Universidade de Aveiro Departamento de Química

Ano 2016

VIVIANE MARISA REIS RUNA

EFEITO DA CONFIGURAÇÃO DO REACTOR NO PERFIL DA FERMENTAÇÃO ACIDOGÉNICA

EFFECT OF REACTOR CONFIGURATION IN THE ACIDOGENIC FERMENTATION PROFILE

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

the jury

president

Doutora Mara Freire Martins

Research coordinator at Department of Chemistry, University of Aveiro

Prof. Doutora Luísa Alexandra Serafim Leal

Assistant professor at Department of Chemistry, University of Aveiro

Doutora Sónia Patrícia Marques Ventura

Assistant Researcher at Department of Chemistry, University of Aveiro

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palavras-chave

Polihidroxialcanoatos, fermentação acidogénica, ácidos orgânicos de cadeia curta, CSTR, SBR, culturas microbianas mistas, extração, surfatantes

resumo

Os polihidroxialcanoatos (PHA) apresentam-se como uma alternativa promissora aos plásticos convencionais, devido à sua biodegradabilidade, biocompatibilidade e propriedades termoquímicas. Várias estratégias têm sido estudadas para reduzir os custos de produção de PHA e ampliar a sua comercialização, como o uso substratos renováveis sujeitos a um pré-tratamento e o desenvolvimento de procedimentos de extração económica e ambientalmente sustentáveis.

A primeira parte do trabalho consistiu na comparação de dois modos de operação de reator distintos para a produção de ácidos orgânicos de cadeia curta (AOCC), substratos preferidos para a produção de PHA. A fermentação acidogénica foi protagonizada por culturas microbianas mistas (CMM), num reator contínuo perfeitamente agitado (CSTR) e num reator descontínuo sequencial (SBR), operados durante 130 dias sob as mesmas condições operacionais. Ambos os reatores atingiram fermentação total (>90%) para a carga orgânica de 10gCOD/L·d, com gama de produtos de fermentação (PF) e respetivas concentrações relativas distintas. Etanol foi o PF dominante no CSTR e ácido acético (HAc) o principal AOCC produzido, enquanto o SBR apresentou dominância pelo ácido butírico (HBu), com concentração pouco acima do HAc. Apesar do CSTR ter tido maior rendimento de AOCC que o SBR - 0.671 e 0.604, respetivamente - o SBR teve uma conversão de substrato em AOCC superior – 48% comparativamente a 38%.

A segunda parte do trabalho incidiu na extração de PHA de culturas puras de *C. necator* e CMM com aplicação de surfatantes e solução enzimática de hidrolisado do fungo *P. variotii*. Inicialmente, foi desenvolvido um protocolo para aplicação dos agentes extrativos em estudo. Após, foi feita a extração sistemática de PHA das culturas microbianas seguindo o protocolo desenvolvido e combinando valores de temperatura e concentração. Dos surfatantes utilizados, o BRIJ 30 apresentou resultados mais promissores com recuperações de polímero acima de 30% para as culturas puras e acima de 20% para as CMM. A solução enzimática permitiu extração de PHA em concentração de 10% mas não em concentrações inferiores, indicando que concentrações mais elevadas deverão ser estudadas.

keywords

Polyhydroxyalkanoates, acidogenic fermentation, short-chain organic acids, CSTR, SBR, mixed microbial cultures, extraction, surfactants

abstract

Polyhydroxyalkanoates (PHA) emerge as a promising alternative to the conventional plastics due to its biodegradability, biocompatibility and thermochemical properties. Several strategies have been studied in order to decrease PHA production costs and enlarge their commercialization, such as the use of pre-treated renewable substrates and the development of economically and environmentally sustainable extraction procedures.

The first part of this work aimed to compare two different reactor operation modes for the production of short-chain organic acids (SCOA), preferred substrates for polyhydroxyalkanoates (PHA) production. Acidogenic fermentation was performed by mixed microbial cultures (MMC) in a continuously stirred tank reactor (CSTR) and in a sequential batch reactor (SBR) operated for 130 days and submitted to the same operational conditions. Both reactors achieved full fermentation (>90%) for an OLR of 10gCOD/L·d, with a different range and relative concentrations of fermentation products (FP). Ethanol (EtOH) was the dominant FP in the CSTR and acetic acid (HAc) the main SCOA produced, while the SBR presented butyric acid (HBu) as dominant FP, slightly higher than the HAc concentration. Although the CSTR showed a higher yield of SCOA than the SBR – 0.671 and 0.604, respectively -, the SBR presented a higher conversion of substrate into SCOA – 48% over 38%.

The second part of the work focused on the PHA extraction from pure cultures of *C. necator* and MMC with surfactants and an enzymatic solution from the hydrolysate of the fungus *P. variotii*. An extraction protocol was developed for application of the extractive agents studied. Afterwards, a systematic extraction of PHA from the microbial cultures was performed and several concentrations and temperatures tested. Among the surfactants used, BRIJ 30 presented the most promising results with recoveries above 30% for the pure cultures and above 20% for the MMC. The enzymatic solution enabled the PHA extraction when used at a concentration of 10% but not in inferior percentages, indicating that application of higher concentrations should be studied.

CONTENTS

ABBREVIATIONS

ADD	Aerobic Dynamic Discharge
ADF	Aerobic Dynamic Feeding
AN/AE	Anaerobic/Anaerobic
BuOH	Butanol
COD	Chemical Oxygen Demand
$CONV_{FP}$	Conversion in Fermentation Products
$CONV_{SCOA}$	Conversion in short-chain organic acids
CSTR	Continuous-stirred tank reactor
EtOH	Ethanol
FID	Flame Ionization Detector
FP	Fermentation Products
GAO	Glycogen accumulating organism
GC	Gas chromatography
HAc	Acetic acid
HB	Hydroxybutyrate
HBu	Butyric acid
HCa	Caproic acid
HHe	Heptanoic acid
HiBu	Isobutyric acid
HiVa	Isovaleric acid
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic Retention Time
HV	Hydroxyvalerate
HVa	Valeric acid
lcl-PHA	Long chain length Polyhydroxyalkanoates
mcl-PHA	Medium chain length Polyhydroxyalkanoates
MMC	Mixed Microbial Cultures
OLR	Organic Load Rate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

P(3HHx-*co*-3HO)	Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)
P(4HB)	Poly(4-hydroxybutyrate)
PAO	Polyphosphate accumulating organisms
pCOD	Particulate Chemical Oxygen Demand
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PP	Propylene
RT	Retention Time
SBR	Sequential Batch Reactor
scl-PHA	Short-chain length polyhydroxyalkanoates
SCOA	Short-chain Organic Acids
sCOD	Soluble Chemical Oxygen Demand
SDS	Sodium Dodecyl Sulphate
SRT	Solids Retention Time
SS	Suspended Solids
SVI	Sludge Volume Index
tCOD	Total Chemical Oxygen Demand
TSS	Total concentration of SCOA
VSS	Volatile Suspended Solids
$\mathbf{Y}_{FP}$	Yield of Fermentation Products
$\mathbf{Y}_{SCOA}$	Yield of Short-chain organic acids

FIGURES

TABLES

1. INTRODUCTION

The increasing awareness on environmental issues heighten the need for the sustainable resources exploitation and residues management.[1] [2] In the last decades, governments strive to address these matters establishing worldwide directives and policies to meet sustainability requirements. A major concern is the exploration of fossil resources and excessive plastic consumption, mostly derived from petroleum. [3] [4]

Petroleum-based plastics are broadly used due to their favorable and versatile properties, such as durability, mechanical integrity, light weight and low cost, which make them suitable for a wide range of applications, from packaging to disposable goods, products of trivial use, electronic equipment and large plastic demand applications.[5] Despite the view of plastic as a useful material, its excessive use is associated with a dreadful environmental impact. Since plastics derive from fossil resources such as petroleum, harmful constituent substances are released to the environment causing severe pollution and negatively affecting ecosystems during their slow degradation process which, in turn, leads to a massive accumulation in landfills.[6]

Bioplastics emerged as an alternative to fossil-based polymers due their biodegradability, biobased origin or combination of both these characteristics that lead to a more sustainable production and management of plastic products.[7] [8] [9] Despite all knowledge about bioplastics properties and processing procedures, they still represent only 1% of the global plastics market and although this percentage is expected to raise 2% by 2017, it still represents a small share of the plastics produced and commercialized globally.[9] [10] Among bioplastics, polyhydroxyalkanoates (PHA) have attracted much attention as a promising substitute to petroleum-based plastics when considering the biopolymer's properties, possible applications and the far more sustainable production.[8] [11] PHA is already successfully applied in several products such as biomedical devices[12], controlled drug delivery[13] and herbicides release[14] and as coating components[15], and shown to be suitable for replacing common plastics in a wide range of applications.[16] However, its production and purification costs are a major drawback which prevents a wider commercialization and application in products of lower added-value.[17] In order to decrease PHA production and obtainment costs, several approaches have been explored during the past years: the use of mixed microbial cultures (MMC) instead of pure cultures which require sterilization[18], expansion of the range of substrates that can be used for PHA production through bacteria, including cheap by-products and wastes[19], and development of alternative downstream procedures more

economically and environmentally sustainable than the usage of hazardous components and time-consuming strategies[20].

This work reviews some strategies applied for reduction of the PHA production costs, specially by the fermentation of renewable feedstocks used as carbon source for PHA production by MMC and by the development of procedures for polymer extraction and recovery. The first part of this work aimed to compare the production of SCOA by a MMC using a synthetic feeding as carbon source through two different reactor operation modes – a continuous stirred tank reactor (CSTR) and a sequential batch reactor (SBR) – submitted to the same operation conditions, namely temperature, pH, volume, solids retention time (SRT), hydraulic retention time (HRT) and organic load rate (OLR). The results allowed for the comparison of the two reactors setups and for conclusions about which one can be more suitable for SCOA production foreseeing their use as substrate for PHA production. Although several combinations of operational conditions and different reactors configuration have already been reported in literature, no comparison of reactor performance uniquely based in the reactor operation mode was performed so far.

The second part of the work focused the extraction of PHA. The downstream processing still counts for a great part of the overall production cost and improvements and innovation are still needed. Moreover, the conventional methodologies often used for PHA recovery rely on the use of hazardous substances with a negative impact on the environment. In this work, the most studied extraction procedures are reviewed - however, literature does not cover this topic extensively and a lot of procedures are protected by patents. The main goal of the laboratory work was to develop a new extraction procedure with application of extractive agents that could contribute for a more environmental and economically sustainable process: surfactants, amphiphilic molecules with similar structure to the cell membrane phospholipids, and an enzymatic mixture, obtained from the fungus *Paecilomyces variotii* hydrolysate by a non-expensive and sustainable way. Preliminary extraction assays were performed for PHA extraction from pure cultures of *C. necator* and MMC applying the procedure previously outlined.

The laboratory work was carried at two different places and contexts. The first part considering the acidogenic fermentation was performed at the Swedish company Anoxkaldnes AB, in Lund, following the work guide lines of the biopolymer group during an internship at the company. The extraction work was carried at the Biological Engineering Laboratory of the Chemistry Department of the University of Aveiro, contributing to the research on the complete process of PHA production.

2. STATE OF ART

2.1 Insight into Polyhydroxyalkanoates

2.1.1. A promising bioplastic

PHA were first observed by Lemoigne, in the 1920s, as intracellular granules in Gram-positive *Bacillus megaterium*. It was composed by hydroxybutyrate (HB) monomers, being the polymer-like material posteriorly called polyhydroxybutyrate (PHB).[21] However, just later, when PHA was perceived as a bioplastic with useful and promising properties and as a potential substitute to petroleum-based plastics, it became a more extensively studied subject. PHA can be synthesized by bacteria through fermentation – biological provenance instead of a fossil and pollutant one – using renewable feedstocks and helping reduce the harmful impact of the reckless use of conventional plastics through a more sustainable production and innocuous degradability.[11 16]

The main advantages related with PHA are their biodegradability and biocompatibility coupled with its versatile properties, as addressed later.[22] Opposing to conventional plastics, which have a long degradation process accompanied by the release of toxic and recalcitrant components into the environment, PHA can be degraded more easily and in a shorter period of time. Depolymerases secreted by microorganisms hydrolyze ester bounds of the polymeric chain, converting the polymer into water-soluble monomers and oligomers: water and carbon dioxide as main end-products under aerobic conditions and methane in anaerobic environment. These smaller components can later be assimilated by microorganisms as a carbon source.[23 24] Several life cycle assessments – analysis of environmental impact considering all stages of a product's life - performed on PHA-based materials show that these materials are more environmentally friendly than other plastics, despite improvements in the production process and energy consumption are needed and adequate policies required.[25 26]

Despite all the advantages associated with PHA and all effort put on research and development during the last decades to valorize the biopolymer, it still not meeting its full potential. PHA commercialization represents a very small share on bioplastics market – around 2% in a market dominated by PLA and other starch-based polymers - and although it meets the requirements to be applied in a wider range of products, the price can be five to six time higher than the price from conventional plastics (4.5€ Kg^{-1} against 1.3€ Kg^{-1}, approximately)[5], which needs to be substantially reduced to compete with conventional plastics.[27] The high price of the biopolymer results from the sum of the high production cost, low process efficiency when considering fermentation, processing

difficulties and downstream procedures, from recovery to purification of the biopolymer, that can count up to 50% of the final cost.[5] Although final PHA concentration can range from a few mg to 200g/L, polymer yields are lower than the yields achieved by the chemical industry. In addition, conversion of the substrate into PHA is usually around 33%. These weaknesses need to be overcome in order to compete with the petroleum-based plastics production, such as polyethylene(PE) and polypropylene(PP), able to achieve final product concentrations of 500g/L and conversions from 90% to 100%.[17] It is also challenging to assure the properties consistency of the PHA produced while a great stability and reliability of conventional plastics properties is achieved.[24]

A dynamic development in technical and economic topics related with PHA still needs to be pursued for a visible penetration in the global plastics market and to make this biopolymer able to compete with petroleum-based plastics. PHA is still a promising bioplastic with a wide range of potential applications and it is anticipated that the demand for a material with such versatile properties and passible to tailor for a specific purpose will grow.[28]

2.1.2. Structure

PHA are linear polyesters composed by chiral hydroxyalkanoic acids (HA) connected by ester bonds (Figure 2a). The HA monomers in microbial polyesters are in the R(-) configuration due to stereospecificity of the PHA synthase, indispensable detail to assure biodegradability and biocompatibility of PHA and granting them optically active feature.[22]

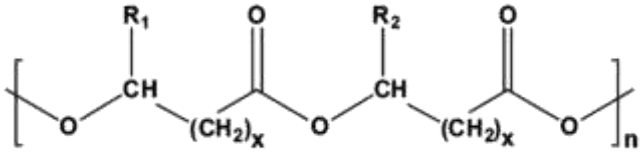


Figure 2a. General structure of PHA: R_1/R_2 = alkyl groups C1–C13, x = 1 – 4, n = 100–30000.

The polymerization degree ranges from 10^5 to approximately 10^7 and molecular weight between 50 000 and 1 000 000 Da.[8] [22] The length of the side chain and functional groups of the monomeric units as well as the more than 150 constituents results in a huge PHA diversity that enables a wide range of applications to be covered by the final PHA-based products.[17]

Structure of PHA can be classified considering the type of monomeric units determined by the monomers combination in the polyester chain and the number of carbon atoms in the side

chain. Homopolymer describes a polymeric chain composed by only one type of monomers – is the case of PHB, which comprises only HB units and is the most commonly PHA produced naturally by bacteria. If the polymeric chain combines different monomers is named heteropolymer or copolymer – for instance, the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) results from the integration of both HB and hydroxyvalerate (HV) units into the polymeric chain.[29 30]

When considering the number of carbon atoms of the side chain, the polymer can be classified as short-chain length PHA (scl-PHA) having 3 to 5 carbon atoms, medium-chain length PHA (mcl-PHA) with 6 to 14 carbon atoms and some authors even consider the classification of monomers with more than 14 carbon atoms in the side chain as long-chain length PHA (lcl-PHA).[31 32] Scl-PHA include poly(3-hydroxybutyrate) (P(3HB)) and poly(4-hydroxybutyrate) (P(4HB)) and are synthesized by several bacterial species, such as *Cupriavidus necator* and *Alcaligenes latus*. [33 34] Bacteria from *Pseudomonas* genus are able to produce mcl-PHA including poly(3-hydrohexanoate) and poly(3-hydroxyoctanoate). Copolymers can be found within these two main classes, for instance P(3HB-*co*-3HV) or poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate) (P(3HHx-*co*-3HO)).[35 36] The physical properties of the final polymer are determined by the structure features cited, mainly by the side chain of monomers and their combination. Scl-PHA can be quite brittle and stiff with high melting points and low glass transition temperatures, being their thermo-plastic properties similar to the ones of propylene (PP).[36] On the other hand, mcl-PHA present low crystallinity and high elongation break as well as lower melting points and glass transition temperatures when compared to scl-PHA. These elastic properties, resemble a rubber behavior, make mcl-PHA suitable for a wider range of novel applications such as cosmetics, medical purposes, paint formulations and other coatings.[32 35] Lcl-PHA are the less studied among PHA and their categorization as well as characterization are debatable. Therefore, it is possible to induce the production of a biopolymer with desired characteristics/monomeric content by selecting a particular microorganism, using specific media components, adjusting molar ratio of two distinct carbon sources and tuning the operation parameters. The view of PHA as promising polymer and all potentialities it delivers are due to the structure versatility and the possibility to design it, crucial features to enhance all polymer's applications.[11 29]

PHB is the most studied PHA since it is the type of PHA commonly produced by bacteria. This specific polyester has a high crystallinity and high melting point, both responsible for restraining the processing conditions that can be applied to produce PHB-based products.[37] The high crystallinity can be diminished by including HV monomers into the polymeric chain, thus

allowing the applications of a wider range of processing conditions and also improving its biodegradability. The co-polyester obtained, P(3HB-*co*-3HV) presents improved properties and a given percentage of HV content can be promoted in order to enhance thermal stability and reduce brittleness.[16] [38] A comparison between PHB, P(3HB-*co*-3HV) with different proportions of HV monomers and PP is resumed in Table 2a.

