 2012). As a higher hydrolysis yield was obtained by acid hydrolysis, this method was selected to perform the work described in the subsequent chapters.

4. Acidogenic fermentation of brewer’s spent grain (BSG): pH and organic load effects

4.1 Introduction

As an alternative to petroleum-based plastics, polyhydroxyalkanoates (PHA) are biodegradable polymers currently industrially produced by pure cultures, depending on expensive substrates which increases the overall process of PHA production (Reis et al., 2003). Being the main goal to reduce PHA production costs, several agro-industrial wastes, largely discharged from agricultural and food processing, can be used as substrate by MMCs to organic acids synthesis towards PHA production (Gouveia et al., 2016). To produce organic acids, which are PHA precursors, a first anaerobic stage is necessary. Acidogenic fermentation is crucial when PHA is being produced by MMCs and is the first of the 3-stage process of PHA production. This stage consists on the conversion of sugars from wastes into organic acids (HOrg) (e.g. acetic, propionic, butyric, lactic and valeric acids), EtOH, H_2 and CO_2 (Lier et al., 2008). Unlike pure cultures, this stage involves microorganisms growing in a mixed consortia, providing high microbial diversity and possibility to treat different types and mixtures of substrates under non-sterile conditions (Perimenis et al., 2016).

In the last few decades some parameters such as type of substrates, pre-treatment, inoculum, temperature, pH, retention time, and organic loading rate (OLR) for fermentation products (FP) production have been studied in order to manipulate FP composition (Li et al., 2017; Duque et al., 2014, Gouveia et al., 2016). At different pH, different organisms are selected, guiding the process through a specific metabolic pathway, resulting in different FP compositions. This in turn directly affect the PHA monomer composition allowing to achieve a large range of polymer compositions (Gouveia et al., 2016). Albuquerque et al. (2007) showed that operational pH during acidogenic fermentation of sugar cane molasses can affect the FP composition and, consequently, the composition of the produced PHA. Operating in continuous mode, higher FP yields are obtained comparing to batch mode, where lower yields are obtained (0.75 - 0.87 gCOD $gCOD^{-1}$ versus 0.59 - 0.60 gCOD $gCOD^{-1}$ (Bengtsson et al., 2008a).

This part of the work focused on brewer's spent grain (BSG) acidogenic fermentation in batch, using anaerobic granules. Due to their rich composition in nutrients, namely sugars, proteins and minerals, BSG is usually reused as animal feed (Mussatto et al., 2006). However, it may have potential to be applied in several other applications, such as PHA production. Batch assays were operated at three different pH (5.0, 5.5 and 6.0) and under three different organic loads (10, 20 and 30 gCOD L^{-1}).

The main goal is to investigate, in batch, the effects of pH and organic load on acidogenic fermentation of hydrolyzed BSG, namely on organic acids profile and concentration.

4.2 Materials and Methods

4.2.1 BSG preparation

BSG was pre-treated according to the 3.2.2 section, by acid hydrolysis and stored in the fridge at 4 °C. Prior to each experiment and after feed neutralization, pH was adjusted using concentrated HCl (HCl 37%, Sigma Aldrich) or NaOH (2 M, Laborspirit).

4.2.2 Experimental set-up

Batch assays were performed in reactors with a working volume of 500 mL (Figure 4.1). Each reactor was inoculated with 150 mL of anaerobic granules, supplied by a Portuguese Brewery Industry, corresponding to about 30% of the reactor volume (Lim and Kim, 2014). The reactors were fed with hydrolyzed BSG in order to apply three different organic loads (10, 20 and 30 gCOD L^{-1}) and tap water was added up to 500 mL.

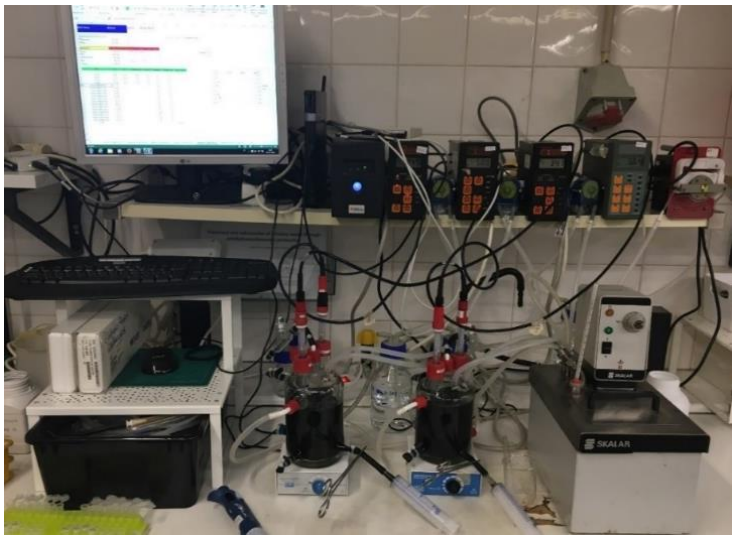

Figure 4.1: Batch assays experimental set-up.

Operational conditions are summarized in Table 4.1, where three organic load (OL) were conjugated with three pH (5.0, 5.5 and 6.0). Reactors were mixed at 300 rpm and the temperature was kept at 30 ± 0.1 °C using a water bath (Skalar). The pH was controlled by the automatic addition of 1 M NaOH and 1 M HCl solutions. Samples were daily taken from the reactor for quantification of phosphate (PO_4^{3-}) and ammonia (NH_4^+), COD, FP, TSS and VSS, sugars and proteins. Analysis of gas was daily performed, namely methane, oxygen, nitrogen, hydrogen and carbon dioxide. Assays were online monitored using a software program developed in the research group, through redox potential and pH data acquisition. All the assays were run in triplicate.

Table 4.1: General operating conditions applied for BSG acidogenic fermentation in batch.

Operational conditions		
10 gCOD L^{-1}	20 gCOD L^{-1}	30 gCOD L^{-1}
pH 5.0		
pH 5.5		
pH 6.0		

4.2.3 Analytical Procedures

4.2.3.1 Total suspended solids (TSS) and volatile suspended solids (VSS)

Biomass concentration, was measured, in duplicate, as volatile suspended solids (VSS) by filtration in a vacuum pump (VARIAN - Filtration System), according to the Standard Methods (ALPHA, 1998). Briefly, after sample filtration, the filter (VWR, Glass fibre filters 21 mm) was dried up overnight at 105 °C (Oven TR 60, Nabertherm, Soquimica). The weight of dried sample contained inside the dish corresponded to the value of TSS. The dried sample was then reduced to ash throughout incubation in a muffle (Nabertherm, Soquimica) for 2 hours at 550 °C. VSS concentration was achieved by the difference between the weight of the sample before and after the step at 550 °C.

4.2.3.2 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) method determines the quantity of organic oxygen necessary to oxidize the organic matter in a sample under controlled conditions. The COD determination was based on dichromate ion ($Cr_2O_7^{2-}$) oxidation of COD material in the sample, resulting on change of chromium from the hexavalent (VI) state to the trivalent (III) state. The COD concentration was measured spectrophotometrically using standard kit tests in the range of 5 - 60 $gO_2\ L^{-1}$ (Hach, Germany) (Figure 4.2), after digestion (Hach Lange HT 200) for 15 minutes (170 °C) according to the Standard Methods (ALPHA, 1998).

Figure 4.2: COD analysis of batch assays samples.