Table 2a. Comparison between approximated values of different thermal and mechanical properties from PHB, P(3HB-*co*-3HV) and PP.[22] [39]

Property	PHB	P(3HB-co-3HV)			Propylene
		3% HV	20% HV	70% HV	
Melting point (°C)	175	169	148	87	176
Glass Transition temp. (°C)	4	-	-1	-13	-10
Young's Modulus (GPa)	3.5	2,9	1,2	-	1,7
Elongation to Break (%)	5	-	50	-	400
Tensile Strength (MPa)	40	38	20	-	34,5

2.1.3. Properties and Applications

The diversity of PHA possible to obtain and knowledge about factors that can be manipulated in order to condition the characteristics of the biopolymer produced are relevant factors when considering the application of the polymer in a specific product and its commercialization. PHA properties (Figure 2b) can determine their value and suitability for processing.[39]

One of the most important attributes of PHA is the molecular weight which heavily affects the polymer behavior. The molecular weight is conditioned by the producer bacterial strain, substrate used as carbon source and medium composition. The extraction procedures applied can also affect the polymer final molecular weight and consequently determine the possible applications.[36] Glass transition temperature and melting temperature, are important thermal properties which define lower and upper temperature limits for the biopolymer processing and further use. The glass transition temperature specifies the temperature at which the polymer transits from a brittle and glassy state below glass transition temperature to a soft and malleable one above glass transition temperature. The temperature point when the polymer transits from a

crystalline phase into an amorphous solid is the melting temperature. Other thermal properties such as heat of fusion and thermal conductivity can also be assessed in order to characterize the polymer with more detail.[40] As mechanical properties, is important to consider the elastic or Young's modulus to measure the polymer stiffness, from a flexible mcl-PHA to a brittle scl-PHA. The elongation to break traduces the capacity of the polymer to be stretched without breaking – this value is inversely proportional to the Young's modulus. For instance, while elastic modulus decreases with the decreasing of HB and increasing of mcl-PHA's monomers content, elongation to break has the opposite behavior, increasing its value.[17] Other properties can also be assessed by mechanical tests, such as tensile strength and crystallinity. In addition to these main properties, other parameters can be determined for a better understanding of the polymer behavior and usage, such as oxygen permeation, biodegradability and toxicity.[41]

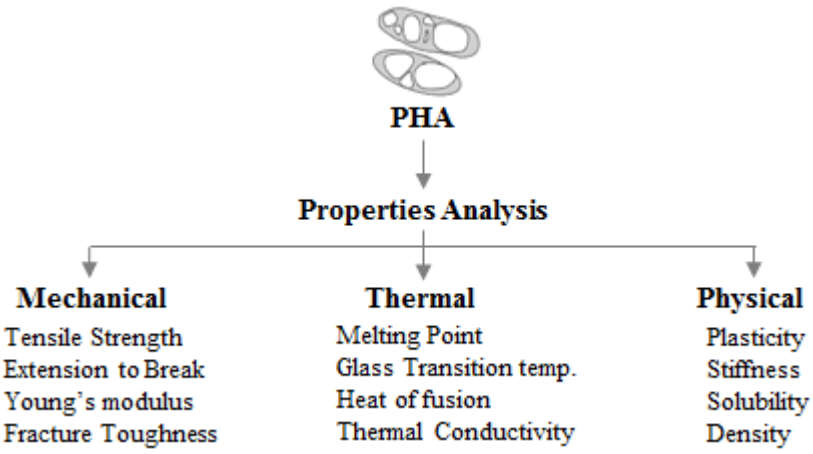


Figure 2b. Sketch of the most significant polymer properties for characterization and applicability of the PHA.

The more information can be gathered about a given polymer, the more efficiently processing conditions and applicability can be determined. PHA can be suitable for a wide range of applications, from trivial plastic containers to specific and high-value added products, and to replace petroleum-based plastics since the biopolymer can have similar properties regarding durability, toughness, low oxygen permeability and moisture resistance.[42]

PHA can not only represent a major ingredient for coatings, packages, films and paints[43] but is also source of small molecules – its chiral monomers – relevant chemicals used as precursors or intermediates in the synthesis of several compounds from pharmaceuticals, food additives, vitamins, antibiotics and biodegradable solvents and that can be combined with other compounds

or nanocomposites for enhanced performance.[44 45] Biomedical field also demands for devices which can be degraded by enzymes into innocuous or organic substances, such as 3-hydroxybutyric acid, a blood plasma constituent. Solutions for biomedical challenges concerning tissue engineering and repair, implants, cardiovascular devices and controlled drug delivery systems also rely of PHA-based materials.[46] [47] [48] Thus, the PHA as component of biomedical devices represents a great commercialization opportunity since medical products have a high added value and can justify the advantages and production costs of the biopolymer.

2.2 Production of Polyhydroxyalkanoates

Although several microorganisms are reported to produce PHA, the synthesis of the biopolymer is mainly performed by bacteria.[49] The polyester is accumulated intracellularly in inclusion bodies (Figure 2c) as a carbon, energy and reducing power storage resource, used by the cell to survive when facing adverse environmental conditions.[50] Accumulation of PHA granules is usually triggered as response to an external stress, such as a nutrient limitation (nitrogen, phosphorus or oxygen), and excess of a carbon source convertible into biopolymer through aerobic conversion.[51]

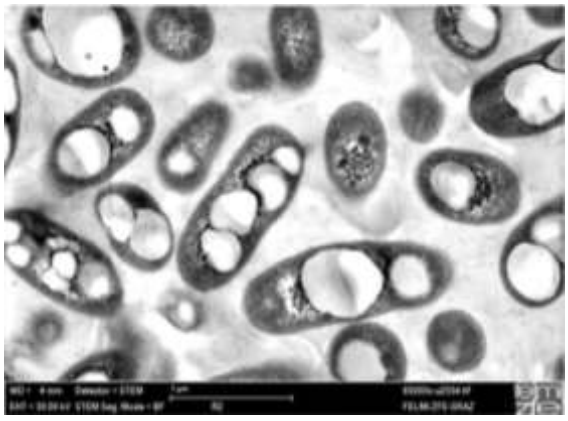


Figure 2c. STEM picture of Cupriavidus necator cells containing PHA in form of inclusion bodies; magnification of 1/65.000.[11]

Different PHA have been observed to be produced naturally, but only PHB, P(3HB-*co*-3HV), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), P(4HB) and P(3HHx-*co*-3HO) in considerable

amounts. Only P(3HB-*co*-3HV), P(4HB) and P(3HHx-*co*-3HO) proceeded to large quantities production.[22]

As stated above, PHA commercialization has still not meet its full potential due to the high costs associated with the production process. In order to turn PHA into a competitive product in the plastics market, production process must have high productivity rates and reduce investment costs. In order to overcome these drawbacks, several strategies were implemented, with highlight to (1) the enhancement of PHA producers resorting metabolic engineering and synthetic biology tools to maximize accumulation potential and productivity, (2) use of MMC instead of pure cultures aiming the process reduction costs and (3) use of renewable feedstocks as cheaper carbon source instead of synthetic substrates.[8 52 53] Since bacteria normally found in nature with the ability to accumulate PHA present low productivity, in production processes is preferable to use well studied and improved strains that will allow for the obtainment of higher quantities of the polymer.[16 54] However, bacterial pure cultures require controlled culture conditions, sterilization of all materials and solutions and use of expensive medium components resulting in an overall high production cost. As alternative to pure cultures, MMC have gained much attention from research on PHA production since they dispense aseptic conditions and are able to use a wider range of carbon sources.[55 56] Although some pure cultures were reported to produce PHA from complex feedstock, such as *C. necator*[57 58], MMC are more capable of using alternative substrates as carbon sources – this feature gains especial interest when the substrate consists of renewable feedstocks. The use of renewable carbon sources can contribute to a reduction of production costs due to their large availability and low cost and the use of industrial by-products not only supports a biorefinery economy but also assures a sustainable process.[53]

Nowadays, and in view of a significant PHA production, priority is given to the use of carbon-rich wastes and effluents from industries or human activities, converting it into value-added products, instead of using substrates such as sucrose, starch or vegetable oils which are expensive and have ethical drawbacks considering human nutrition and animal feeding.[11] Although pure cultures of PHA-producers still assure large-scale production of the biopolymer, research is oriented to the use of MMC susceptible to selection and enrichment on species of interest.[56]

2.2.1 Production by Pure Cultures

Although a few hundred bacterial species have been stated as PHA producers, the majority is not suitable for a large scale production due to the low amount of biopolymer they are able to

accumulate.[59] However, the actual commercialization of this biopolymer still relies on production by pure cultures, resulting in an expensive final product due to the high cost of the carbon source, low yield and productivity of PHA and downstream processing.[28 60]

The basis for the PHA accumulation lies in the limitation of an essential nutrient to the cell growth with excessive carbon source, inhibiting biomass reproduction and promoting the storage of reserve compounds.[50] This concept is studied in several bacterial species like *C.s necator*[61], *Rhodopseudomonas palustris*[62] and *Methylbacterium organophilum*[63]. However, PHA synthesis can occur alongside bacterial growth as observed in *Alcaligenes latus*[22] and recombinant *E. coli* with PHA biosynthetic genes[64]. At a laboratory level, it is common to apply a two-stage strategy to induce accumulation: the first stage is designed to promote bacterial growth and achieve a high biomass concentration while the following stage induces PHA accumulation within the cells through a nutrient limited fermentation. The time at which the nutrient is depleted has a relevant impact on the overall biopolymer accumulation.[16]

Bacteria use specific metabolic pathways to produce PHA from different carbon sources. When considering an aliphatic carbon source, synthesis of PHA begins with glycolysis of sugar that resulted from the decomposition of the carbon sources, to pyruvate. Latter, pyruvate is converted to acetyl-CoA through the oxidation by pyruvate dehydrogenase (PDH). By the action of β-ketothiolase, coded by the *pha A* gene, two molecules of acetyl-CoA are condensed into acetoacetyl-CoA. In turn, acetoacetyl-CoA is reduced by acetoacetyl-CoA reductase (*phaB*) to form (R)-3-hydroxyacyl-CoA which is the building block for PHA synthesis. Ultimately, carboxylate group of the monomers are polymerized into PHA through the catalysis by PHA synthase (p*haC*), that catalyzes the formation of ester bonds between neighboring monomers. The polymerizing enzyme only accepts (R)-isomers as substrates. All the main three enzymes involved in the metabolic pathway, coded by the genes *phaA*, *phaB* and *phaC* included in the operon, are located in the cytosol.[16]

Pure cultures generally produce a homopolymer, namely PHB, as a natural feature. However, the production capacity can be further enhanced by genetic manipulation, as reported for *Rhodobacter sphaeroids*[65] and *E. coli*[66]. It is also reported that some strains are able to produce copolymers when complex substrates are fed as carbon source.[67]

Striving to decrease production costs, several alternative feedstocks have been applied for PHA production by pure cultures, despite resulting in low productivities.[68] Facing the high productivities from single-carbon substrates, this approach still needs to be developed and the necessity of sterilization, also contributing for the final cost, cannot be dismissed. Although

innumerous attempts to develop a more economic and environmental process for PHA production using pure cultures, the imbalance between cost and productivity is still a huge drawback to overcome.

2.2.2 Production by Mixed Microbial Cultures

Facing the major drawbacks stated in the section above on PHA production using pure cultures, MMC can be preferred from both environmental and economic perspectives. MMC are natural inocula with a huge diversity of bacterial species and were first observed to produce PHA by Wallen and Rohwedder, in 1974, in a wastewater treatment plant.[69] When considering the adjoining production costs in a process using MMC, expenses can be reduced due to exemption of sterilization, with less equipment and process control needed, the ease of these cultures in using complex and low-cost substrates and adapt between changes on the substrate. In addition, continuous process is possible without strain degeneration.[18 70 71]

Although MMCs metabolism for PHA production is assumed to be similar to the pure cultures, the former ones are able to metabolize a wide variety of complex feedstocks rich in nutrients. Thus, the polymer production is not triggered by nutrient limitation, enabling the use of feedstocks with undefined composition as substrates.[17] However, PHA production by MMC also presents some weaknesses that compromise the viability of the process, such as low storage capacity, process instability due to the complex culture and synergy of operational parameters.[28 22]

Research on PHA production by MMC focus on the improvement of the PHA storage capacity of bacteria, on the use of cheap and available carbon sources and also on the development of a process suitable to be performed at a pilot or industrial scale. As an alternative substrate, several wastes and industrial by-products have been successfully fed to MMC aiming PHA production, as addressed later in subchapter 2.2.2.3. Using such complex carbon sources also extends the range of PHA produced. The resulting polymer often contains a mixture of different monomers – these copolymers can present properties more convenient and suitable for posterior processing.[72 73] In addition, a PHA production by MMC can be integrated in wastewater treatment processes, allowing for the use of already existing facilities and valuing the wastewater treatment.[74]

2.2.2.1 Metabolism for PHA production by MMC

Although the metabolism for PHA production is well established for pure cultures, metabolic studies are still needed in order to understand what occurs in MMC. Thus, metabolism for PHA synthesis in MMC is assumed to be similar to the one in pure cultures with the same carbon source.[19] [16]

Three main metabolic pathways for PHA synthesis can be highlighted: **I** with sugars as a carbon source, **II** which involves degradation of fatty acids and **III** with the biosynthesis of fatty acids (Figure 2d). These pathways can have several types of PHA as a final product, considering the composition of the feeding, with a unique substrate or mixture of substrates, usually sugar-based compounds or SCOA. [75] In Pathway **I**, as also reported to pure cultures, PHA synthesis begins with glycolysis of sugar, which resulted from the decomposition of the carbon sources in the feedstock, to pyruvate. Later, pyruvate is converted to acetyl-CoA through oxidation by pyruvate dehydrogenase (PDH). Acetyl-CoA can then be used as precursor for PHA or driven to bacterial growth metabolism and reducing power. By the action of β-ketothiolase, coded by the *pha A* gene, two molecules of acetyl-CoA are condensed into acetoacetyl-CoA. In turn, acetoacetyl-CoA is reduced by acetoacetyl-CoA reductase (*phaB*) to form (R)-3-hydroxyacyl-CoA which is the building block for PHA synthesis. Ultimately, carboxylate group of the monomers are polymerized into PHA through the catalysis by PHA synthase (p*haC*), that catalyze the formation of ester bonds between neighboring monomers. The polymerizing enzyme only accepts (R)-isomers as substrates. The intake of conjugated bases of SCOA, such as propionate, butyrate and valerate, with 3, 4 and 5 carbon atoms, respectively, also result in PHA through Pathway **I**. Propionate can originate several PHA precursors: acetyl-CoA by descarboxylation, HV, hydroxymethylbutyrate and 3-hydroxy-2-methylvaleryl-CoA by condensation of two porpionyl-CoA molecules. Butyrate and valerate are directly converted into respective HA, monomers for PHA; however, valerate can also be susceptible of entering Pathway **II**. Acetyl-CoA resulting from glycolysis can be shifted to intervening in malonyl-CoA and malonyl-acyl carrier protein (malonyl-ACP), involved in pathway **III** with *de novo* fatty acids biosynthesis. Malonyl-CoA is later converted into 3-hydroxyacyl-ACP and 3-hydroxyacyl-CoA, PHA precursor.[55] [76] [77] Longer-chain fatty acids follow the Pathway **II** of β-oxidation, being converted into (R)-3-hydroxyl-CoA which is precursor of mcl-PHA. In the other hand, this longer-chain fatty acids can also be driven to Pathway **I**: they can be converted in only acetyl-CoA if they have an even number of carbon atoms or into acetyl-CoA and propionyl-CoA when having an odd number of carbons.[77]