4.2.3.3 Fermentation products and sugars analysis by High performance liquid chromatography (HPLC)

Concentration of sugars (glucose, xylose and arabinose), organic acids (acetic, butyric, lactic, propionic, valeric and isovaleric acids) and EtOH were determined by HPLC method as described in section 3.2.3 using a standard calibration curve (31 - 1000 mg L^{-1} of each sugar, organic acid and EtOH) (Duque et al., 2014).

4.2.3.4 Ammonia and phosphate content

Ammonia (NH_4^+) and phosphate (PO_4^{3-}) concentration were determined by colorimetry, as implemented in a flow segmented analyzer (Skalar 5100, Skalar Analytical, Netherlands) (Carvalho et al., 2007). The samples were diluted with mili-Q water and filtered (VWR, spin filter pore size 0.2 µm). Ammonium chloride (NH_4Cl 99%, Sigma) and ortho-phosphoric acid (H_3PO_4 85%, Panreac) were used as standard at concentrations ranging from 5 to 20 mg L^{-1}.

4.2.3.5 Total sugars content

Total sugars content was measured colorimetrically by the Morris method (Morris, 1948) with modifications by Koehler (Koehler, 1952), Baily (Bailey, 1958) and Gaudy (Duque et al., 2014). Briefly, samples were digested (Hach Lange LT 200) with anthrone ($C_4H_{10}O$ 97%, Sigma Aldrich) solution (0.125 g anthrone in 100 mL sulphuric acid) at 100 °C for 14 minutes. Absorbance was measured at λ = 625 nm and total sugars concentration was calculated through a standard calibration curve of D-glucose monohydrate ($C_6H_{12}O_6$, Scharclau, Spain) (0 - 100 mg L^{-1}).

4.2.3.6 Total protein content

The protein content was determined according to the spectrophotometrically method described by Lowry (Lowry et al., 1951). This method was based on a digestion with sodium carbonate (Na_2CO_3), potassium sodium tartrate (NaK), sodium hydroxide (NaOH), cupric sulphate ($CuSO_4$), sulfuric acid (H_2SO_4) and Folin phenol solution (Folin, Panreac AppliChem, Spain) for 30 minutes at room temperature, in the dark. Absorbance was measured at λ = 750 nm and total protein concentration was calculated through a standard calibration curve of bovine serum albumin (BSA 98%, pH 7.0, Sigma Aldrich) (0 - 100 mg L^{-1}).

4.2.3.7 Gas chromatography (GC)

To evaluate the composition of the gas produced, namely in methane (CH_4), carbon dioxide (CO_2), oxygen (O_2), nitrogen (N_2) and hydrogen (H_2), gas chromatography (GC) was performed. GC (GC Thermo, Trace GC Ultra model) was equipped with a TCD detector and 30 meters of Carboxen 1010 Plt column. The mobile phase was constituted by helium at a flow rate of 1 mL min^{-1} and conducted with isothermal runs during 50 minutes at 35 °C. The injector temperature was 200 °C.

4.3 Calculations

Sugars and FP concentrations were measured by HPLC and converted to COD units, and biomass concentration (gVSS L^{-1}) was calculated as volatile suspended solids (VSS).

The degree of fermentation (DF in gCOD-FP $gCOD^{-1}$) was calculated based on Eq 4.1, by dividing the amount of fermentation products (FP) produced (difference between the amount of the total detected outlet fermentation products (TFP_{out}) and the total inlet fermentation products (TFP_{in})), converted to COD units, by the influent COD (COD_{in}).

$$DF = \frac{TFP_{out} - TFP_{in}}{COD_{in}} \qquad \text{Eq 4.1}$$

FP yield on substrate ($Y_{FP/S}$) was calculated as described on Eq 4.2, where COD_{out} is the outlet effluent COD (in gCOD L^{-1}).

$$Y_{FP/S} = \frac{TFP_{out} - TFP_{in}}{COD_{in} - COD_{out}} \qquad \text{Eq 4.2}$$

Volumetric substrate uptake rate ($-r_S$ in gCOD L^{-1} h^{-1}) and volumetric FP production rate (r_{FP} in gCOD-FP L^{-1} h^{-1}) were calculated by linear regression of the substrate concentration (S = COD_{in}

– TFP_{out} in gCOD L^{-1}) or TFP_{out} concentration (in gCOD L^{-1}), respectively, versus the time over the batch experiments (t in hours) (Eq 4.3 and 4.4).

$$-r_S = \frac{\Delta S}{t} \qquad \text{Eq 4.3}$$

$$r_{FP} = \frac{\Delta FP}{t} \qquad \text{Eq 4.4}$$

The division of the volumetric rates by the biomass concentration (gVSS L^{-1}) results on specific substrate uptake ($-q_S$, gCOD $gVSS^{-1}$ h^{-1}) and production rates (q_{FP}, gCOD-FP $gVSS^{-1}$ h^{-1}) represented in Eq 4.5 and 4.6.

$$-q_S = \frac{-r_S}{VSS} \qquad \text{Eq 4.5}$$

$$q_{FP} = \frac{r_{FP}}{VSS} \qquad \text{Eq 4.6}$$

Protein removal (in % (g g^{-1})) was calculated by dividing the amount of proteins consumed (difference between the inlet ($TPro_{in}$) and the outlet ($TPro_{out}$) protein concentration) by the inlet protein concentration (Eq 4.7).

$$\text{Total protein removed (\%)} = \frac{TPro_{in} - TPro_{out}}{TPro_{in}} \times 100 \qquad \text{Eq 4.7}$$

4.4 Results and Discussion

As mentioned above, brewer's spent grain can be a substrate for production of organic acids by MMCs due to its reach composition in nutrients (mainly sugars and proteins). In this section, batch assays were performed in order to study pH and organic load effects on acidogenic fermentation of hydrolyzed BSG. Batch assays were conducted at different operating conditions, namely three different pH (5.0, 5.5 and 6.0) and three OL (10, 20 and 30 gCOD L^{-1}), and all were run for 7 days. Sugars and FP profiles along one batch assay (pH 5.0, OL 20 gCOD L^{-1}) is presented in Figure 4.3.