Pathways from **IV** to **VIII** are known to be related with the synthesis of different copolymers. For instance, pathways **V** and **VII** result in production of P(4HB) monomers, pathway **VI** in 4,5-hydroxyacyl-CoA precursors and pathway **VIII** in 6-hydroxyacyl-CoA precursors.[75]

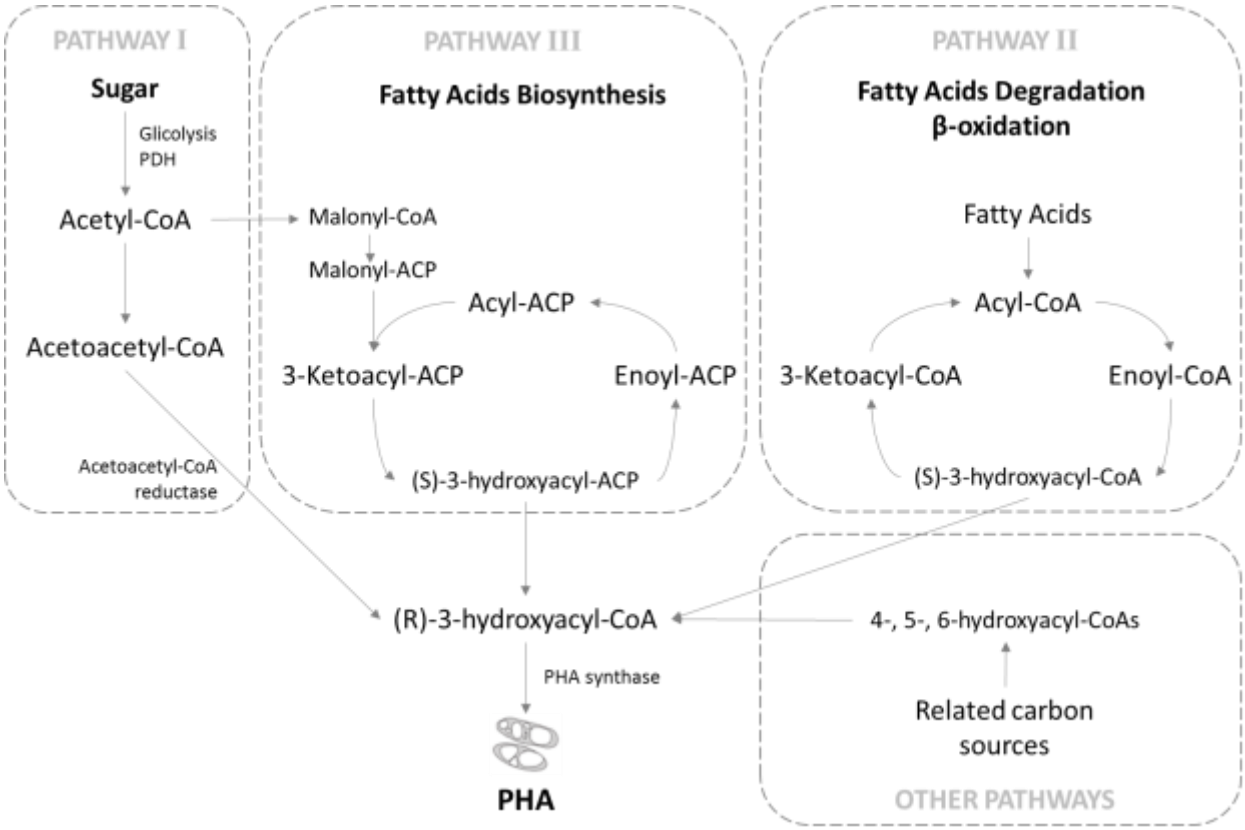


Figure 2d. General scheme of the metabolic pathways for PHA biosynthesis from sugar catabolism, fatty acids biosynthesis, fatty acids β-oxidation and other related carbon sources.[16 55 75]

SCOA became a more relevant substrate for PHA production during last years, since they can be obtained through bacterial fermentation of organic wastes and by-products, thus representing a less expensive and environmental approach for the polymer synthesis. This topic is later detailed in section 2.3.

2.2.2.2 A three-stage process

Based on wastewater treatment and biological removal of nutrients and organic matter, activated sludge/MMC were explored as cultures amenable to selection and enrichment of PHA producers – thereby, is possible to couple waste treatment with obtainment of the biopolymer as

added-value product. First suggested by Takabatake et al in 2002[78], a three-stage process has been repeatedly reported as an effective way to produce PHA through MMC. This **three-stage process** comprises (1) acidogenic fermentation of by-products used as ubstrate, (2) culture enrichment by selection of PHA-produing organisms and (3) PHA accumulation (Figure 2e).

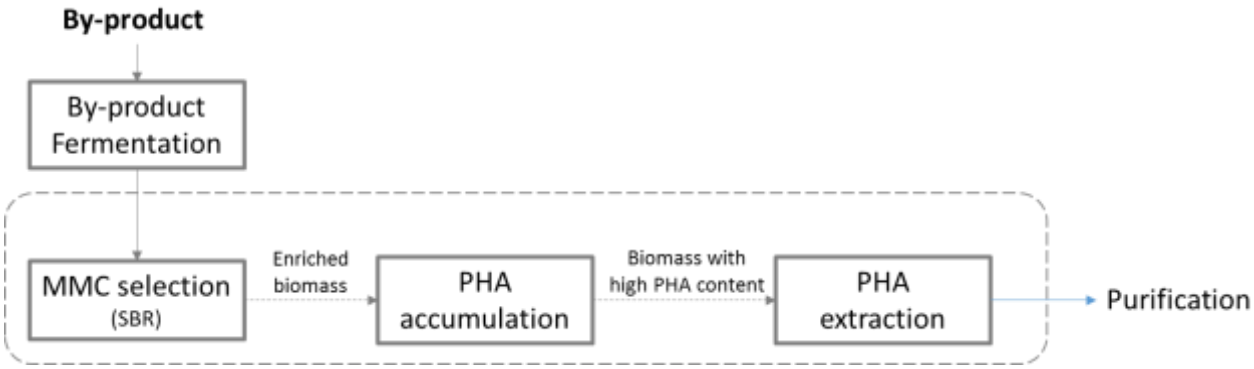


Figure 2e. Representation of a three stage process for PHA production by MMC, including the pre-treatment of the feedstock, selection of PHA storing organisms and PHA accumulation with further extraction and purification of the polymer.[70]

The first stage consisting of (1) **anaerobic acidogenic fermentation** of the feedstocks aims the pre-treatment of the waste, degrading its organic matter into simpler readily fermentable compounds. This step is necessary for upstream treatment of high carbohydrates content substrates, since MMC, unlike the pure cultures, convert carbohydrates such as glucose or starch into glycogen (polymer of glucose) rather than PHA.[79] [80] The fermentation is usually performed by a MMC under anaerobic conditions and the resulting compounds are mainly SCOA. The respective effluent, rich in compounds readily convertible by MMC into PHA, is then fed to the second stage of the process.[80] Acidogenic fermentation, as main topic of this research work, will be further discussed in section 2.3.

The (2) **culture enrichment stage** intends to select bacteria from the MMC that are able to produce and store PHA to the detriment of other existent microorganisms. In addition, the culture homogeneity is an advantage for the production and downstream processes, since non polymer storage organisms contribute to decrease the yield and increase extraction and recovery costs.[19] The selection can be achieved submitting the culture to dynamic conditions regarding the carbon supply and oxygen availability.[71] Two strategies aiming the enrichment in bacteria with PHA-storage capacity have been reported. One of them, termed “feast and famine” or Aerobic Dynamic

Feeding (ADF), refers to a strategy that comprises a phase of feeding and availability of substrate that favors cell growth and PHA storage followed by famine conditions with exhaustion of substrate, repeatedly. Only microorganisms able to produce PHA during the abundance of substrate will be able to assure bacterial growth and maintenance by using this storage compound and medium ammonia in order to survive during substrate limitation.[18 81] The ratio of lengths of feast and famine phases, F/F ratio, reflects the efficiency of microbial selection: a low ration indicates a good selection of microorganisms able to physiological adapt and significantly store PHA during the feast phase – this is, with a competitive advantage. Thus, feast phase must correspond to a short feeding phase followed by a longer famine phase.[82] Although these conditions are not growth limiting, the biopolymer accumulation response is dominant over cell growth. The fact that PHA storage requires less enzymes and RNA than cell growth is reported as justification for this phenomena.[83] This dynamic feeding can be achieved using a SBR or a series of reactors, being all the process carried under aerobic conditions. The use of SBR is the more reported reactor configuration when studying a three-stage process. The configuration itself impose transitory substrate availability and allows the easy adjustment of operational conditions, from length of feeding phase and cycle to parameters such as HRT or SRT, influencing culture behavior and productivity of the overall process.[84] The use of SBR for the enrichment step of the MMC was reported for synthetic feeding[85] as well as for multiple substrates such as sugar molasses[80 86] and olive oil mill effluent[87]. Recently, a new enrichment strategy was reported, named Aerobic Dynamic Discharge (ADD). The ADD process improves the selection of microorganisms considering the physical selective pressure in addition to the ecological selective pressure. It is assumed that intracellular storage of PHA will increase the cell density, therefore a physical selective pressure exists and can be used to improve MMC selection.[88 89] Chen et al reported that the use of ADD improved the accumulation performance since a culture with high PHA accumulation capacity was obtain in a three times shorter time than in systems with only conventional ADF strategies applied. Although ADD seems a promising strategy for selection of PHA producing organisms, further research is needed.[89]

For enrichment of MMC in PHA-storing microorganisms, is also reported the manipulation of oxygen availability in the process, alternating anaerobic and aerobic phases (AN/AE). In this case, the culture is enriched in different types of organisms when comparing to the ADF strategy.[19 90] When the substrate is fed during the anaerobic phase, a class of organism named polyphosphate accumulating organisms (PAO) are selected. PAO are responsible for phosphorus removal in waste treatment, being able to store PHA, polyphosphate and glycogen when a carbon source is fed under

anaerobic conditions. The latter two reserve polymers, mainly polyphosphate, are amenable to be hydrolyzed for obtainment of energy, it means, ATP.[91] Besides PAO, AN/AE also promotes the enrichment in glycogen accumulating organisms (GAO) which have been reported to accumulate PHA along with glycogen. GAO will also hydrolyze mainly glycogen for ATP production.[92] Both these phenotypes of anaerobic organisms will be able to survive during the aerobic phase, using the stated reserve polymers stored in the previous anaerobic feeding phase. PHA is also hydrolyzed in aerobic conditions: not only to assure growth but also to restore storage of polyphosphate and glycogen.[90] Considering that GAO are often related with inefficient phosphorus removal - since they compete with PAO for the carbohydrates without absorbing any phosphorus - they are promising for PHA production when a feed poor in nutrients and rich in carbon is used in the process. In addition, GAO are assumed to synthesize a higher PHA content during the anaerobic phase in order to maintain a redox balance within the cell.[93] However, and based in research carried so far, ADF appears to be the better strategy for culture enrichment aiming PHA production. ADF enables to achieve higher productivities as well as sludge with higher PHA content than the AN/AE process.[18] [71]

As final goal, it is intended that the selective pressure favors PHA-storing organisms and a more homogenous culture follows to further steps of the process. The culture enrichment stage has a relevant impact in the productivity and yield of the overall process, since only the organisms able to adapt and maintain a high cell growth rate facing the dynamic conditions and to store higher PHA contents will be selected. Although MMC may achieve higher PHA content and storage yield, they are also related with lower volumetric productivity when comparing to pure cultures. Thus, is important to have a high cell density in the enriched culture, since the more concentrated biomass inoculum will be, the higher volumetric productivity will be achieved.[70] These considerations will also impact the downstream processing: a more homogeneous culture will require simpler cell lysis strategies and simplify the PHA recovery process from all medium components.[90]

As third stage of the process, (3) **PHA accumulation** intends to maximize the polymer synthesis using the enriched culture. The efficiency of the accumulation not only relies on the optimization of the stage itself and storage capacity of bacteria, but is also dependent on how efficiently the enrichment step was performed previously. Moreover, the physical separation of the process stages allows for the establishment of optimal conditions for each step, maximizing their potential.[19] Generally, accumulation is induced under nutrient limiting conditions to promote storage until saturation is reached. Batch and fed-batch essays are perform to promote PHA accumulation – the feeding is added in pulses and dissolved oxygen level measured in order to

monitor when a new pulse must be added (the dissolved oxygen uptake rate is related with PHA storage – the decreasing of dissolved oxygen uptake rate indicates depletion of external substrate and the need of a new feeding pulse). If carbon and nutrient sources are continuously provided, the culture will respond with a progressively increasing growth, therefore limiting PHA content to lower values than the maximum storage capacity. [85]

The process described is followed by downstream procedures to extract and recover the biopolymer. Although some strategies have been reported to be successful in the obtainment of a polymer with high quality and purity, they are often time consuming, expensive and require the use of hazardous substances. Thus, downstream processing still need to be developed considering PHA properties and sustainability, as addressed in section 2.4.[20 94]

2.2.2.3 Use of Renewable Feedstocks

The great interest in using MMC for PHA production also relies in the wide variety of alternative feedstocks that can be used instead of defined synthetic substrates, and the lower impact they have in MMC when comparing with pure cultures.[22] Alternative feedstocks may refer to complex and renewable carbon and nutrient sources such as industrial by-products and different types of waste. Their use can allow for reduction in process costs while helping in waste management and treatment and produce added-value products, contributing to an overall process valorization. Renewable feedstocks are less expensive than pure medium components used in synthetic feeding; however, they also contain a lot of undesirable compounds that affect medium rheology, process control and that can even be toxic to bacterial cultures.[19] Is also important to take into account the seasonality and availability of the waste, as well as the unstable composition which will affect the final biopolymer obtained. Since monomeric composition is defined by the carbon sources in the medium, the instability of final PHA structure can make the application at an industrial scale a key challenge.[95 96]

Facing these main drawbacks, a **pre-treatment** step can be crucial for a more efficient PHA production process. Solids and major compounds can be separated through physical methodologies and dilution can attenuate toxic effects of some constituents. When it comes to the complex media components, a previous fermentation of the feedstock can allow for treatment of undesired compounds and the conversion of bigger molecules, mainly carbohydrates, to simpler particles and monomers easily taken by microorganisms in further stages of the process. Furthermore, is possible

to manipulate the operational conditions of the pre-treatment stage, manipulating the scope of simpler molecules, such as SCOA, and consequently influence the polymer monomeric composition and also cell content. [55 71 97]

Production of PHA with MMC using renewable feedstocks as substrate has been often reported. The use of papermill wastewater[74 98], hardwood sulphite spent liquor[99 100], crude glycerol[101], sugar cane molasses[80 86 102] and olive oil mill pomaces[103] are some examples. Is to note that all these references applied an ADF strategy for enrichment of the MMC in PHA-storing organisms.

2.3 Acidogenic Fermentation

Acidogenic fermentation is part of the anaerobic digestion, process applied with the final aim to obtain biogas, composed by methane and carbon dioxide, while treating wastewater and organic wastes.[104] The anaerobic treatment presents some advantages over the aerobic processes since it results in less sludge production, high removal rates of organic matter, lower nutrient requirements, lower reactor volume and requires less energy-input, coupled with the production of energy (biogas). However, it may require alkalinity addition and be sensitive to adverse effect of operation conditions such as temperatures.[105 106] Several reports confirm the advantages of the anaerobic digestion and its successful implementation in operational large-scale treatment facilities during the last decades.[107] The anaerobic digestion can be segmented in four stages: (1) hydrolysis, (2) acidogenesis or acidogenic fermentation, (3) acetogenesis and (4) methanogenesis.

In the first stage, complex organic molecules are hydrolyzed through catalyzes of hydrolytic enzymes secreted by the microorganisms. As products from the hydrolysis, monosaccharides, fatty acids and amino acids are obtained, along with other compounds that can be very diverse depending on the waste being treated. In the following stage, acidogenesis, these monomers are fermented into other products such as carbon dioxide, alcohols, hydrogen and organic acids. Thus, both hydrolysis and acidogenic fermentation are involved in the production of SCOA, being suitable for a pre-treatment of a stream posteriorly driven to PHA production. Although both are considered different steps of the anaerobic digestion, they occur simultaneously - acidogenic occurs as soon as hydrolysis products are available in the medium - and are conducted in the same reactor. If the waste is not rich in readily fermentable products, acidogenesis will be hindered by the hydrolysis rate and longer times will be required for the process. [106 108] In the acetogenesis, the products from the two previous stages are converted into acetic acid, hydrogen and carbon dioxide by acetogenic

bacteria. As the final stage of the process, methanogens, which are strict anaerobes, convert the acetic acid and hydrogen in methane that, together with the carbon dioxide, will compose the biogas.[106]

The aerobic digestion is performed by MMC including strict or facultative anaerobes. Acidogenic fermentation, coupled with hydrolysis, can play a major role in a PHA production process, consisting in the feedstock pre-treatment. After the acidogenic fermentation, is intended to obtain an effluent rich in preferable compounds for PHA-storing microorganisms, which can be easily assimilated.[106] Several reports show that MMC store carbohydrates – abundant in most wastes - in form of other polymers, such as glycogen or polyglucose, rather than PHA. However, when comparing carbohydrates, namely glucose and starch, with acetate, the later one results in a significant PHB accumulation. Therefore, SCOA such as acetate, propionate, butyrate and valerate are preferred substrates for PHA production through MMC.[79] [109] Acidogenic fermentation also allows to overcome the concentration variations of the surplus used as feeding, since different compounds are converted to simpler SCOA. Thus, a higher stability can be achieved in the process by using a more regular feedstock composition in the enrichment stage of a PHA production process.[71] SCOA profile in the treated waste can also be manipulated by adjusting operational conditions during the acidogenic fermentation – this topic is address in detail in section 2.3.3. This possibility of manipulation will also enable the production of a tailored PHA, enriched in the monomers of interest, and the valorization of the final polymer for specific applications.

2.3.1 SCOA as substrate for PHA production

SCOA are fatty acids containing six or fewer carbon atoms which can be distilled at atmospheric pressure, i.e. which are volatile. The most significant are acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and valeric acid (HVa).[110] Streams derived from the acidogenic fermentation and rich in SCOA are a valuable feedstock with potential to be applied in the production of added-value products. For instance, in the production of biopolymers[111], building blocks for chemical industry[112], production of energy in form of biogas[113] [114], hydrogen[115] and biodiesel[116], among numerous applications reported. In addition to these advantages, SCOA-rich stream derive from fermentation of renewable feedstocks, supporting the biological waste treatment.[110]

Several wastes have been applied for SCOA production, such as lignocellulosic biomass[117] and mainly wastewater[118] [119]. Although some wastes rich in proteins or lipids are reported as

suitable substrates for acidogenic fermentation, the commonly used renewable resources present a dominant concentration of carbohydrates. In Figure 2f is schematized the metabolic pathways used by MMC for production of some organic acids, ethanol and other metabolites from fermentation of carbohydrates. These are primarily converted in pyruvate through glycolysis. Pyruvate will be the main precursor of the organic acids, liable to follow different metabolic pathways to the final fermentation products. The productions of adenosine triphosphate (ATP) and reducing power in form of NADH during glycolysis are used in the further conversions.[120] Is to highlight that, using a diverse consortium of microorganisms and a complex feedstock, other fermentation products will be produced in different yields and at different rates.

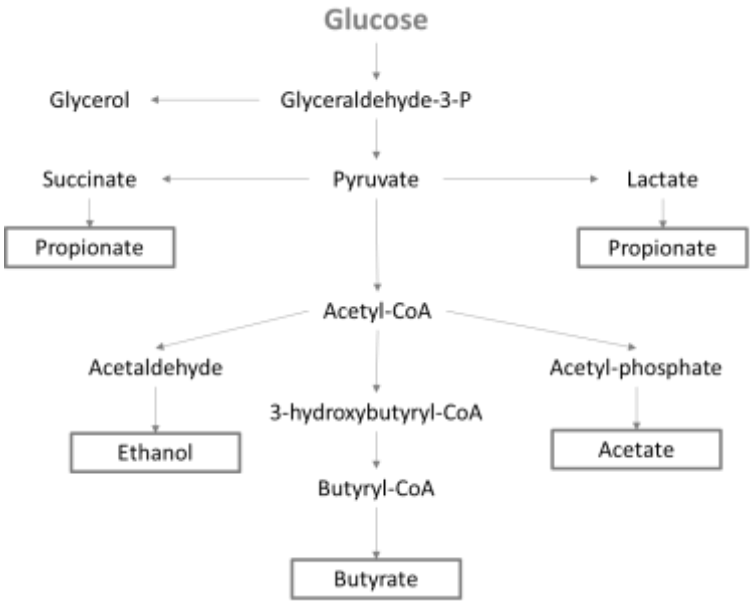


Figure 2f. Schematic representation of the metabolic pathways and intermediates involved in acidogenic fermentation by MMC, with production of ethanol, HAc, HPr and HBu.[121]

As stated above, SCOA are preferred carbon sources for PHA production through MMC. SCOA-rich streams can be used for enrichment of a MMC in PHA-storing organisms as well as in the accumulation by an enriched culture, having a major significance in the PHA production process.[122] Similar to what occurs for other substrates, SCOA will also affect the polymer composition. Thus, arises the possibility of predicting the final PHA and to tailor the polymer by controlling which SCOA are present in the feeding and their ratio.[123] Figure 2g shows the metabolic pathways followed by several SCOA in order to be converted into PHA monomers as final products. HAc will be converted into acetyl-CoA which, in turn, can be directed to tricarboxylic acids cycle and consequently to

bacterial growth or be combined with other acetyl-CoA molecule and reduced to a HB monomer. HPr is further converted in propionyl-CoA and follow three different paths: be combined with a molecule of acetyl-CoA and result in HV or 3-hydroxy-2-metylbutyrate (3H2MB) in less extend, be combined with another propionyl-CoA molecule and form monomers of 3-hydroxy-2-methylvalerate (3H2MV) or decarboxylated into acetil-CoA.[124]

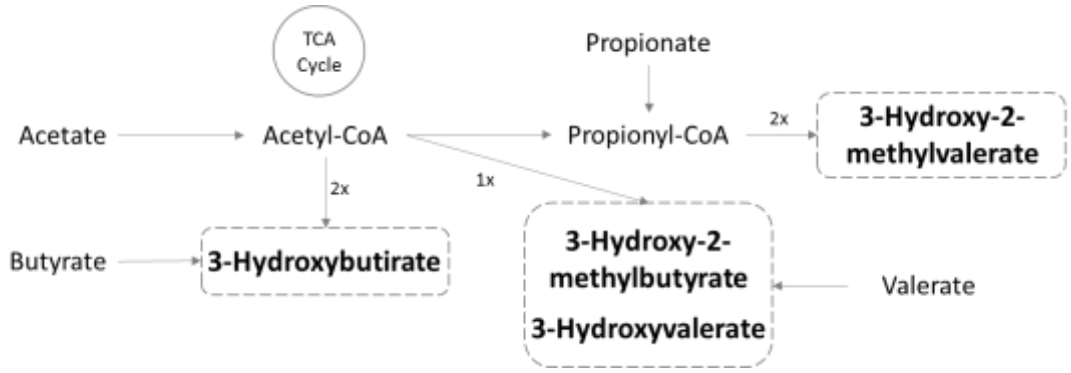


Figure 2g. Schematic representation of the metabolic pathways involved in PHA production through conversion of SCOA, namely HAc, HPr, HBu and HVa.

However, when using complex feedings, the prediction can be inaccurate and is important to considering other factors with impact in the process, such as the presence of inhibitors or compounds that can influence different metabolic pathways from the intended ones.[110] Is also widely reported that operational conditions influence the properties of the final polymer, as discussed later in section 2.3.3. Research is still needed on the production of SCOA, especially when assessing the suitability of a given complex waste for an efficient acidogenic fermentation and also in the parameters influencing the composition of the effluent, which can be further used for production of added-value products. Fundamental studies using synthetic feeding still valuable for understanding the acidogenic fermentation as a process and help to extrapolate and comprehend the results using complex feedings.

2.3.2 Reactor Configuration

Several strategies and reactors configuration have been developed during the last decades aiming the anaerobic treatment of wastes (Figure 2h). As stated above, the anaerobic treatment presents some advantages over aerobic approaches, being a preferred treatment for large and full-scale processes.[105] Anaerobic treatment can be performed by systems with (1) suspended growth,

(2) attached growth and (3) sludge blanket. These strategies, along with others less relevant such as covered anaerobic lagoon or separation using membranes, are constantly being developed and enhanced.[110]

Three types of processes rely on biomass suspended growth: the complete-mix suspended growth anaerobic reactor in which the hydraulic and solids retention times are the same; the anaerobic contact process which includes the recirculation of biomass separated by gravity to the reactor, resulting in a SRT superior to the HRT; and the anaerobic sequence batch reactor where reaction and separation occur in the same reactor, with establishment of a settling phase.[105] Anaerobic treatments with attached growth, consisting of a reactor with porous support for biomass growth, differ in the packing and degree of bed expansion. Packed-bed reactors have a fix packaging as immobilization system, which can be ceramic, silica or plastic-derived beads, as surface for biomass growth, in which the waste circulates upflow or downflow through the interstices. Although it inhibits biomass wash-out and is suitable for high influent flow rates, if the influent has high solids concentration, clogging is likely to compromise the process.[125 126] If fine grain is used as support, the system is named expanded bed reactor, with recycle/recirculation of the stream for promoting upflow and bed expansion, with consequently bigger growth surface. A fluidized bed reactor also uses a fine grain for an increase in surface area, with recycled stream and mixing of the packaging. Conceived to avoid clogging related with packed bed reactors, allows for the treatment of high OLR with short HRT and does not constrain gas flow.[125 127]

Lastly, processes with sludge blanket (which can also be considered suspended growth) include the baffled reactor with upflow through a series of sludge blanket reactors; the migrating blanket reactor, similar to the previous one but with mechanical mixing and keeping the biomass without settling or packing; and the upflow anaerobic sludge blanket. The last one is efficient on the treatment of wastes with high Chemical Oxygen Demand (COD) contents due to the promoted growth of dense granulated sludge with great settling properties. The influent travels upflow through the sludge blanket resulting from the biomass sedimentation. Reactor design is planned considering gas recovery, capture of less dense granules and efficient effluent withdraw and several improvements in geometry have been added to the original configuration. However, the formation of granulated sludge can take a long time, delaying the process start-up.[105 128]

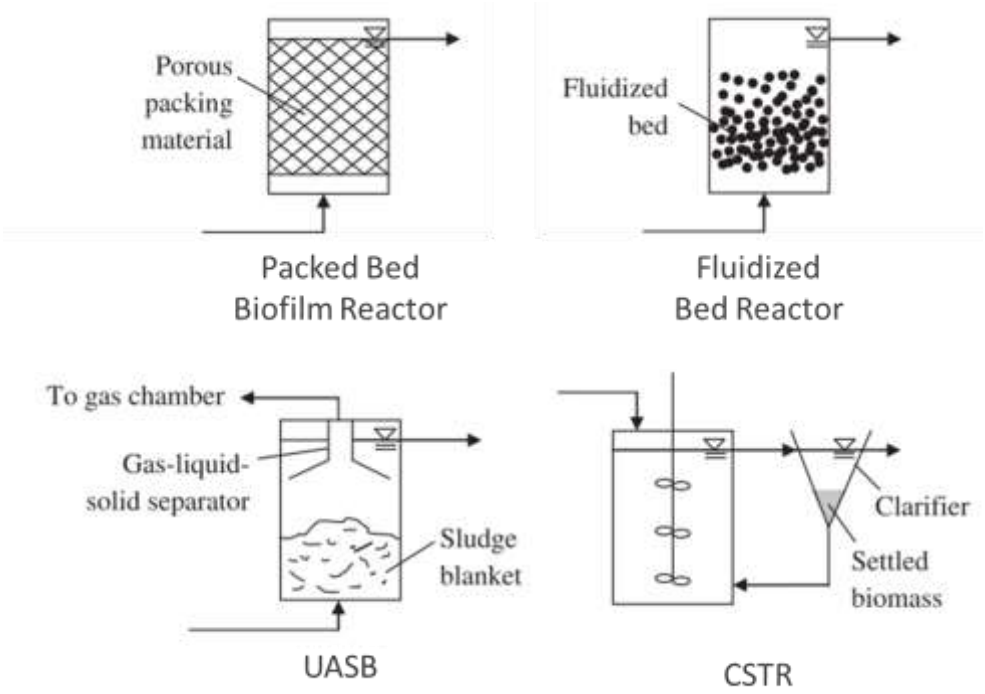


Figure 2h. Representation of the reactors commonly used for anaerobic treatment of waste with SCOA production.[105 110]

All these strategies listed are applied in large scale and proved to be efficient in the complete wastewater treatment with coupled energy production. When focusing on the first two steps of the anaerobic treatment – hydrolysis and acidogenesis - which are the steps involved in SCOA production, simpler approaches are taken. However, the geometry of the reactor stills influences significantly the biological process.[110]

Laboratory research usually uses a CSTR or SBR to produce SCOA. Both CSTR and SBR are biological reactors with suspended biomass. The CSTR is usually operated under continuous mode - chemostat - which is only suitable for processes with low retention times. Therefore, CSTR can be converted into a batch or semi-continuous reactor with intermittent feeding and withdraw for slow reactions. For an increase in the SRT, and consequently higher biomass concentration in the reactor, the CSTR can have a recirculation stream to guide settled biomass of the effluent to the reactor. For the SBR, is included a settling phase before effluent withdrawal in each batch, so the biomass will be kept in the reactor. For both configurations, is desirable to have a granulated biomass with good settling properties, which will have a direct impact in fermentation performance and productivity. During the reaction, an efficient mix of waste and biomass is desirable and can be enhanced by geometry of the reactor and adequate agitation without damaging the biomass.[105]

A CSTR for production of SCOA and alcohols was used by Temudo et al[121] in order to assess the product spectrum resulting from the MMC fermentation as function of the pH value and relate it with biochemical and thermodynamics consideration. Yang et al[129] also applied a CSTR to study the fermentation products resulting from an hydrolyzed activated sludge. Several SBR were used by Valentino et al[130]to study the effect of feeding frequency in the range of SCOA and consequent PHA produced. Jiang et al[131] also used a batch reactor for food waste fermentation in order to study the effect of pH, temperature and OLR on SCOA range, suspended solids and COD. A SBR was also applied by Tamis et al[132] to study a SCOA production process by granular sludge at low pH. Simon et al[133] applied both configurations for acidogenesis of industrial wastewaters in order to study the impact of retention time (RT) and pH in the SCOA production. The examples stated study the influence of several operational parameters in the acidogenesis performance and profile. However, the influence of the reactor configuration in the production of SCOA, independent of operational conditions, is not fully explored, shown by the lack of literature on this topic. Thus, further study of the effect of reactor operation mode in the production of SCOA is relevant and necessary to clarify the implications of this parameter in the process and achieve the full potential of the acidogenic fermentation.

2.3.3 Operational Conditions for VFA Production

When targeting the production of SCOA, has been widely shown that the operational conditions play a determinant role in the process. Parameters such pH, temperature, OLR, redox potential, RT and presence of additives have been reported to greatly influence the range of fermentation products and productivity by interfering in the biomass development.[110] Although numerous studies show the effect of these variables in the acidogenic fermentation, extrapolation of results can be inaccurate when considering different waste-based feedstocks or disregarding the synergy effect of all parameters combined.

pH

The pH value during acidogenic fermentation will directly impact the diversity of the MMC culture and is often related with the profile of acidogenic fermentation, i.e., the range and ratio of FP produced, especially the SCOA range.[115] pH is one of the most studied parameters due to the significant impact it has in the acidogenesis process. However, the optimal value is not unanimous and although acidogenic fermentation is usually carried under acidic conditions of pH from 5.0 to

6.0, it can be found in literature evidence of enhanced SCOA production in alkaline pH, especially for protein-rich wastes.[134] [135] However, pH values between 5 and 6 do not require addition of alkali that increase the process costs and are not acidic enough to inhibit the acidogenic fermentation.[133] Is also reported that shifts in the pH during the acidogenesis process can alter the microbial composition and therefore the scope of SCOA produced. Temudo et al[121] reported that a production of butyrate(HB), acetate (HAc) and molecular hydrogen at pH between 4.0 and 6.5 can shift to HAc, ethanol (EtOH) and formate at higher pH from 6.5 to 85. However, when using different substrates, a different behavior depending on the feedstock was observed by Simon et al.[133] Comparing the optimum pH for acidogenic fermentation, cheese whey presented an optimum pH between 5.5 and 6.0 while the paper mill effluent between 5.25 and 5.5. An increase in pH also results in different shifts in the SCOS spectrum and concentration: while an increase in HPr and decrease of HAc and HBu was observed for the paper mill effluent, cheese whey presented an increase of HBu and HPr. Jiang et al[131] also reported an optimum SCOA production from food waste at pH 6.0 and that the control of pH during the process is crucial for a significant production of acids.