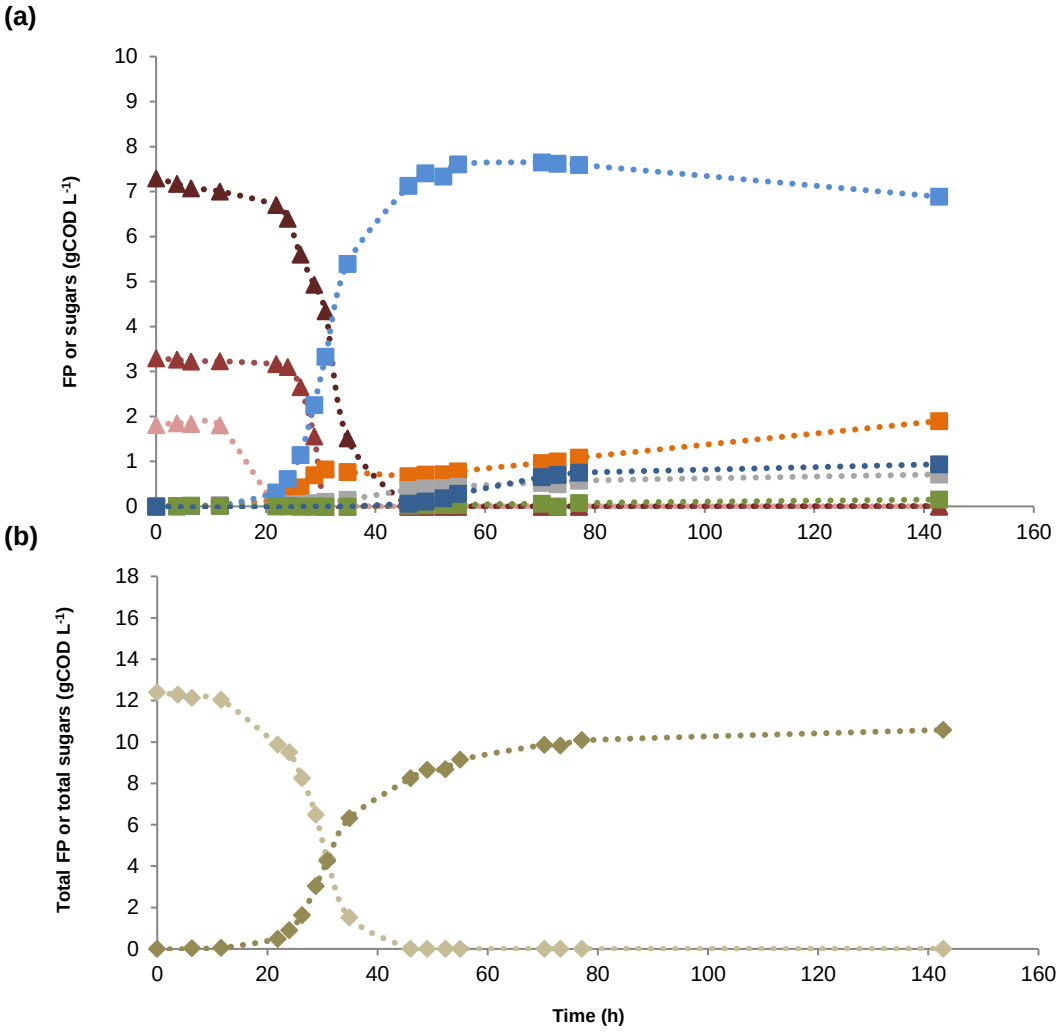

Figure 4.3: Example of one batch assay (pH = 5.0, Organic Load = 20 gCOD L^{-1}): (a) detected FP (HAc (■), HProp (■), HBut (■), HVal (■), HIsov (■) and sugars (glucose (▲), xylose (▲) and arabinose (▲), (b) total profile of detected FP (HOrgs) (♦) and consumed sugars (glucose + xylose + arabinose) (♦) are presented.

As can be depicted from Figure 4.3, all the sugars (glucose, xylose and arabinose) were totally consumed during the exponential phase. During the same phase, the greatest amount of product was obtained. The main HOrg detected in all batch assays were acetic (HAc), propionic (HProp), butyric (HBut), valeric (HVal) and isovaleric (HIso) acids. No lactic acid and ethanol production was observed. In Figure 4.4 the average FP composition obtained at the end of the experiments is shown for each assay.

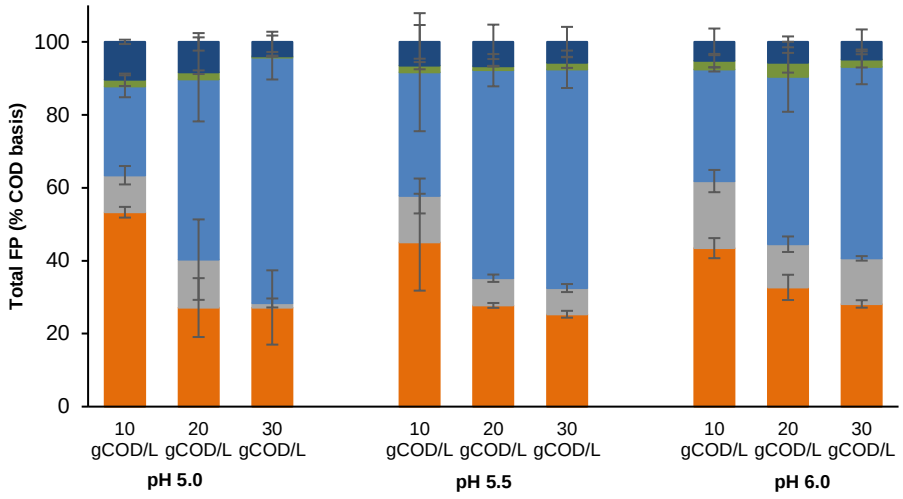

Figure 4.4: Fermentation products profile obtained at the three different pH (5.0, 5.5 and 6.0) and organic loads (10, 20 and 30 gCOD L^{-1}) tested. HAc (■), HProp (■), HBut (■), HVal (■) and HIsov (■) are presented. Error bars represents standard deviation.

The fermentation products spectra demonstrate that, regardless of pH or organic load, the more concentrated species were HBut and HAc in all experiments (46.8 ± 13.61% and 34.5 ± 9.6%, respectively). These results are in agreement with those observed by Vergine et al. (2015) where under acid conditions (3.9 < pH < 5.2), FP composition consists mostly on HAc and HBut. HProp, HVal and its isomer (HIso) were also present, but in lower concentrations. According to Figure 4.4, for the range of pH tested, increasing OL, HAc and HProp decreased whereas HBut increased. Higher concentrations of HVal were obtained at 20 gCOD L^{-1} and pH 5.0 and 6.0. HIso concentrations decreased with OL increase at pH 5.0 whereas lower concentrations were achieved under the highest OL (30 gCOD L^{-1}) at pH 5.5 and 6.0.

On the other hand, when the same organic load at each different pH tested is compared, there was no general tendency. For 10 gCOD L^{-1}, HAc and HIso decreased, whereas HProp and HVal increased with pH increase. HBut have higher concentrations at pH 5.5. When organic load was increased to 20 gCOD L^{-1}, HAc increased and HIso decreased from pH 5.0 to 6.0. HProp and HVal, under the same OL, reached lower concentrations at pH 5.5. At 30 gCOD L^{-1} of OL, HProp and HVal increased with pH increase. At the same pH range, HBut decreased. HIso and HAc reached lower concentrations at pH 5.0 and 5.5, respectively. Bengtsson et al. (2008a), who have studied the acidogenic fermentation of cheese whey, observed that HProp concentrations increased with pH increase from 5.0 to 6.0. This is in accordance with the results obtained at 10 and 30 gCOD L^{-1} of OL. A study on the dynamic effect of pH on cheese whey acidogenesis have shown that HVal production is enhanced at higher pH values (Gouveia et al., 2016). This was also observed in the present study, HVal increased with pH, reaching higher concentrations at pH 6.0. Furthermore, the production of short chain fatty acids such, as HBut, is favoured by operation at lower pH (Zoetemeyer et al., 1982, Albuquerque et al., 2007). Its higher concentration was obtained at pH 5.0. These results showed that pH guide FP production through specific and different metabolic pathways, resulting in different fermentation products profile. In order to

evaluate the overall batch assays performance, stoichiometric and kinetics coefficients were calculated and listed in Table 4.2. All coefficients were calculated in the exponential phase for each batch assay. The yield of biomass per substrate ($Y_{X/S}$) was not calculated due to the absence of cell growth. As no growth was observed, an average biomass concentration value was assumed for each batch assay, in order to calculate both specific substrate uptake and FP production rates ($-q_S$ and q_{FP}, respectively).

Table 4.2: Stoichiometric and kinetic coefficients calculated for each batch assay.

	pH 5.0			**pH 5.5**			**pH 6.0**		
	Organic Load (gCOD L^{-1})								
	10.0	**20.0**	**30.0**	**10.0**	**20.0**	**30.0**	**10.0**	**20.0**	**30.0**
Biomass concentration (gVSS L^{-1})	15.77 (1.08)	17.88 (1.98)	19.64 (2.36)	15.69 (1.05)	20.56 (1.07)	19.59 (1.07)	16.32 (1.40)	18.61 (1.43)	19.32 (2.49)
Total FP (gCOD L^{-1})	6.09 (0.81)	11.31 (1.73)	13.18 (0.41)	6.23 (0.97)	11.77 (0.99)	16.49 (2.23)	6.12 (0.67)	12.32 (1.36)	17.76 (2.15)
$Y_{FP/S}$ (gCOD-FP $gCOD^{-1}$)	0.66 (0.12)	0.69 (0.12)	0.51 (0.004)	0.63 (0.03)	0.57 (0.01)	0.60 (0.03)	0.63 (0.03)	0.61 (0.04)	0.55 (0.03)
$-r_S$ (gCOD L^{-1} h^{-1})	0.40 (0.08)	0.78 (0.15)	1.29 (0.02)	0.30 (0.05)	0.87 (0.04)	0.92 (0.04)	0.24 (0.02)	0.61 (0.04)	1.15 (0.13)
r_{FP} (gCOD L^{-1} h^{-1})	0.27 (0.01)	0.53 (0.03)	0.67 (0.03)	0.19 (0.04)	0.50 (0.03)	0.55 (0.01)	0.16 (0.01)	0.38 (0.02)	0.63 (0.03)
$-q_S$ (gCOD $gVSS^{-1}$ h^{-1})	0.025 (0.004)	0.044 (0.009)	0.064 (0.003)	0.019 (0.003)	0.042 (0.004)	0.047 (0.001)	0.015 (0.002)	0.033 (0.002)	0.060 (0.008)
q_{FP} (gCOD-FP $gVSS^{-1}$ h^{-1})	0.017 (0.001)	0.030 (0.003)	0.034 (0.001)	0.012 (0.003)	0.025 (0.002)	0.028 (0.002)	0.009 (0.000)	0.020 (0.001)	0.032 (0.003)
DF (gCOD-FP $gCOD^{-1}$)	0.26 (0.06)	0.30 (0.04)	0.18 (0.01)	0.36 (0.04)	0.29 (0.06)	0.33 (0.02)	0.39 (0.04)	0.39 (0.02)	0.36 (0.09)
Protein Removal (%)	19.80 (9.00)	17.60 (7.00)	17.20 (8.50)	42.10 (10.80)	35.60 (14.00)	26.00 (9.40)	43.58 (4.08)	40.33 (1.46)	43.40 (15.00)

It was observed that the highest total FP concentration was reached at the highest organic load (30 gCOD L^{-1}), as the quantity of organic matter subjected to fermentation is higher in the case of 30 gCOD L^{-1} than 10 or 20 gCOD L^{-1} (Table 4.2). For all the OL tested, total FP concentration increased with pH increase, reaching higher concentrations at pH 6.0 (Table 4.2). Jiang et al. (2013), who have studied acidogenesis of food waste, reported that the maximum volatile fatty acids (VFA) was obtained at pH 6.0 derived from the optimal activities of hydrolytic enzymes at this pH. Regardless of the pH range tested, volumetric and specific rates increased with OL. Generally, they were higher at pH 5.0 and lower at pH 6.0. On the other hand, maintaining OL at 10 gCOD L^{-1} and increasing pH, volumetric and specific rates decreased. The same was verified at 20 gCOD L^{-1}, expect volumetric substrate uptake rate that were higher for pH 5.5. The lower volumetric and specific rates at pH 6.0 for 10 and 20 gCOD L^{-1} could be explained by the fact that the microorganisms could slowly grow or by high biomass concentration which affect the values. At 30 gCOD L^{-1}, both volumetric and specific rates were higher at pH 5.0 and lower at pH 5.5.

The DF, defined as the capacity of the system to produce acids from a substrate (COD_{in}) degradation (Dahiya et al., 2015), did not suffer major changes with organic load at pH 5.5 and 6.0. However, at pH 5.0 it decreased at 30 gCOD L^{-1}, as other inhibitory compounds could also increase with OL. Furthermore, sugars could be inhibitory at a certain concentration under this pH or feed oscillations can also be related to this decrease. DF increased with pH for the same organic load (e.g. 0.18 ± 0.01 to 0.36 ± 0.09 gCOD-FP $gCOD^{-1}$ for 30 gCOD L^{-1}, at pH 5.0 and 6.0, respectively).

The average yields of FP/S obtained for pH 5.0, 5.5 and 6.0 are presented in Table 4.2. The highest yield was obtained at pH 5.0 and 20 gCOD L^{-1} of OL. Generally, FP yield was not significantly affected by pH but slightly affect by the organic load. The lowest yields of FP on substrate were obtained at 30 gCOD L^{-1}. The fermentation yields obtained (0.69 - 0.51, 0.63 - 0.57 and 0.63 - 0.55 gCOD $gCOD^{-1}$ at pH 5.0, 5.5 and 6.0, respectively) were in the same range of those reported for cheese whey by Gouveia et al. (2016) (0.59, 0.58, and 0.55 - 0.68 gCOD $gCOD^{-1}$ at pH 4.5, 5.0 and 6.0) and by Tamis et al. (2015) for glucose (0.66, 0.60 and 0.59 gCOD $gCOD^{-1}$, at pH 4.5, 5 and 5.5, respectively).

During the assays, protein concentration decreased. This demonstrated that anaerobic bacteria were able to use protein as N and P source. Regardless of organic load, protein removal increased with pH increase, reaching the maximum value of 43.40 ± 15.00% at pH 6.0 and 30 gCOD L^{-1}. Duque et al. (2014), who have investigated the acidogenic fermentation of cheese whey at pH 6.0 under ca. 13 gCOD L^{-1} reported ca. 41 - 48% of total amount of protein removed using an anaerobic membrane (AnMBR) reactor.

The final step of anaerobic digestion is the conversion of organic acids to gas, namely methane. However, gas production was not the purpose of the present study. Contrarily, the main aim of this study was to produce organic acids, thus stopping the process before gas production. Therefore, gas analysis was performed along the experiments. The mainly detected gases were nitrogen (N_2) and carbon dioxide (CO_2). Hydrogen (H_2) was detected at pH 5.5 and 5.0. Minor amounts of oxygen (O_2) and methane (CH_4) were also detected. As such, the right conditions were applied to the batch assays in order to reach organic acids production (acidogenesis), without methane production (methanogenesis).

As mentioned above, acid hydrolysis can enhance the degradation of sugars resulting in several inhibitory compounds. Despite of the low concentrations of furfural in the hydrolyzed BSG, it was verified what happens with acidogenic fermentation. In the beginning of the exponential fase of each batch assay, furfural was present. In the end of the exponential phase, furfural was not detected. This can be to the fact that under anaerobic conditions, some bacteria can convert this compound into less inhitory compounds (Monlau et al., 2014). According to Hahn-Hägerdal (2000), furfural can be metabolised by *S.cerevisae* and reduced to furfuryl alcohol during acidogenic fermentation under the conditions indicated above.