Some correlations between pH value and SCOA spectrum can be established and explained; however, if using a complex feedstock as substrate for the acidogenic fermentation, its composition will greatly influence the process. Thus, an optimal pH value can vary with the waste used.[122]

Temperature

Although temperature is a significant parameter influencing the SCOA production, its effects are not considered as relevant as the ones deriving from the pH. An increase in temperature within a mesophilic range – between 20°C and 50°C - is usually reported to improve the SCOA production, namely the concentration[136] [137], production rate[138] and yield[136]. This behavior was observed by Xiong et al[138] for activated sludge, with a substantial increase of SCOA concentration when temperature was increased form 40°C to 50°C and decreased when temperature was raised to 60°C. Zhang et al[137] reported consistent results for activated sludge, with an increase of SCOA concentration following the raising of temperature from 10°C to 35°C. Kim et al[139] operated three different CSTR digesters for SCOA production, conducted with two independent variables, HRT and temperature, analyzed with response surface methodology. Although the quantity of SCOA produced was more affected by changes in the RT, temperature showed to have a more significant impact on the bacterial community structure. However, a balance between optimal condition for enzymatic activity, favorable temperature for the MMC and energy costs for heating and temperature maintenance is required to design an efficient and sustainable process.[138]

Retention Time

Two different retention times can be distinguished when characterizing a reactor: the HRT which concerns the medium volume and the time it is kept in the reactor while de SRT is related with the biomass and the time it is retained in the reactor. Thus, HRT defines the time that the substrate or waste spends in the reactors and the SRT refers to the microbial community.

The HRT presents a major effect in the acidogenic fermentation of complex substrates, such as wastes or sludge.[140] Although some authors defend that higher HRT favor the SCOA production, when using complex feedstocks, prolonged HRT hinders the SCOA production due to the presence.[141] This was observed by Simon et al[133] who reported that the HRT significantly affected the SCOA production: for an increase of HRT over 10h, the HBu production was shifted to HPr synthesis. Fang and Yu[141] also showed that the increase of the HRT from 4h to 12h was followed by an increase of SCOA production from a dairy wastewater; however, further increase of the HRT resulted in a very little SCOA production improvement.

When considering the SRT, lower values are reported to enhance SCOA production from wastewater.[140] [142] Xiong et al[143] studied the SCOA production for a range of SRT from 1 to 13 days. A SRT of 5d allowed for the maximum SCOA production that decreased significantly after the SRT was progressively increased to 13d. It was suggested that lower SRT prevents the methanogens presence, which consume the SCOA, since they have a lower growth rate when compared with acidogenic bacteria. However, SRT needs to be long enough to allow for a proper sludge hydrolysis and fermentation. Vanwonterghem et al[144] reported that shortening the SRT and increasing the temperature could be beneficial for SCOA production. Furthermore, a shift in the community composition was observed, indicating that these parameters can be selected and manipulates towards a production of specific SCOA of interest.

More research is needed about both retention times since they have a great influence over the SCOA production process and can be crucial for optimization. As for the other parameters explored, is also necessary to considering the cumulative effect with other operational conditions defined and substrate used.

Organic Load Rate

The OLR represents the rate at which the substrate is fed into the reactor. The literature about the OLR effect over SCOA production is not totally consistent and several values are reported. Jiang et al[131] reported that an increase of ORL to a certain level – similar to what is observed for

temperature – is beneficial for SCOA production. However, a further increase in the OLR can change the medium rheology, thus affecting the overall fermentation. For an OLR of 5gTS/L·d, SCOA production increased for the first 7 days and stabilized; for the OLR of 11gTS/L·d a steady-state was reached by the 14th day; in opposite, the ORL of 16gTS/L·d resulted in a significant decrease in SCOA concentration

The OLR can also influence the composition of SCOA in the effluent. Yu[145] studied the effect of the OLR in the SCOA profile using an upflow sludge blanket reactor for SCOA production from starchy wastewater. From 2 to 26gCOD/L·d. HAc was the dominant SCOA in the medium, followed by HPr. However, when the maximum OLR was achieved, the fermentation profile shifted to a fermentation with HBu as main product. Is to highlight that not only the ORL affects the SCOA range, and several parameters must be considered to take assumptions.

Additives

Several additives have been applied to acidogenic fermentation processes in order to improve SCOA production. However, it is important to consider the costs associated with the addition of these substances since they are usually expensive and might turn the process economically unsustainable. In addition, additives can affect the efficiency of the process and quality of final products since it can interact with metabolites and medium compounds, requiring more steps on further purification of the products intended.[122 146]

A way to improve SCOA productivity in anaerobic digestion is by inhibiting the activity of methanogens, organisms that consume the SCOA, through the addition of chemicals that will interfere with the microorganisms enzymes.[147] Some surfactants, amphiphilic molecules that decrease surface tension, such as sodium dodecyl sulphate (SDS) and sodium dodecylbenzene sulfonate, can be added to the fermentation medium in order to dissolve extracellular polymeric substances and promote the sludge hydrolysis and consequent SCOA production.[148 149]

2.4 Extraction and Recovery of Polyhydroxyalkanoates

As stated above, the extraction and recovery of PHA contributes to a significant part of the final product cost. In order to enable a wider commercialization of this biopolymer and design an environmental and economically sustainable process, research and innovation considering the downstream processing is still needed. Extraction procedures regarding PHA are not extensively

covered by literature, protocols are usually undetailed and the results not always reproducible. Moreover, some of the efficient and detailed extraction procedures are protected by patents.[94]

The fact that the PHA is stored in inclusion bodies inside the cell (Figure 2c) is the main obstacle to the extraction. Moreover, the polymer is accumulated in intracellular granules coated by proteins, such as phasins which contribute to the synthesis and stability of PHA[150], and phospholipids. Thus, in order to release the polymer, is necessary to disrupt the cell wall and membrane as well as the storage granule.[151] In addition, cell debris and other small cellular components, such as lipids or DNA, are released during extraction and can represent a contaminant in further steps of the downstream that also needs to be removed. [152]

When using a pure culture as PHA producer, the polymer recovery can be easier since all cells have the same structure and the extraction procedure can target the specific microorganism. For instance, *C. necator* has two thin phospholipid layers with peptidoglycan placed between them, feature of the Gram-negative bacteria, and are the type of bacteria easier to disrupt.[153] However, a Gram-positive bacteria such as *Bacillus megaterium*, also a PHA producer, presents a thick cell wall composed by peptidoglycan covalently bonded to polysaccharides and the disruption of the structure can be more challenging.[154] Opposing to the pure cultures, a MMC is a way more complex consortium with different types of microorganisms, distinct in size, morphology and physiology, and which composition can vary along the process. Thus, a procedure that affects all the different features of the multiple PHA-containing microorganisms is required to achieve an efficient extraction. In addition, MMC used for PHA production are usually fed with waste feedstocks as carbon sources, which contain a lot of different components that can be difficult to separate/remove from the biomass and constitute another hindrance for the extraction of PHA.[94]

For the extraction to be considered efficient, it not only needs to enable high recovery yields but also to assure high purity and minimal damage of the polymer molecule. Moreover, in order to improve the PHA recovery, extraction methodologies are often combined or preceded by biomass pre-treatments.

2.4.1 Pre-treatments of Biomass

When aiming the PHA extraction, pre-treatments can be applied to the biomass prior to the extraction per se, in order to destabilize and weaken the cellular membranes and favor the following steps. However, these methodologies can be useful ate a laboratory scale, but unsuitable for a larger, industrial scale. The biomass pre-treatment usually consists of oscillations in (1) temperature, (2) osmotic pressure and (3) pH value.

Considering (1) changes in temperature, application of heat can promote protein and other thermos-labile components denaturation, outer membrane destabilization and decrease the cytoplasmatic viscosity. It can also conveniently lead to the denaturation of depolymerases that could potentially damage the PHA polymeric chain and reduce the purity of the final product recovered.[155] Cycles of freezing/thawing cause expansion of the cell content (mainly water) that, together with the formation of water crystals, damage the cell membranes and allows for the leakage of intracellular compounds. Although this method is not suitable for an industrial scale, it can be easily applied in laboratory research.[156] The addition of a salt, such as sodium chloride, to the biomass medium will cause a change in the (2) osmotic pressure and induce cell fragility and plasmolysis. However, the efficiency of this method depends on the type of organism and cell structure.[157] A change in the (3) pH of the medium can also be considered a pre-treatment of the biomass since it can affect cell behavior and integrity. For instance, the use of an alkali solution such as sodium hydroxide is a strategy often reported as a significant step in the PHA extraction process.[158] [158]

2.4.2 Extraction of Polyhydroxyalkanoates

Organic Solvents

The application of organic solvents for PHA extraction is the most commonly used procedure due to its high recovery and purity (both over 95%) of the final polymer, and simplicity of the process. Organic solvents not only disrupt the cells efficiently but some are also able to solubilize the PHA and allow for an easy recovery of the polymer by solvent evaporation or precipitation.[94] However, most organic solvents used in large quantities, such as chloroform and 1,2-dichloromethane, are chlorinated hazardous compounds with a huge negative impact on the environment. [94]

Some non-halogenated solvents less harmful to the environment have been applied as alternative to the halogenated substances. Is the case of the Fiorese et al[159] that extracted PHB from *C. necator* applying 1,2-propylene carbonate. The extraction was carried at 130°C for 30min, resulting in a recovery of 95% and purity of 84%. Samorí et al[95] extracted PHA from *C. necator* applying dimethyl carbonate for 1h at 90°C, achieving a recovery of 63% and a polymer purity of 98%. Subjecting the biomass to a prior treatment with sodium hypochlorite improved the recovery to 82% (although decreasing polymer purity). The team also showed that a second extraction cycle allowed to recover remaining PHA in the biomass and that the dimethyl carbonate was also able to

extract PHA from MMC. However, the application of these greener solvents often requires high temperatures that might affect the polymer structure, decreasing its molecular weight, and represent additional costs for the overall PHA production process.

Mechanical Disruption

Mechanical forces are a conventional and former method applied to disrupt the cells and promote the release of the PHA granules. Equipment such as bead mill and vertical grinding chamber use the shearing action of solid beads to damage the biomass.[157] Although these methods do not present a negative environmental impact neither the damaging of the polymer, mechanical procedures are time consuming and expensive, and consequently not suitable for large scale applications

More recently, high pressure homogenization was considered for PHA extraction. In this method, cell suspensions of high density can be disrupted by constriction action of a pump. Among the processes described by Ghatnekar et al[160] to extract PHB from *Methylobacterium* sp. V49, high pressure homogenization, in the presence of 5% SDS, led to the best performance with a recovery of 98%. However, the process can promote thermal degradation of the desired product. Extraction by high pressure homogenization was also studied by Koller et al[161]: culture broth of PHA-rich *C. necator* as submitted to high pressure homogenization, achieving recoveries higher than 90%. Furthermore, the team reported that higher recoveries were achieved when combined with biomass pre-treatment with 1% SDS and sodium hydroxide. The process is suitable for scale-up and, even without biomass pre-treatment, high recoveries can be achieved.

Sonication corresponds to the application of ultrasounds to disrupt biomass, based on the impact of sound waves. This method is widely used for lysis of biomass in biotechnological processes, including PHA extraction. The most referenced PHA extraction method using ultrasonic disintegration was applied to *Haloferax mediterranei* by Hwang et al[162]. The disruptive efficiency of the sonication was attested by scanning electron microscopy and several parameters influencing the cell disruption fraction were assessed.

Chemical Disruption

Some chemicals, such as sodium hypochlorite, are successfully applied for PHA extraction. These compounds are able to solubilize the non-polymeric cell material and release the cell

content, leaving the PHA precipitated in the solution. However, they are strong oxidants and affect the polymer structure, decreasing its purity and molecular weight.[20] A particularity of this method is that can be applied directly in environmental samples without lyophilization needed, simplifying and reducing the process cost. The extraction with sodium hypochlorite if often combined with chloroform: while the sodium hypochlorite dissolves the cell membranes and releases the cell content, the chloroform simultaneously solubilizes the polymer protecting it from the oxidant effect of the sodium hypochlorite. Applying this strategy, Hahn et al[163] were able to extract PHB from *Alcaligenes eutrophus*, recovering 91% of the polymer with a purity over 97%.

Some surfactants – amphiphilic molecules that adsorb to surfaces when present in low concentration and with similar structure to the cell membrane phospholipids – are also reported to be used in PHA extraction procedures. Surfactants disintegrate the cell wall by incorporating themselves into the phospholipid bilayer, promoting cell disintegration and further solubilization of non-polymeric cell materials, without degrading PHA granules.[164] In addition, they can be applied in high cell density cultures and in the extraction of any type of PHA, regardless of monomer composition, and achieve high recovery yields.[165] Kim et al[166] combined heat treatment with addition of SDS for PHB extraction, in different SDS/biomass ratio. When the SDS/biomass ratio was higher than 0.4, purity of recovered polymer was between 95% and 97%. The polymer recovery was superior to 90% regardless the cell concentration and degradation of the polymer by the SDS was negligible. A recent article from Samorí et al[167] described an extraction procedure using switchable anionic surfactant. Ammonium carboxylate of lauric acid was applied for PHA extraction in high concentrated *C. necator* slurry and stirred for 3h at 90°C, achieving recovery over 99% and purity over 90%. Moreover, the team successfully recycled 98% of the ammonium laurate by decreasing the pH by carbon dioxide addition – the high recovery and purity obtained with the possibility to reuse the extractive agent turns the sustainable process a very promising procedure. Although no PHA extraction using biosurfactants was reported, these compounds can also have potential for a sustainable extraction process.[168]

Enzymatic Disruption

The cell disruption by enzymes presents an alternative to some chemicals since enzymes do not degrade the PHA structure. Although high recovery levels can be achieved, the enzymatic disruption is an expensive procedure – due to the price of pure enzymes – and so is often combined with the application of surfactants and chelates. Several lytic enzymes, such as lysozyme or alcalase, promote the extraction of PHA by damaging the cell membrane components. As example, Yasotha

et al[169] reported the use of enzymatic digestion for mcl-PHA extraction from *P. putida* combined with surfactant chelator. Nearly 90% of recovery was achieved using alcalase to digest denatured proteins, SDS to assist solubilization, ethylene diamine tetra acetic acid (EDTA) to complex cations and lysozyme to digest the peptidoglycan wall. The purity was about 92%, requiring further purification or post-treatment if PHA is intend to use for biomedical or other high purity requiring applications. A mixture of different enzymes acting synergistically can be a solution for an efficient cell disintegration. *Zeneca Process* is often referenced as an enzymatic method developed by Imperial Chemical Industries (UK), to extract PHB from *C. necator*. It comprises the use of proteolytic enzymes in addition to hydrolytic enzymes such as nucleases, phospholipases and lysozyme that harshly affect biomass but negligible damage in the PHA. Biomass is submitted to a thermal pre-treatment and then submitted to enzymatic disintegration. Optimized application requires addition of SDS and further bleaching to increase purity of the PHB extracted. However, these steps result in a demanding and expensive process.[170]

Supercritical Fluids

Supercritical fluid is a substance at temperature and pressure above critical point, in which distinct liquid and gas phases do not exist. The application of supercritical fluids as extraction methodology has been developed during the last decade and extended to the extraction of PHA. Since the substances used as supercritical fluid are usually water and carbon dioxide (non-expensive with low reactivity and toxicity), and despite the initial investment required for the equipment, the method is seen as an environmentally and economically sustainable process. In addition, the fluid can be easily removed by evaporation at the end of the process.[161]

Heijazi et al[171] used carbon dioxide as supercritical fluid to extract PHB from *C. necator*. The optimal conditions were 100 minutes of exposure to a pressure of 200atm at 40 ºC and with 20 mL of methanol as modifier achieving a recovery of 89%. Khosravi-Darani et al[172] reported the extraction of PHB from *C. necator* using carbon dioxide as supercritical fluid (200bar at 30°C) combined with application of a 0.4% sodium hydroxide solution. A recovery of 81% was achieved. The team also showed that pressure and temperature of operation as well as biomass cultivation method affects the efficiency of the extraction with supercritical fluid.

Cell fragility

Cell fragility can be affected by medium composition. Thus, supplementing the medium with specific components can decrease cell wall strength and enhance PHA extraction. For instance, Page and Cornish[173] reported that the addition of fish peptone to the medium led to pleomorphism of the *Azotobacter vinelandii* UWD cells, with increase of the osmotic sensitivity. Thus, the production of P(3HB) with high molecular weight was promoted and the posterior polymer extraction had a recovery of 96% with a simple extraction method with ammonia. For this method, however, it crucial to balance the cell wall fragility and integrity in order to promote microbial growth with high PHA content without causing cell lysis untimely.

Some PHA producing strains can be manipulated not only to enhance the productivity and storage capacity of the bacteria but also to promote a lysis system based on phage lysis.[174] This system is considered a cell fragility extraction method by some authors and was already applied in E. coli[174] and *C. necator*[175] Although biological lysis systems seem attractive extraction procedures to reduce downstream processing costs, they have not been applied in pilot or industrial PHA production processes.[94]

2.4.3 Recovery and Purification of Polyhydroxyalkanoates

After extraction of the PHA with the methodologies described in the previous section, except for the solvent extraction, a mixture of polymer, cell debris, biological molecules and the extractive agent is obtained. Thus, a further step for PHA recovery and purification is often needed to obtain the final product with the required purity for a specific purpose. The purification can resort to separation techniques, such as centrifugation, precipitation of the polymer and selective dissolution of PHA. Solutions of acetone or sodium hypochlorite can also be used to briefly wash the final bioplastic and remove some remaining contaminants.[94] The characterization of the PHA obtained, in terms of thermal, mechanical and physical properties is also relevant to understand the impact that the extraction procedure might have in the polymer structure. It also enables to assess the possible applications of the biopolymer and if it meets the requirements for particular purposes.[36]

3. MATERIALS AND METHODS

3.1 Acidogenic Fermentation

3.1.1 Inoculum and Fermentation Medium

The MMC used to inoculate the two reactors studied in this work came from a conventional municipal activated sludge treatment plan in Malmö, Sweden. The CSTR and SBR were inoculated once – at the start of the operation - with 600mg and 840mg of settled activated sludge, respectively.

The study on acidogenic fermentation was carried using synthetic feed with glucose as carbon source with influent concentration of 10g COD/L during the first 80 days of operation and 5g COD/L for the remaining days of the experiment. The composition of the medium was adapted from Tamis et al.[132] and is summarized in Table 3a.

Table 3a. Composition of the synthetic feeding used during the experimental work, for both influent concentration of 5gCOD/L and 10gCOD/L, adapted from Tamis et al..[132]

Compound	mg/L	
Glucose	5,000	10,000
NH_4Cl	678.00	
KH_2PO_4	127.00	
$MgSO_4 \cdot 7H_2O$	7.20	
$MnCl_2 \cdot 4H_2O$	2.88	
$CoCl_2 \cdot 6H_2O$	0.72	
$NiCl \cdot 6H_2O$	0.36	
$ZnCl_2$ ($ZnSO_4$)	0.39	
$CuSO_4 \cdot 5H_2O$	0.15	
$FeSO_4 \cdot 7H_20$	3.00	
BH_3O_3	0.156	
$NaMoO_4 \cdot 2H_2O$	0.66	
$Na_2SeO_3 \cdot 5H_2O$	0.342	
$Na_3WO_3 \cdot 2H_2O$	0.42	
$CaCl_2 \cdot 2H_2O$	4.64	

The feed was prepared by weighting the glucose and dissolving it in deionized water. Nutrients stated in Table 3a were added measuring the respective volume from different stock solutions: (1) nitrogen from a stock solution of 95.5g NH_4Cl, (2) phosphorus from a stock solution of 56.0g/L KH_2PO_4, (3) trace elements from a stock solution with 4.80g/L $MgSO_4 \cdot 7H_2O$, 1.60g/L $MnCL_2 \cdot 2H_2O$, 0.48g/L $CoCl_2 \cdot 6H_2O$, 0.24g/L $NiCl_2$, 0.26g/L $ZnCl_2$, 0.10g/L $CuSO_4 \cdot 5H_2O$, 5.20E^{-4} g/L BH_3O_3, 2.20E^{-3}g/L $Na_2MoO_4 \cdot 2H_2O$, 1.14E^{-3} $NaSeO_3 \cdot 5H_2O$, 1.40E^{-3} $Na_3WO_3 \cdot 2H_2O$, 1.44g/L $FeCl_2 \cdot 4H_2O$ and (4) a second trace elements solution with 5.80g/L $CaCl_2 \cdot 2H_2O$. During reactors operation, the feeding was kept at 10°C by placing the containers inside a refrigerator set to this temperature.

3.1.2 Experimental Set-up and Sampling

In order to study the profile and efficiency of acidogenic fermentation as function of the reactor operation mode, two glass reactors of 1L of working volume each were operated one as a Chemostat (CSTR) and the other as SBR. Both reactors were operated simultaneously for 130 days uninterruptedly, with agitation by magnetic stirrer at around 400rpm. The temperature was maintained at 22°C by circulation of water through glass jackets from a water bath coupled to a heating/cooling system. The feed had a concentration of 10gCOD/L during the first 80 days of the experiment and then was decreased to 5gCOD/L for the remaining time. The experimental set-up is schematized in Figure 3a.

The CSTR was operated with continuous feeding at a flow rate of 2L/d and recirculation of the biomass. The reactor was connected to a settler and recirculation of settled biomass occurred during 1 min at each 7 min with a resulting average flow rate of 5.4L/d. Withdrawing occurred by overflow. The SBR was operated with a cycle length of 6h. Each cycle comprised a feeding phase of 15 min, 5.5h of stirring, 10 min of settling (stirring turned off) and 5 min of withdrawing. The flow rates for the feeding and withdrawing were 48L/d and 145L/d, respectively. The pumps for feeding, withdraw and stirring were all controlled by programmable sockets. A volume of 0.5L of substrate was fed each cycle. Thus, both reactors had HRT of 12h.

The pH of both reactors was controlled at 5.5 by a pH controller Endress+Hauser Liquiline, connected to pH sensors from the same manufacturer and peristaltic pumps for dosing sodium hydroxide solution. A NaOH solution of 1M was used during the first 9 days of operation, being increased to 1.5M, 2M and 4M in days 14, 22 and 44 of operation, respectively. The HRT was maintained at 12h during all experiment and the SRT monitored. A stability phase could be determined when both reactors met the same SRT value. In the case of SBR, between days 73 and

85 of operation, the SRT was controlled by manual purge. The volume to collect was calculated based on total suspended solids (TSS) in the reactor and effluent, measured daily (section 3.4.1). The SRT targeted for the first phase of operation, with substrate concentration of 10gCOD/L, was 4d and, after decreasing the substrate concentration to half, the targeted SRT expected was 8d.

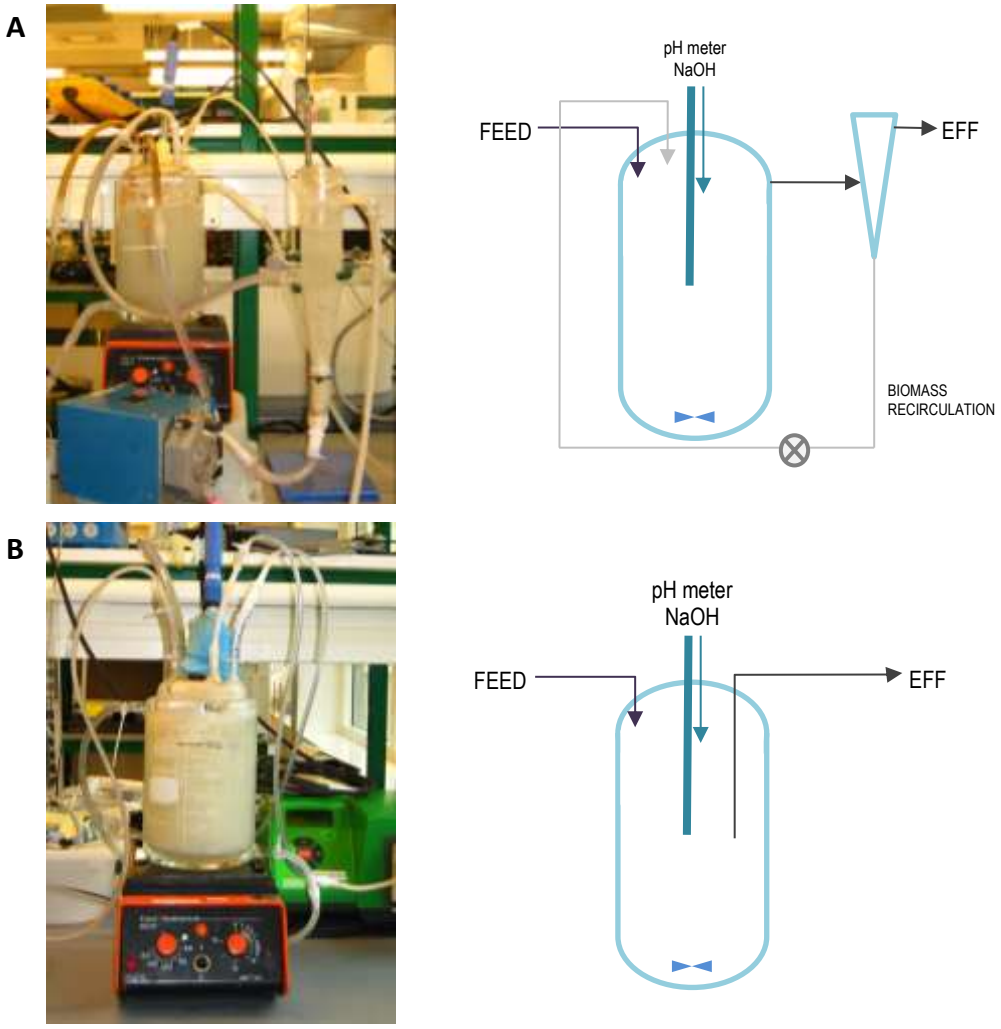


Figure 3a. Picture and schematization of the experimental set up for the CSTR (A) and the SBR (B).

Samples were collected daily throughout all the operation period. The organic content was indicated by COD, the biomass concentration through suspended solids (SS) and settling properties by calculation of the Sludge Volume Index (SVI).

Samples from the reactor medium were taken from the CSTR and from the SBR, before the settling phase at the end of the cycle, and the total COD (tCOD), soluble COD (sCOD), SCOA concentration, TSS, VSS and SVI determined. Samples from the respective effluents were also

collected in order to determine tCOD, sCOD, TSS and VSS. The tCOD, sCOD, SS and SVI were directly analyzed based on the procedures described from section 3.2.1 to 3.2.3. Samples for SCOA were filtered using 0.45µm pore syringe filter sartorius and stored at -4°C and posteriorly analyzed as described in section 3.2.4. Samples from the substrate of each reactor were also collected daily to assess the pH, sCOD and SCOA concentration, in order to verify if any fermentation was occurring.

For both reactors, it was also registered the pH value and temperature indicated in the controller as well as measured manually using an Hanna pH meter HI991001 for comparison and assure proper regulation/measurement from the controller. The consumption of NaOH was also monitored daily by reading the volume dosed during 24h from a graduated cylinder.

3.1.3 Microscopic Examinations

The microbial culture from both reactors were observed with a microscope three times a week after the 69th day of operation and twice a week after the 121st day until the end of the experiment. The microscope used was an Axioskop 2 Plus with a camera AxioCam HRc coupled - all the equipment manufactured by Carl Zeiss. Observations were performed with an ocular of 10x magnification and objectives Plan-NeoFluca of 10x and 20x magnification. Images were capture using AxioVision SE64 software. Length measurements were conducted using an objective micrometer of 1mm with steps of 10µm as reference. The observations aimed to characterize the cultures morphologically as well as determining the abundance, shape and size of flocks. A semi-quantitative scale (from 0 to 5) was defined in order to enable a comparison along the reactors operation. The methodology to characterize the biomass was based on Jekins.[176]

3.1.4 Cycle Studies

In order to follow the culture and fermentation progress, several cycle studies for the SBR were performed, namely at the 43rd, 55th, 62nd and 112th days of operation. The first three cycles' studies targeted the 6h of a complete operation cycle. Samples were taken at each 15 min for the first hour and then each 30 min to register pH value and collect samples for SCOA analysis. The organic content was determined through sCOD at each 15min for the first hour of the cycle, each half an hour between 1h and 3h of the cycle and at each hour for the rest of the cycle study. The biomass concentration was assessed through the SS at 0h, 1h and afterwards at each hour and a

half until the end of the cycle. The influent sCOD as well as the effluent sCOD and SS were analyzed at the end of the cycle.

The last cycle study was extended for 24 hours with no feeding, settling or withdraw phases during that time – just the stirring activated. Samples were taken as described for the shorter cycles' studies until the 8th hour of operation; two samples were taken next day at 23.25h and 23.75h of operation for sCOD, SCOA concentration and SS determination. Simultaneously, the feeding of CSTR was stopped for 24 hours in order to evaluate the fermentation evolution. The same sampling procedure as for the SBR was followed. Samples on the next day were collected at 23.25h and 24h of the experiment, for measurement of the sCOD and SS. The sCOD from the influent was determined as well as the sCOD and SS from the effluent.

The NaOH consumption was followed in all cycles' studies. Determination of sCOD, SCOA and SS were determined as described in sections 3.2.1, 3.2.2 and 3.2.4, respectively.

3.1.5 Analytical Methods

Chemical Oxygen Demand

Determination of COD of the samples from both reactors was made using Hach Lange spectrophotometric kits, namely LCK114 (150-1000mgO_2/L), following the package instructions: shake vigorously the cuvette with the digestion mixture, add 2.0mL of sample with the proper dilution, shake again and incubate for 2h at 148°C. The incubation was carried in a block heater LT200 from Hach Lange. After incubation, cuvettes were softly shaken and left to cool down and settle the precipitate. When the cuvettes reached the room temperature, COD was measured in a spectrophotometer DR3900 from the same manufacturer. The respective blank was prepared with 2.0mL of distilled water instead of sample. For determination of sCOD, samples were previously filtered with glass microfibers filters with 55mm pore; for tCOD, no treatment was made.

Suspended Solids

The SS were determined based on what is defined by APHA. For each sample, 5mL were vacuum filtered through a weighted glass microfiber filter with pore of 47mm diameter, previously burned for 24h at 550°C. Subsequently, the filters were left in the oven for 24h at 105°C before being weighted again, in order to determine the TSS. A final weighing was made after burning the filters for 1h at 550°C in the muffle. The filters were placed in a desiccator before each weighing for temperature and weight stabilization. The TSS and VSS were calculated according to Equations 1

and 2, with the VSS corresponding to the biomass concentration on the sample. Both SS concentrations were converted from gSS/L to gCOD/L through Equation 3.

The VSS was the main parameter used to monitor biomass concentration of both reactors since systematic error can be reduced by using large sample volume. However, when plotted with particulate COD (pCOD), corresponding to the biomass on the reactor, against time, both variables presented similar evolution, confirming the accuracy of the measurements (section 4.1.2).

Sludge Volume Index

The SVI was calculated in order to assess the settling properties of the biomass. The SVI was determined daily by allowing 100mL of well mixed reactor suspension to settle for 30min in a 100mL graduated cylinder. The settled sludge volume was measured and applied for SVI calculations as described in Equation 6.

Gas Chromatography

Gas chromatography (GC) was used for determination of the concentration of EtOH, butanol (BuOH) and several acids: HAc, HPr, iso-butiric acid (HiBu), HBu, isovaleric acid (HiVa), HVa, caproic acid (Hca) and heptanoic acid (HHe). A control sample with a mixture of VFA of known concentration was also ran in each measurement in order to assess reliability of the results. In all runs, the accuracy for the detected components was always higher than 90%, attesting the reliability of the results.

Standards were prepared in vials with 100µL of a solution of 25% of formic acid and 3g/L of acrylic acid – the internal standard – and 900µL of a standard solution (Appendix A). Standard samples were diluted in order to enable measurements within the range of 0.01g/L to 0.1g/L as low range and from 0.1g/L to 1.00g/L as higher range for all acids; for EtOH, concentration for standards ranged from 0.005g/L to 0.06g/L for low range and from 0.05g/L to 0.60g/L to higher range; calibration curve for BuOH was designed just after the 115^{th} day of operation – when identification of this alcohol was made through chromatograms analysis – covering concentrations from 0.01g/L to 1.0g/L. Filtered samples from the substrates of each reactor were prepared in vials with 100µL of a solution of 25% of formic acid and 3g/L of acrylic acid and 900µL of the sample without dilution. For reactor samples, vials were prepared with the same procedure, including a dilution of 1:2 of the sample.

The stated compounds were quantified with a Clarus 400 gas chromatograph (GC) from Perkin Elmer and the data treated using TotalChrom Navigator software. The GC was equipped with

a split injector (split 1:20) for an injection volume of 0.5µL through an Elit FFAP column (length: 30m, inner diameter: 32mm; film thickness 0.25µm) and a flame ionization detector (FID). Helium was used a carrier gas at flow rate of 1.8mL/min. Temperatures of injector and detector were 220°C and 250°C, respectively. The column temperature was initially 85°C for 0.5min; temperature was firstly ramped to 105°C by 25°C/min and followed by a second ramp of 7°C/min until reaching 200°C; a last ramp was programmed at a rate of 20ºC/min to reach 240°C and kept at this temperature for 1min.