Since volumetric and specific rates increases with organic load, biomass concentration is not limitant. The relation between total FP produced and the three organic loads applied at different pH is presented in Figure 4.5.

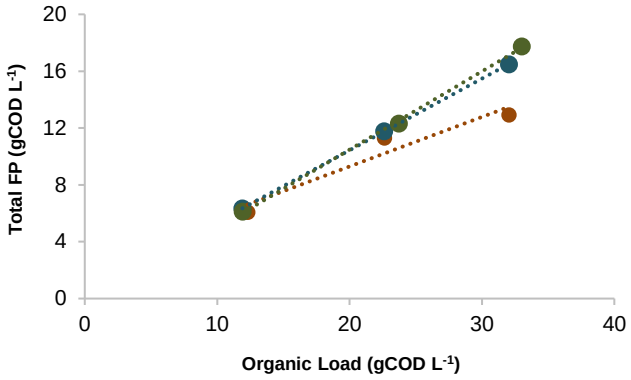

Figure 4.5: Linear regression between total fermentation products obtained for the three applied organic loads at different pH (5.0 (●), 5.5 (●) and 6.0 (●)) for the same biomass concentration.

As the organic load increased, total FP also increased. This showed that there was no substrate saturation, which means that higher organic loads may be applied for the same biomass concentration.

Since different conditions were applied, different FP concentrations, profiles and yields were obtained. It was possible to understand that organic load was related to the total HOrg concentration, as its concentration increased with organic load increase. On the other hand, pH affects the HOrg profile. Consequently, variable HOrg in FP composition generated, is directly related to the composition of the produced polymer (Vergine et al., 2015; Gouveia et al., 2016). For instance, HAc and HBut enhance hydroxybutyrate (HB) monomers synthesis whereas hydroxyvalerate (HV) monomers production depends on HProp presence (Bengtsson et al., 2008a). However, the production of other HOrg can promote the synthesis of different homopolymers or copolymers with improved mechanical properties than P(3HB), which is characterized by its limited use due to their relatively rigidity and fragility (Suriyamongkol et al., 2007, Lemos et al., 2006). PHA copolymers synthetized from other HOrg are generally more flexible and resistant, providing its application in a wide range of applications (Suriyamongkol et al., 2007).

In summary, the manipulation of the acidogenic fermentation stage operating conditions, such as organic load and pH, allow the production of different HOrg concentrations and profiles from BSG. Consequently, this will allow the manipulation of PHA composition.

5. Acidogenic fermentation of BSG in an expanded granular sludge bed reactor (EGSB)

5.1 Introduction

Nowadays, anaerobic treatment of several industrial wastewater has been operated in high-rate anaerobic systems. Within the possible configurations, the most common are the upflow anaerobic sludge blanket reactors (UASB), operated in the absence of mixing devices and equipped with a set of gas liquid solid separator (GSS) promoting the granules/effluent separation (Lim and Kim, 2014). It is characterized by liquid upflow velocity (V_{up}) ranging from 0.5 m h^{-1} to 1 m h^{-1} (Lim and Kim, 2014, Kato et al., 1999). Expanded granular sludge bed reactors (EGSB) belongs to the UASB reactors family, which have been developed with additional effluent recirculation and a higher height (Figure 5.1). Operated under V_{up} higher than 6 m h^{-1} it provides efficient granules expansion and hydraulic mixing, improving better sludge/substrate contact and reducing dead space in the reactor (Lim and Kim, 2014, Lier et al., 2008).

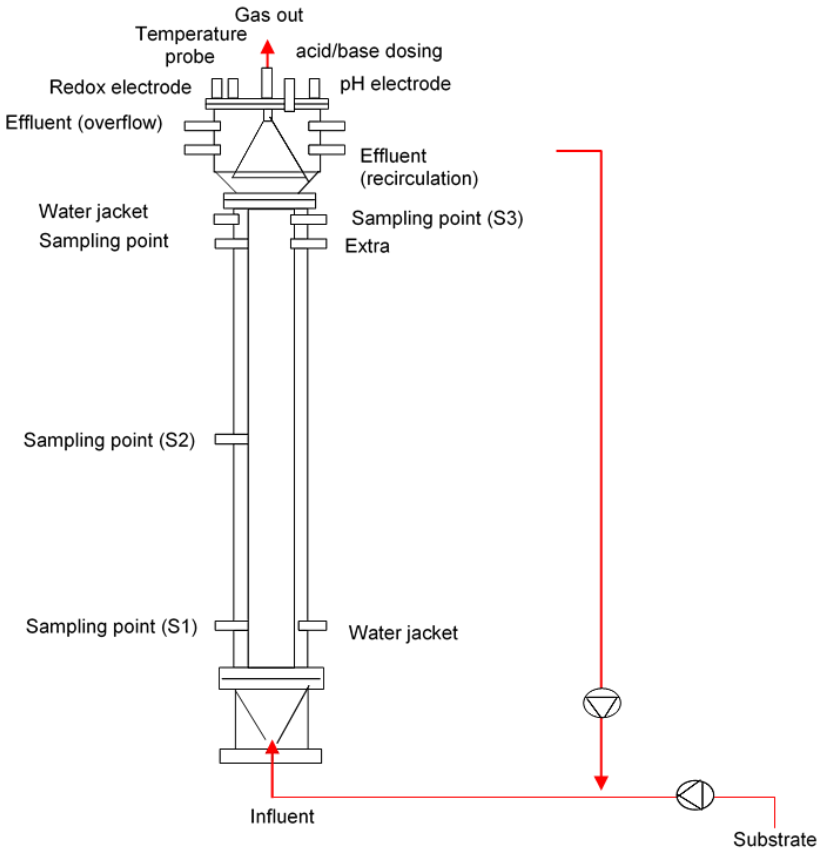

Figure 5.1: Schematic representation of EGSB reactor.

EGSB has been used to treat effluents such as palm oil mill (Zhang et al., 2008), oleic acid (Pereira et al., 2002), for energy production such as biohydrogen through fermentation of molasses (Guo et al., 2008) or biogas production from potato-juice (Fang et al., 2011) and to treat wastewaters composed by biodegradable organics, which are toxic at high concentrations (Dinsdale et al., 2000). This configuration is widely used due to its more stable performance, namely a less sensibility to fluctuations of several the environmental parameters (e.g., acidity, HRT) than a CSTR (Lim and Kim, 2014). This chapter focused on hydrolyzed BSG acidogenic fermentation in continuous mode using an expanded granular sludge bed (EGSB) reactor.

5.2 Materials and Methods

5.2.1 BSG preparation

Brewer's spent grain (BSG) was supplied by a Portuguese Brewery Industry and pre-treated by acid hydrolysis according to chapter 3. Hydrolyzed BSG was used as feed and prepared by diluting this feedstock with tap water at a final concentration of 20 gCOD L^{-1}. The medium was kept at 4°C in a continuously stirred vessel. Due to its high protein content, BSG was not supplemented with nutrients.