High Pressure Liquid Chromatography

High Pressure Liquid Chromatography (HPLC) was used to determine the concentration of lactate (HLa) in the samples collected from the reactor and cycle studies. Before the analysis, the samples were filtered with 0.45µm syringe filter sartorius and added to the vials with the proper dilution. A lactate standard solution was prepared and diluted, allowing for a determination ranging from 0.05g/L to 0.8g/L (Appendix B). The content of each vial was injected by an Auto-sampler Hitachi L-2200 in an anion exchange column (RezexTM ROA – Organic Acid H^+ (8%), Phenomenex, 300x7.8mm) connected to a refraction index detector Hitachi RI L-2490. The injection volume was 20µL and the column was kept at 65°C in a Gecko 2000 external oven. A 0.005N H_2SO_4 solution, prepared with Milli-Q water, was used as eluent with a flow rate of 0.5mL/min(Hitachi L-2130).

3.1.6 Calculations

To determine TSS and VSS from a 5mL sample, the following equations were applied:

$$TSS\left(\frac{g}{L}\right) = \frac{m_{oven}(g) - m_{filter}(g)}{5mL} \times \frac{1000mL}{1L} \qquad \text{Equation 1}$$

$$VSS\left(\frac{g}{L}\right) = \frac{m_{oven}(g) - m_{muffle}(g)}{5mL} \times \frac{1000mL}{1L} \qquad \text{Equation 2}$$

with m_{filter} corresponding to the mass of the filter prior to filtration, m_{oven} the mass of the filter after 24h at 105°C in the oven and m_{muffle} the mass of the filter after 1h at 550°C in the muffle. The concentration of VSS in g/L can be further converted in gCOD/L:

$$VSS\left(\frac{gO_2}{L}\right) = VSS\left(\frac{g}{L}\right) \times \frac{1.48gO_2}{gVSS} \qquad \text{Equation 3}$$

where 1.48gO_2/gVSS is the conversion factor of weight of biomass into grams of COD.

The SRT was calculated considering the suspended solids in the reactor, TSS_R, and the flow rate of TSS on the effluent, Q_{TSSE}. The calculation can be translated to:

$$SRT(d) = \frac{TSS_R\ (g)}{TSS_E\left(\frac{g}{L}\right) \times Q_E\left(\frac{L}{d}\right)} = \frac{TSS_R(g)}{Q_{TSS_E}\left(\frac{g}{d}\right)} \qquad \text{Equation 4}$$

If manual purge was applied, the biomass removed manually is considered for the SRT calculation:

$$SRT(d) = \frac{TSS_R\ (g)}{TSS_E\left(\frac{g}{L}\right) \times Q_E\left(\frac{L}{d}\right) + TSS_R\left(\frac{g}{L}\right) \times Q_P\left(\frac{L}{d}\right)} \qquad \text{Equation 5}$$

where Q_P is the purge flow rate.

The SVI was calculated by the following equation:

$$SVI\left(\frac{mL}{g}\right) = \frac{SSV_{30min}\left(\frac{mL}{100mL}\right) \times 10}{TSS_R\left(\frac{mg}{L}\right)} \times \frac{1000mg}{1g} \times \frac{1000mL}{1L} \qquad \text{Equation 6}$$

where SSV_{30min} is the settled sludge volume during 30 min in a 100mL graduated cylinder and TSS_R the TSS in the reactor.

To allow the comparison of COD measurements with EtOH, BuOH and SCOA concentration values, the latter ones were converted from g/L to gCOD/L using the specific conversion factors (*c.f.*). The *c.f.* were determined based on the following oxidation equation:

$$\alpha C_x H_y O_z + \beta O_2 \longrightarrow \gamma CO_2 + \delta H_2 O \qquad \text{Equation 7}$$

in which α, β, γ and δ are the stoichiometric coefficients and is assumed that no nitrification occurs. The *c.f.* is calculated according to the following equation:

$$c.f.\left(\frac{gCOD}{g}\right) = \frac{\beta \times M_{O_2}}{\alpha \times M_{C_x H_y O_z}} \qquad \text{Equation 8}$$

where α and β are the number of moles of oxygen and the compound to be oxidized respectively, based on the chemical equation 6 and *M* the molar mass. Table 3b summarizes the conversion factors calculated for EtOH, BuOH and acids quantified. The conversion factor is multiplied by the concentration in g/L in order to obtain the compound concentration in gCOD/L.

Table 3b. Conversion factors (*c.f.*) to convert concentration of different compounds in g/L into gCOD/L.

	EtOH	BuOH	HLa	HAc	HPr	HiBu	HBu	HiVa	HVa	HCa	HHe
c.f.	2.083	2.59	1.066	1.07	1.51	1.81	1.81	2.04	2.04	2.2	2.34

Yields were calculated considering the organic load entering the reactor and produced fermentation products (FP), namely EtOH, BuOH and several SCOA, highlighting HLa, HAc, HPr, HiBu and HBu. The yield of FP (Y_{FP}) was calculated by the following equation, based on Simon et al[133]:

$$Y_{FP} = \frac{\sum FP_E \left(\frac{gCOD}{L}\right) - \sum FP_I \left(\frac{gCOD}{L}\right)}{\left[\left(sCOD_I \left(\frac{gCOD}{L}\right) - \sum FP_I \left(\frac{gCOD}{L}\right)\right] - \left[sCOD_E \left(\frac{gCOD}{L}\right) - \sum FP_E \left(\frac{gCOD}{L}\right)\right]} \quad \text{Equation 9}$$

The equation translates the substrate consumed to produce the FP, where FP_I and FP_E represent the SCOA in the influent and in the effluent, respectively, and $sCOD_I$ and the $sCOD_E$ is the influent and effluent sCOD.

When considering just the SCOA, the yield calculation follows the equation:

$$Y_{SCOA} = \frac{\sum SCOA_E \left(\frac{gCOD}{L}\right) - \sum SCOA_I \left(\frac{gCOD}{L}\right)}{\left[\left(sCOD_I \left(\frac{gCOD}{L}\right) - \sum SCOA_I \left(\frac{gCOD}{L}\right)\right] - \left[sCOD_E \left(\frac{gCOD}{L}\right) - \sum SCOA_E \left(\frac{gCOD}{L}\right)\right]} \quad \text{Equation 10}$$

which relates the amount of SCOA produced with the amount of substrate consumed. $SCOA_I$ and $SCOA_E$ represent the SCOA in the influent and in the effluent

The biomass yield can also be calculated considering the substrate COD converted into biomass:

$$Y_X = \frac{X_E \left(\frac{gCOD}{L}\right) - X_I \left(\frac{gCOD}{L}\right)}{\left[\left(sCOD_I \left(\frac{gCOD}{L}\right) - X_I \left(\frac{gCOD}{L}\right)\right] - \left[sCOD_E \left(\frac{gCOD}{L}\right) - X_E \left(\frac{gCOD}{L}\right)\right]} \quad \text{Equation 11}$$

where X_I and X_E are the biomass in the reactor and in the effluent, respectively.

In order to assess the fraction of sCOD converted into all FP or just SCOA, Equations 12 and 13 can be used:

$$CONV_{FP} = \frac{\sum FP_E \left(\frac{gCOD}{L}\right)}{sCOD_E \left(\frac{gCOD}{L}\right)} \quad \text{Equation 12}$$

$$CONV_{SCOA} = \frac{\sum SCOA_E \left(\frac{gCOD}{L}\right)}{sCOD_E \left(\frac{gCOD}{L}\right)} \quad \text{Equation 13}$$

The percentage of conversion can be obtained by multiplying Equation 12 or 13 by 100%. This equation indicates the profile and potential of the fermentation process. However, this last parameter do not reflect the efficiency in converting the influent COD in the organic acids.

3.2 Extraction of Polyhydroxyalkanoates

3.2.1 Biomass containing Polyhydroxyalkanoates

Both pure and MMC containing PHA were used in the extraction experiments.

As pure culture, *Cupriavidus necator* DSM 545 was the bacterial strain selected to produce PHA, obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen. 100mL pre-inoculates of *C. necator* DMS 545 were grown in Luria-Bertani (LB) medium (Table 3c), at 28°C and 180rpm agitation. Cell density and culture growth were monitored through spectrophotometry, as described in section 3.5.1.

Table 3c. Composition of the LB medium used for growth of the *C. necator* pre-inocula.

Compound	g/L
Tryptone	10.00
Yeast Extract	5.00
NaCl	10.00

When the culture presented exponential growth, a defined volume of cell suspension (Equation 14) was transferred to 250mL inoculates of defined medium (Table 3d) and kept at 28°C and 180rpm agitation. Cell density and culture growth were also monitored through spectrophotometry. When the culture achieved the stationary phase, the medium was centrifuged at 5000rpm and at 4°C for 20min and the supernatant discarded. The biomass was further washed

three times with 0.9% NaCl solution, at 5000rpm and 4°C for 15min each time. Centrifuged biomass samples containing PHA were stored at -80°C and posteriorly lyophilized and kept dry in a desiccator. Both mediums and trace elements solution, as well as all material used to grow and manipulate the pure cultures were autoclaved for 20min at 121°C.

Table 3d. Composition of the defined medium with nitrogen limitation, used for growth of the *C. necator* and PHA accumulation.

Compound	g/L
KH_2PO_4	2.30
Na_2HPO_4	2.30
NH_4Cl	1.00
$MgSO_4 \cdot 7H_2O$	0.50
$NaHCO_3$	0.50
$CaCl_2 \cdot 2H_2O$	0.01
$C_6H_{14}N_2O_7$	0.022
$C_6H_{12}O_6$	20.00
$FeCl_3$	0.03
Trace elements solution	5.00 mL

Table 3e. Composition of the trace elements solution supplemented to the defined medium described in Table 3d.

Compound	g/L
$ZnSO_4 \cdot 7H_2O$	0.10
$MnCl_2 \cdot 4H_2O$	0.03
H_3BO_3	0.30
$CoCl_2 \cdot 6H_2O$	0.20
$CuCl_2 \cdot 2H_2O$	0.10
$NiCl \cdot 6H_2O$	0.02
Na_2MoO_4	0.03

The MMC containing PHA was obtained from a SBR used for selection and enrichment of PHA-storing microorganisms using hardwood sulphite spent liquor, a by-product of the pulp and paper industry, as carbon source and operated by other members of the research group.[100] The selected MMC was submitted to accumulation assays in order to maximize their PHA content.

Biomass samples from the MMC containing PHA were centrifuged at 5000rpm for 1h at 4°C and then washed three times with 0.9% NaCl solution, at 5000rpm and at 4°C for 40min each time. As for the pure culture, the centrifuged biomass samples containing PHA were stored at -80°C and posteriorly lyophilized and kept dry in a desiccator.

3.2.2 Extractive Agents

Four different surfactants were applied for PHA extraction: olyethylene glycol alkyl ether BRIJ 30, polyoxyethylene sorbitan monooleate TWEEN 80, and polyethylene glycol octylphenyl ethers TRITON X-110 and TRITON X-114. All surfactants were purchased from Sigma-Aldrich.

For the enzymatic extraction of PHA, an enzymatic mixture obtained from the fungus *Paecilomyces variotii* NRRL-1115 was used. The inoculum was obtained from *P. variotii* plaque cultures, by covering the fungus with 10mL of ME medium and scraping the fungus surface to detach the cells. The liquid was collected and the cell concentration determined by filtration and drying of a sample. A 300mL suspension of ME medium was inoculated with the adequate volume to have an initial biomass concentration of 0.07g/L. The *P. variotii* was grown at 28ºC with 180rpm agitation and the pH was monitored. When the pH achieved a stable value, the suspension was filtrated by vacuum filtration and the biomass stored at 4°C. The medium and all material used to manipulate the fungus were autoclaved at 121°C for 20min.

The extraction of the intracellular enzymes was made by sonication. 1.00g of dry fungus biomass was suspended in 10mL of buffer solution with NaCl 100mM and SDS 1% (m/v) at pH 7. The suspension was placed in a container with ice and subjected three times to 2min sonication periods, each one followed by 1min break. The hydrolysate was centrifuged at 5000rpm and 4°C for 20min. The supernatant was collected, consisting of the enzymatic solution applied in extraction assays.

3.2.3 Development of the extraction procedure

Samples of each biomass batch were submitted to a PHA extraction using chloroform as control. Lyophilized biomass was dissolved in chloroform - 1:50(m/v) and left overnight with stirring. Afterwards, the solution is vacuum filtered using glass microfiber filters with a 47mm diameter and the filtrate left to evaporate. After complete evaporation of the chloroform with inert

gas, namely nitrogen, the polymer film is weighted and the biomass PHA content estimated, assuming that 97% of the total PHA is extracted with chloroform.

Surfactants and enzymatic solution were the extractive agents applied in the lyophilized biomass of pure and MMC for PHA extraction. Since these compounds were never reported for PHA extraction (except for some biomass pre-treatments), it was necessary to develop and continuously improve a new extraction procedure. Several steps were considered: the reaction for cell lysis and release of PHA, separation of PHA from the remaining compounds and medium and finally the polymer recovery. For each extractive agent, concentrations of 2%, 5%, 7% and 10% (v/v) were tested at different temperatures, namely 30°C, 40°C and 50°C, and the reaction time for all essays defined as 24h. To separate the PHA from the medium and remaining extraction residues, a selective dissolution for the polymer was studied applying hexane, acetone, propylene carbonate and dimethyl carbonate. Finally, phase separation with posterior evaporation were applied for the polymer recovery. Efficiency of the procedure and suitability of the studied parameters were assessed by calculation of the percentage of the polymer recovered (Equation 15).

3.2.4 Analytical Methods

Spectrophotometry

All spectrophotometry measurements to monitor cell density of the pure cultures and for determination of protein content by the Biuret test (section 3.5.3) were made using a spectrophotometer UVmini-1240 from STIMADZU and acrylic cuvettes. Blank and necessary dilution of pure culture samples were made using the respective medium.

Gas Chromatography

The PHA concentration of the biomass samples was determined by GC-FID, using a Clarus 480 from Perkin Elmer equipped with a column SGE BP20 (WAX) (length: 60m; inner diameter: 0.32mm; film thickness: 0.5µm) and using a method adapted from Lemos et al.[123] The lyophilized biomass was incubated at 100°C for 3.5h with heptadecane solution (internal standard), dissolved in chloroform (1:1) and 20% acidic methanol. After digestion, the organic fraction of each sample was extracted and injected in the GC coupled to a FID. The HB and HV monomers concentration were calculated using P(HB-*co*-HV) (88%-12%) standards. The column temperature

was initially 50°C and was firstly ramped to 100°C by 16°C/min and followed by a second ramp of 9°C/min until reaching 220°C and kept at this temperature for 3min.

Biuret Test

The Biuret Test was applied for protein quantification of the enzymatic solution obtained from *P. variotii* lysis. To 1mL of five different samples with albumin concentrations from 2.0g/L to 10g/L, it was added 5mL of Biuret reagent and left to react for 30min. Then, the samples absorbance was measured at 540nm and the calibration curve determined (Appendix C). The same procedure was applied for different dilutions of the enzymatic solution and the protein concentration determined.

3.2.4 Calculations

The volume of *C. necator* culture in LB medium used to inoculate each 250mL defined medium for PHA accumulation was calculated targeting an absorbance of 0.200:

$$V_{LB} = \frac{0.200 \times 250mL}{Abs_{LB}} \qquad \text{Equation 14}$$

where Abs_{LB} is the absorbance measured for the culture in LB medium.

The percentage of polymer extracted, used to assess the extraction assays performed during the experimental work, was calculated by the following equation:

$$\%\ PHA = \frac{m_{PHA}(g)}{fr_{PHA} \times m_X(g)} \times 100 \qquad \text{Equation 15}$$

with m_{PHA} corresponding to the mass of the polymer film obtained, fr_{PHA} the PHA fraction of the biomass (determined by GC or chloroform extraction) and m_X the mass of lyophilized biomass.

4. RESULTS AND DISCUSSION

4.1 Reactors General Performance

4.1.1 Defined Parameters

Two reactors aiming the production of SCOA were operated simultaneously during 130 days. The final objective was to compare the effects of operation mode – in this study, CSTR and SBR – in the production of SCOA, preferable substrates for a further PHA production. Both reactors were submitted to the same operational parameters, namely pH, temperature, OLR and HRT, allowing for conclusions just based on the different operation mode. Comparison of performance between the reactors was based in the assessment of the biomass properties and in the production profile of the FP.