5.2.2 Experimental set-up

BSG acidogenic fermentation was performed in an expanded granular sludge bed (EGSB) reactor, operated under anaerobic conditions with a working volume of 3 L (Figure 5.2). The reactor was inoculated with anaerobic granules supplied by a Portuguese Brewery Industry at ca. 20% (v/v) (Lim and Kim, 2014). The anaerobic reactor was operated in a continuous mode, at pH 4.5 and 30 ± 0.1 °C. pH was controlled by automatic addition of 2 M NaOH. Hydrolyzed BSG feed solutions flow rates were adjusted to keep the reactor HRT at 2 days with an organic load of 10 gCOD L^{-1} d^{-1}. The hydraulic retention time (HRT) was controlled by influent flow rate. Samples were periodically taken from the reactor for quantification of PO_4^{3-} and NH_4^+, COD, FP, TSS and VSS, sugars and proteins. Gas was measured weekly and pH and redox were online monitored. The general conditions applied to the reactor are presented in Table 5.1.

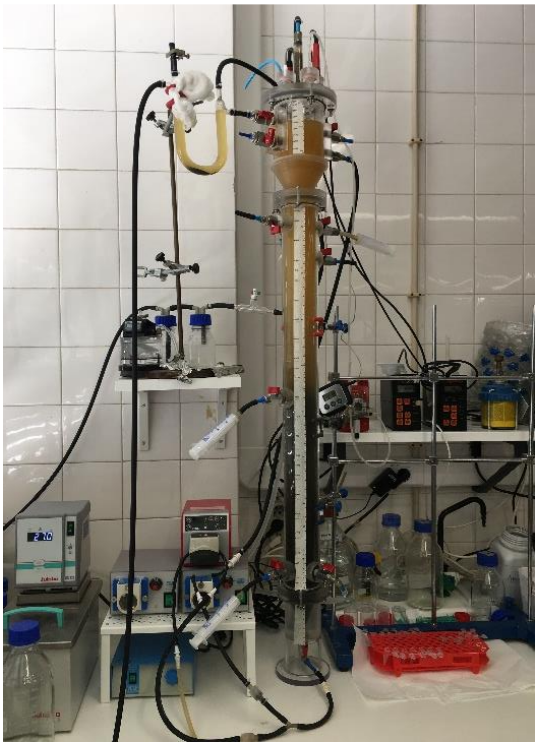

Figure 5.2: Acidogenic fermentation set-up.

Table 5.1: General operating conditions applied to the EGSB for BSG acidogenic fermentation.

pH	4.50 ± 0.08
Temperature (°C)	30.0 ± 1.00
HRT (days)	2.60 ± 0.23
OLR (gCOD L^{-1} d^{-1})	7.88 ± 1.26
Superficial upflow velocity (m h^{-1})	6.0
C:N:P ratio (C-mol:N-NH_4^+-mol:P-mol)	100:27:1

5.2.3 Analytical Procedures

Biomass concentration was measured as VSS concentration according to Standard Methods (ALPHA, 1998). Concentrations of sugars (glucose, xylose, arabinose), organic acids (acetic, butyric, lactic, valeric, isovaleric and propionic acids) and EtOH were determined by HPLC as mentioned in section 3.2.3 through a standard calibration curve (31 - 1000 mg L^{-1} of each sugar, organic acid and EtOH) (Duque et al., 2014).

Chemical oxygen demand (COD) was measured spectrophotometrically using standard kits tests (Hach-Lange, Germany) according to the Standard Methods (ALPHA, 1998).

The protein content was quantified according to the spectrophotometrically method at λ = 750 nm described by Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as standard (0 - 200 mg L^{-1}). Total sugars content was measured colorimetrically at λ = 625 nm by the Morris method (Morris, 1948) with modifications by Koehler (Koehler, 1952), Baily (Bailey, 1958) and Gaudy (Duque et al., 2014) by a standard calibration curve of D-glucose monohydrate (0 - 100 mg L^{-1}).

Ammonia and phosphate concentration were determined by colorimetry, as implemented in a flow segmented analyzer (Skalar 5100, Skalar Analytical, The Netherlands) (Duque et al., 2014). GC (GC Thermo, Trace GC Ultra model) was performed to evaluate the composition of the gas produced.

5.3 Calculations

The degree of fermentation (DF in gCOD-FP $gCOD^{-1}$) was calculated by dividing the total detected outlet fermentation products (TFPout in gCOD L^{-1}), converted to COD units, by the influent COD (COD_{in} in gCOD L^{-1}) according to Eq 5.1.

$$DF = \frac{TFP_{out}}{COD_{in}} \qquad \text{Eq 5.1}$$

The yield of fermentation products on substrate ($Y_{FP/S}$ in gCOD $gCOD^{-1}$) was based on Eq 5.2, where COD_{out} is the outlet effluent COD (in gCOD L^{-1}).

$$Y_{FP/S} = \frac{TFP_{out}}{COD_{in} - (COD_{out} - TFP_{out})} \qquad \text{Eq 5.2}$$

The yield of biomass on substrate ($Y_{X/S}$) was calculated according to Eq 5.2 but replacing the TFP_{out} by the amount of biomass produced measured as VSS (in gVSS L^{-1}). Volumetric substrate uptake ($-r_S$ in mgCOD-S L^{-1} h^{-1}) and FP production rate (r_{FP} in mgCOD-FP L^{-1} h^{-1}) were calculated by diving the substrate consumed (in mgCOD L^{-1}) and the FP (in mgCOD L^{-1}), respectively, by the HRT (in hours or days) (Eq 5.3 and 5.4).

$$-r_S = \frac{\Delta S}{HRT} \qquad \text{Eq 5.3}$$

$$r_{FP} = \frac{\Delta FP}{HRT} \qquad \text{Eq 5.4}$$

Specific substrate uptake ($-q_S$, gCOD $gVSS^{-1}$ h^{-1}) and FP production rates (q_{FP}, gCOD-FP $gVSS^{-1}$ h^{-1}) were obtained by dividing the volumetric rates by the biomass concentration (gVSS L^{-1}).
Biological protein removal efficiency (BPRE in %) was calculated as the difference between total inlet protein concentration ($TPro_{in}$) and the total reactor concentration ($TPro_{out}$) divided by the total inlet protein concentration (Eq 5.5).

$$BPRE\ (\%) = \frac{TPro_{in} - TPro_{out}}{TPro_{in}} \times 100 \qquad \text{Eq 5.5}$$

5.4 Results and Discussion

Acidogenic fermentation in an expanded granular sludge bed reactor (EGSB) was investigated in this chapter. The reactor operation started with an acclimation period, as biomass was stored at 4 °C, with a superficial upflow velocity (V_{up} in m h^{-1}) of 2 m h^{-1}. During this period, V_{up} was gradually increased over the days up to 6 m h^{-1}. EGSB reactor was operated for 17 days with V_{up} = 6 m h^{-1} and OLR of 7.88 ± 1.26 gCOD L^{-1} d^{-1}. Similarly to Kato et al. (1999), who have studied the anaerobic treatment of low-strength brewery wastewater in EGSB reactor, observed an expansion of the bed over the whole reactor height as a result of the applied high velocity. The consumption of sugars and the total fermentation products produced during the acidogenic fermentation of BSG are presented in Figure 5.3.

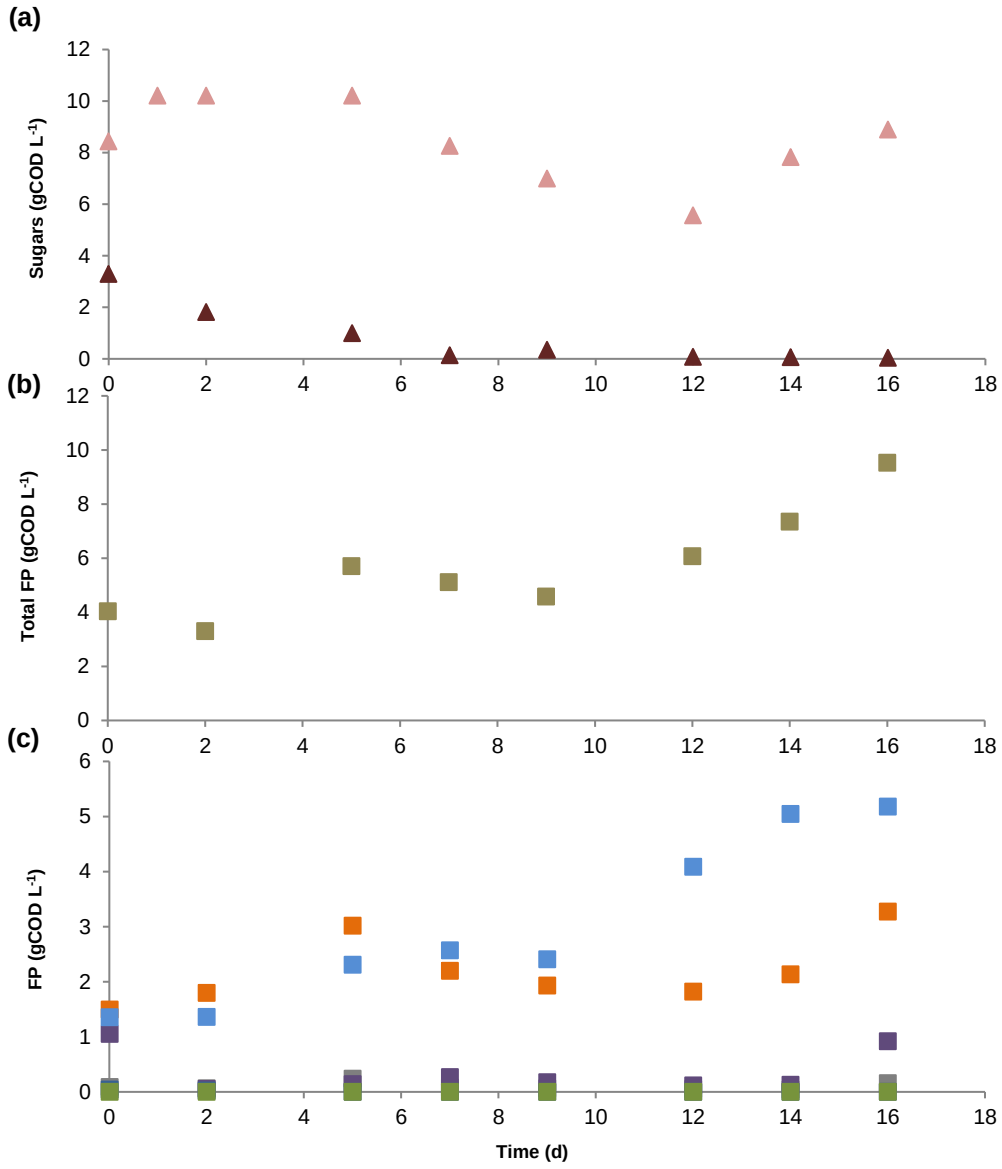

Figure 5.3: EGSB overall operation: (a) total sugars (out (▲) and in (▲), (b) total detected fermentation products (■) and (c) detected FP (HAc (■), HProp (■), HBut (■), HVal (■), HIsov (■) and EtOH (■).

After 7 days of operation, all sugars started to be consumed in the reactor. HBut and HAc were the dominant fermentation products during reactor operation, with average values of ca. 51% and ca. 40% (COD basis), respectively. Small amounts of EtOH, HProp, HIsov and HVal were detected. No lactate was detected. Gouveia et al. (2016), that studied the acidogenic fermentation of cheese whey in a CSTR at pH 4.0, 4.5 and 5.0, reported that only HLac, HAc and lower concentrations of EtOH were produced. HLac was presented in higher concentrations, as cheese whey was constituted mainly by lactose. Besides the FP mentioned above, H_2 was also detected with 40.7 ± 3.5% mol. O_2, N_2 and CO_2 were also identified at 1.4 ± 0.02% mol, 5.9 ± 0.1% mol and 52.1 ± 3.5% mol, respectively. No methane was detected. Similarly to the results obtained in the batch assays, the presence of H_2 and CO_2 was expected as are FP. On the other hand, N_2 and O_2 were detected probably due to a linkage of the system that allowed the entrance of some air. However, the presence of N_2 and O_2 did not affect acids production.

To evaluate the overall reactor performance, stoichiometric and kinetic coefficients were calculated and summarized in Table 5.2.

Table 5.2: Stoichiometric and kinetic coefficients calculated during the EGSB operation. Average values ± standard deviations were presented.

Biomass concentration (gVSS L^{-1})	11.68 ± 2.22
FP concentration (gCOD L^{-1})	5.72 ± 1.84
$Y_{FP/S}$ (gCOD-FP $gCOD^{-1}$)	0.58 ± 0.12
$Y_{X/S}$ (gVSS $gCOD^{-1}$)	0.45 ± 0.25
r_{FP} (mgCOD-FP L^{-1} h^{-1})	90.5 ± 24.9
$-r_S$ (mgCOD L^{-1} h^{-1})	328.8 ± 39.5
q_{FP} (gCOD-FP $gVSS^{-1}$ h^{-1})	0.008 ± 0.003
$-q_S$ (gCOD $gVSS^{-1}$ h^{-1})	0.030 ± 0.008
Degree of fermentation (gCOD-FP $CODin^{-1}$)	0.27 ± 0.06
BPRE (%)	30.5 ± 5.6

Despite of the increase on FP concentration in the last days of operation, the average FP concentration was around 5.72 gCOD L^{-1}. Gouveia et al. (2016), that studied the acidogenic fermentation of cheese whey in a CSTR with ca. 15 gCOD L^{-1} h^{-1} of OLR, reported a total FP of 11 gCOD L^{-1}. This higher FP concentration was approximately the double to the obtained in EGSB reactor once the OLR was also two times higher (15 gCOD L^{-1} h^{-1} for Gouveia et al. (2016) versus 7.88 ± 1.26 gCOD L^{-1} h^{-1}). Volumetric rates were lower than those obtained by Gouveia et al. (2016), but the substrate uptake rate almost double.

The fermentation yield obtained (0.58 gCOD-FP $gCOD^{-1}$) was in the same range to the reported by Tamis et al. (2015) at pH 4.5 when using glucose as substrate (0.58 versus 0.66 gCOD-FP $gCOD^{-1}$ at pH 5.5 and 4.5, respectively). At the same pH, Gouveia et al. (2016) reported similar fermentation yield using cheese whey as substrate (0.59 gCOD-FP $gCOD^{-1}$).

The DF obtained was low (0.27 gCOD-FP $gCOD^{-1}$), probably due to the lower concentrations of FP obtained compared to feed concentrations, which were higher (5.72 ± 1.8 versus 20.5 ± 2.8 gCOD L^{-1}).

During the entire operational period, biomass concentrations were measured at different heights (H_1, H_2 and H_3) in the EGSB reactor. A clear stratification was observed as shown in Figure 5.4.

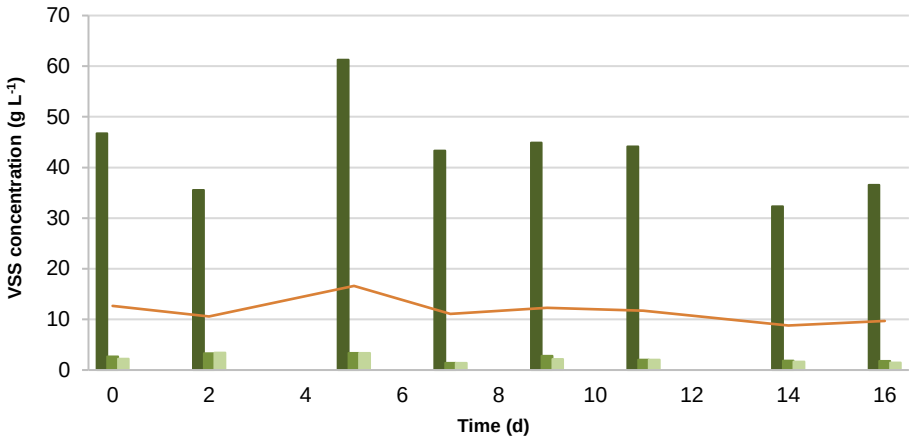

Figure 5.4: Biomass concentration distribution in the bottom (H_1 (■)), middle (H_2 (■) and top (H_3 (■)) parts of the reactor. Average value is represented by a orange dashed line.

High biomass concentrations were achieved in the bottom of the reactor (H_1), whereas in the top of the reactor lower concentrations were present (H_3). The SRT was not controlled. Instead, the biomass amount inside the reactor was manually controlled based on its expansion height. After 5 days of operation, the reactor was purged. Consequently, on day 7, a biomass concentration decrease from 16.61 gVSS L^{-1} to 11.10 gVSS L^{-1} was observed (data not shown). From day 7, biomass concentration remained stable. It was expected that purge could decrease the reactor performance. In Figure 5.5 are presented volumetric and specific rates of sugars consumption and FP production during EGSB operation.

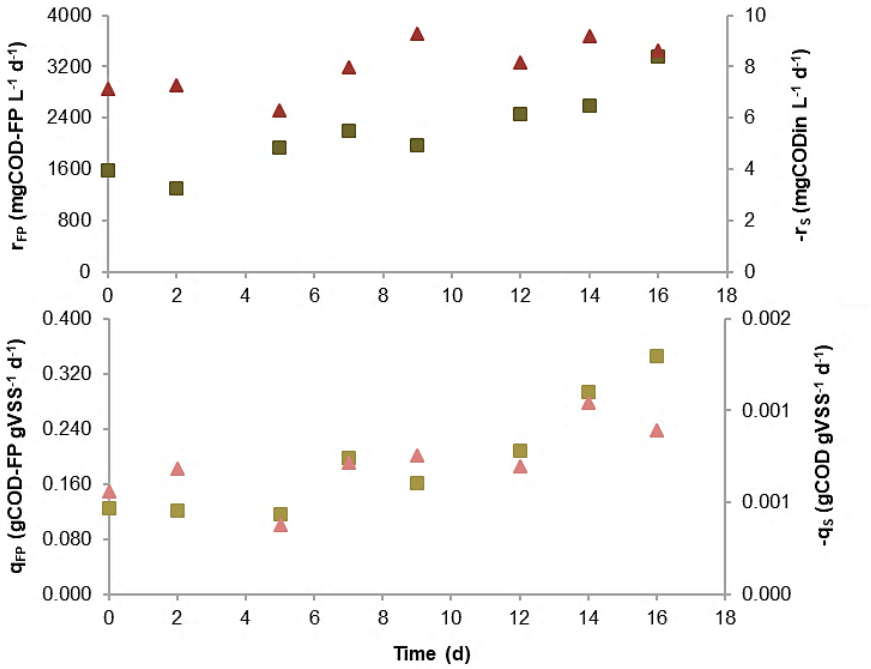

Figure 5.5: Shematic representation of volumetric rates. FP production (■), substrate uptake (▲)) rates and specific FP production (■) and substrate uptake (▲) rates, are presented.

From day 5, volumetric rates increased. Volumetric FP production rate increased with FP production increase. On the other hand, volumetric substrate uptake rate may vary slightly due to feed oscillations. Higher values were obtained at days 9 and 14, as a result of high feed concentration. Lower specific rates were obtained at day 5, where biomass reached maximum concentrations. From day 5, specific FP production rate increased according to the volumetric FP production rate, since no significant variation of biomass concentration was observed (Figure 5.4). Specific substrate uptake rate also, generally, oscillated with volumetric substrate uptake rate, except at day 14 where it increased due to a slight decrease of biomass concentration.

Despite of the obtained results, the reactor was not operated long enough to reach stability. However, sugars consumption with concomitant organic acids production demonstrated the possibility to perform acidogenic fermentation of hydrolyzed BSG using an EGSB reactor. Although pH control was examined during the operation, no base was added. This proves that working at a lower pH reduces the necessity of pH control by base addition, resulting in costs reduction and improving the economic feasibility of large scale implementation (Tamis et al., 2015). To operate the EGSB under the operating conditions described above, a high stock of acid hydrolyzed BSG was necessary. Although it is interesting to apply higher organic load and low HRT, the BSG pre-treatment is still a limiting step.

6. Conclusion and future perspectives

In this work, acidogenic fermentation of brewer's spent grain was shown. Acid hydrolysis was demonstrated to be an efficient method for sugar extraction from BSG, although producing small concentrations of inhibitory compounds. However, pre-treatment with acid is not an environmental friendly method. Subcritical water was tested as an eco-friendly alternative involving the utilization of water at high temperatures and pressure. The results showed that using the conditions applied (temperatures and water flow), the extracted sugars were not at the same concentrations in the extract as obtained by acid hydrolysis. Due to the low yield of SCW, the subsequent work was carried out with acid hydrolysed BSG.

From acidogenic fermentation in batch, different profiles and concentration of fermentation products were obtained at different organic loads and pH, showing that operational conditions manipulation results in different fermentation products, which in turn can result in different polymer composition. In a continuous mode, acidogenic fermentation of hydrolyzed BSG in an expanded granular sludge bed reactor was investigated. Despite of the reduced operating time, different organic acids were obtained from sugars consumption.

As future work, several suggestions arise. The main focus should be given to the strategy used for sugar extraction from BSG, which represents the main limiting step of the process. The proposed future work focus on:

1. Using SCW as a BSG pre-treatment method, other temperatures and water flow should be tested to check differences;
2. To explore BSG hydrolysis using phosphoric acid once it is less aggressive than sulfuric acid;
3. Test other greener BSG pre-treatment methods as an alternative to acid hydrolysis;
4. To investigate which is the highest substrate concentration that can be used for the same volume of biomass, batch tests should be performed under higher organic loads: a) test first hydrolysate with ca. 60 gCOD L^{-1} of organic load and b) apply directly the mix between the two hydrolysates (BSG mix) which organic load is approximately 55 gCOD L^{-1};
5. Operate an EGSB for a longer period in order to study its stability;
6. To study the effect of HRT and organic load on the BSG acidogenic fermentation in EGSB reactor. Evaluate the effects on organic acids concentration and profiles;
7. To study the effect of other pH values on organic acids profile and concentrations.

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