The pH selected was 5.5, based on several studies which describe a successful acidogenic fermentation with a pH of 5.5 with significant production of SCOA, such as recent articles from Tamis et al.[132] and Zheng et al.[177]. Besides, pH 5.5 is low enough to not require a huge amount of NaOH to maintain a stable value – relevant if considering an extrapolation for a large scale process – and acidic enough to inhibit activity of methanogens and, subsequently, production of undesirable methane at the given temperature and SRT. For both reactors, the pH was measured through a regulator as well as manually (as described in section 3.1.3) in order to attest the accuracy of the measurements. Figure 4a shows the progress of pH value for the CSTR and for the SBR. For the CSTR, the pH value was maintained stable along all the process, presenting an average of 5.51 with a standard deviation of 0.33 (less than 10%). When considering the SBR, the pH was also stable along the operation, with an average of 5.60 and a standard deviation of 0.21. These results guarantee that both reactors maintained a stable and similar pH along the process, with variation lower that 10%, enabling the comparison between them. However, in Figure 4a is possible to observe a slight increase in the measured pH after changing the feeding concentration. This fact is later explained, regarding the fermentation progress and NaOH consumption. The pH of daily substrate samples was also measured and can be followed in Figure 4a. Although the value is mainly between pH 3.00 and pH 4.00, an inconsistency is observed. Since the substrate was prepared with a periodicity of each two or three days and the containers washed, the pH is higher in the days in which fresh substrate was fed. The following decrease is likely to derive from residual fermentation, decomposition of glucose and other easily degradable compounds and the low buffer capacity of

the solution, which cannot be totally avoided by keeping the substrate at 10°C and wash the containers frequently.

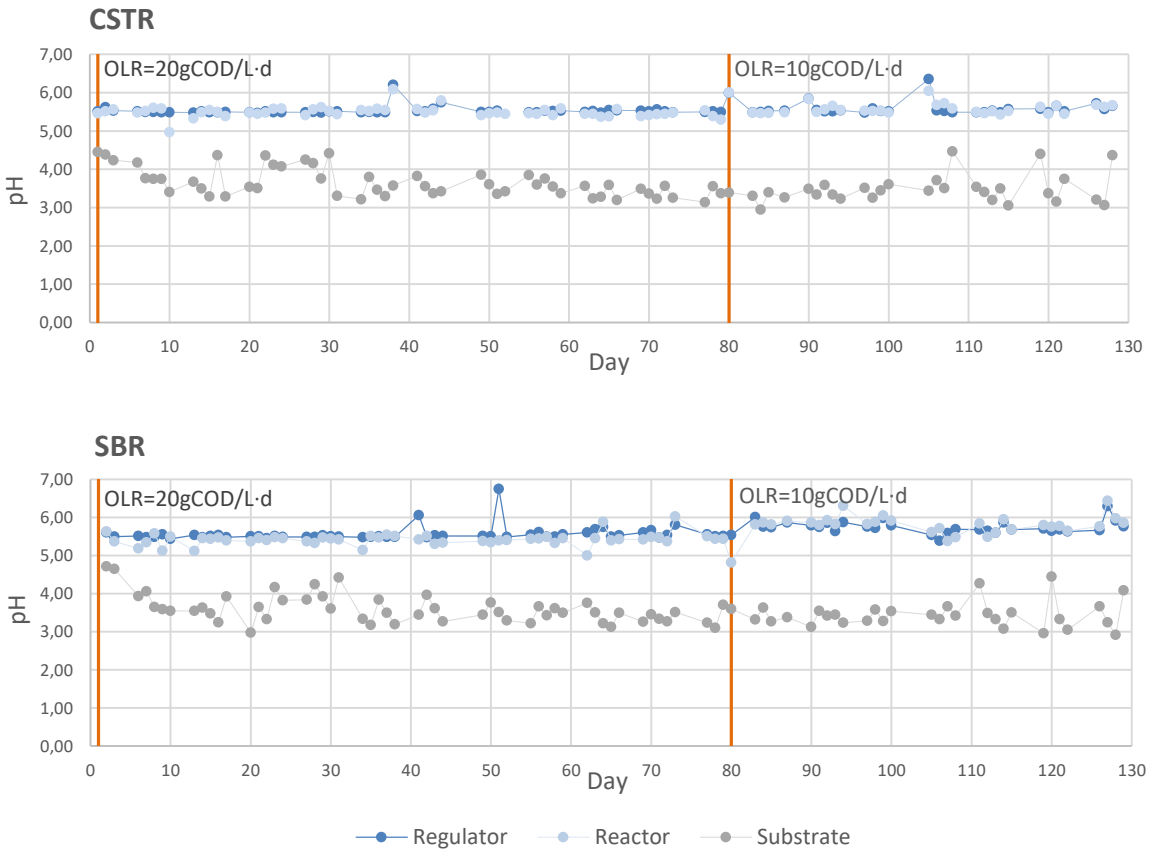


Figure 4a. Registered pH values, by the regulator and measured manually, for the reactor and substrate samples. Vertical lines indicate the change in the OLR.

The temperature value stablished was 22.0°C. The temperature measured by the controller was constantly 22.1°C except for an irrelevant number of days over the operation period. Thus, the temperature was maintained equal in both reactors, assuring a reliable comparison.

The HRT was set at 12h. Since the influent and effluent flow rates were established to meet a HRT of 12h and kept constant through all the experiment, plus a constant work volume of 1L in both reactors, the same HRT for the CSTR and SBR was assured. For the SRT value calculation, the concentration of biomass inside the reactor as well as the biomass withdrawal in the effluent were considered, as shown in Equation 4. Thus, the biomass concentration was monitored in order to calculate the SRT along the reactors operation. When both reactors exhibited similar SRT, it was possible to compare fermentation performance based only on the operational set ups. As shown in Table 4a, two time frames were selected in which both reactors have similar SRT, one for the OLR of 20gCOD/L·d and another for the OLR of 10gCOD/L·d. The first time frame is comprised from day

64 to 80, with an average SRT of 3.206d and 2.837d for the CSTR and SBR, respectively, with correspondent standard deviations of 0.56d and 0.66d. The comparison was also possible within days 100 and 114. CSRT and SBR present an average SRT of 5.130d and 5.512d, respectively, with standard deviations of 1.61d and 1.60d.

Table 4a. Selected time frames from the overall operation time in which the average SRT value is similar in both reactors. Two different periods were selected, one for each of the OLR used during the experiment.

Time Frame	OLR	CSTR		SBR	
		SRT (d)	*σ (d)*	SRT (d)	*σ (d)*
64 – 80	20gCOD/L·d	3.206	0.56	2.837	0.66
100 – 144	10gCOD/L·d	5.130	1.61	5.512	1.60

In the beginning of the experiment, the OLR was set to 20gCOD/L·d and maintained for 80 days, until a stability in COD of the reactor, biomass concentration and SCOA production was observed (see section 4.1.2 and 4.2.1). At the 80th day of operation, it was decided to change the OLR to half – 10gCOD/L·d – in order to evaluate the adaptation of the MMC, effects on fermentation profile and also to promote a total conversion of substrate. When a stable biomass concentration was observed, a measurement of orto-phosphate and ammonium in the effluent was performed, using Hach Lange spectrophotometric kits (LCK 349 and LCK 303, respectively), to determine if these essential nutrients for bacterial growth were available in excess. Both phosphorus and ammonia were detected, assuring that no lack of neither these nutrients was restricting the MMC growth.

4.1.1 Chemical Oxygen Demand and Suspended Solids

Samples from the reactor and effluent were submitted to tCOD and sCOD analyses as well as the substrate to sCOD. In Figure 4b the COD evolution in the reactor is represented. In Figure 4c, COD referring to the effluent and substrate samples during all the experiment for the CSTR and SBR, is depicted.

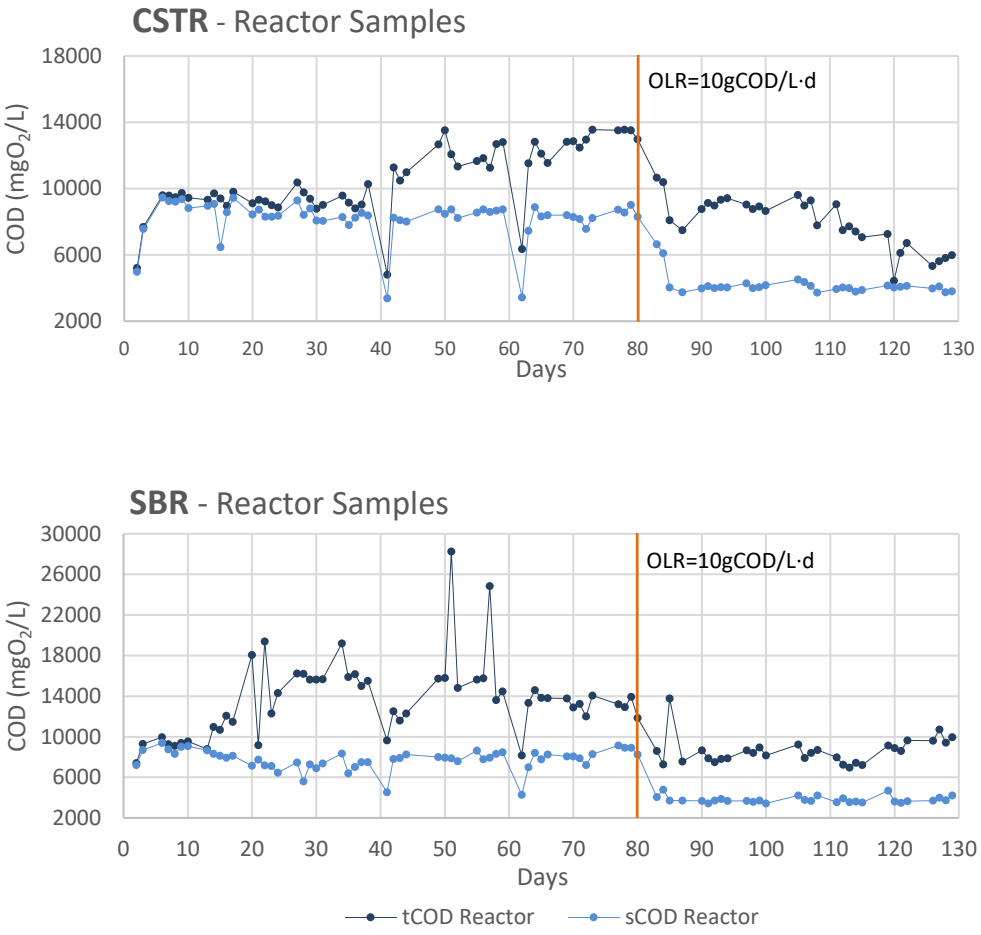


Figure 4b. Evolution of tCOD and sCOD of reactor samples from the CSTR and SBR, during the entire experiment.

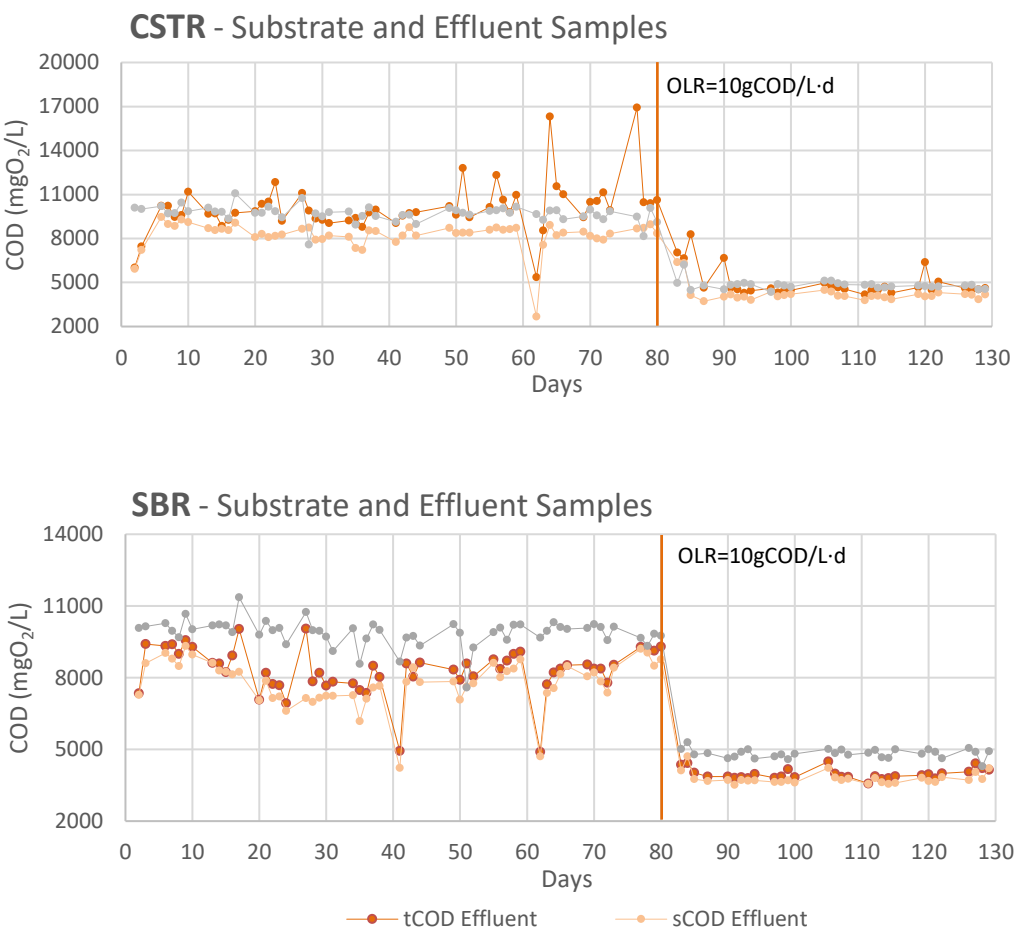


Figure 4c. Evolution of sCOD of the substrate fed to each reactor and evolution of tCOD and sCOD for the CSTR and SBR effluents during the entire experiment.

The substrate fed to the CSTR presented a stable value of sCOD along all the experiment. The mean sCOD value for the feeding was 9.70gCOD/L in the first stage of the experiment and 4.89gCOD/L after the change in the OLR. The standard deviation was 0.53gCOD/L and 0.31gCOD/L, respectively, and resulted from inaccurate volumes measured in plastic cups for feeding preparation. When observing the data referring to reactor samples from the CSTR, an increase in both tCOD and sCOD was observed in the first 4 days of operation while the initial deionized water inside the reactor was replaced by the feeding and the bacterial growth became significant. After day 4, the difference between sCOD and tCOD started to progressively increase – this difference corresponds to the particulate COD (pCOD=tCOD-sCOD). The pCOD corresponds to particles of organic material that are filtered in the sCOD samples and that correspond mainly to cells and flocks of biomass. Thus, the increasing drift can be attributed to MMC growth. After the 37th day of operation, a stable value of sCOD was observed along with a sharp increase of tCOD until the 65th day. Both sCOD and tCOD were constant after the 65th day until the OLR was changed to 10gCOD/L·d: sCOD and tCOD decreased for a constant value around 4.00gCOD/L and 9.00gCOD/L, respectively, after 6 days of adaptation. After the 109th day of operation, a decrease in tCOD, and consequently in pCOD, was observed until the end of the experiment. When focusing on the effluent, a small difference between tCOD and sCOD was observed, which is almost insignificant during the last 37 days of the experiment. This showed that a very low pCOD, it means, biomass, is present in the effluent – around 0.10gCOD/L. Some tCOD values drift indicating a high concentration of organic matter. This fact can be justified by the growth of mold on the settler surface and in the tube of the effluent. To avoid the disturbance it caused in the measurements, samples from the effluent were taken from the top of the settler after the 88th day, instead of at the end of the effluent tube.

When considering the SBR, the sCOD of the substrate presented the same behavior as for the CSTR. The average concentration was 9.88gCOD/L and 4.84gCOD/L, with standard deviations of 0.55gCOD/L and 0.19gCOD/L, respectively, for both OLR used. As for the reactor COD, an initial adaptation is perceptible during the first days of operation. A stable value of sCOD is observed especially between the 42nd and the 80th days of operation, except for a few points which measurements could have been influenced by procedure or random errors. However, the tCOD oscillated throughout the operation period with an OLR of 20gCOD/L·d – thus, for comparison of reactors performance, the period between the 63rd and 80th days of operation was selected, when the tCOD was more stable. After decreasing the OLR to 10gCOD/L·d, the tCOD evolution was more stable, indicating a fast adaptation to the new OLR, as also observed for the CSTR. As for the SBR

effluent, and similar to what was observed to the CSTR, the values of sCOD and tCOD were close, indicating that almost no biomass was withdrawal – just 0.4g pCOD/L in average, corresponding to 6% of the tCOD - and the COD was mainly sCOD. For both reactors, it was also perceptible that the sCOD in the reactor and in the effluent are within the same range of values. This attests the reliability of the measurements.

Similar to what happens in larger and full-scale reactors, no nitrogen was provided to the CSTR not to the SBR. Thus, the reactors were sealed and any air leakage prevented; all tubes used were made from materials impermeable to air. For the SBR, a valve was coupled and activated during the feeding and withdrawal phases so a possible vacuum would not compromise the liquid flow. In order to determine if any of the reactors presented a significant COD loss, a COD balance was calculated considering the COD consumed over the COD in the influent. A COD loss of 13.5% and 16.4% were calculated for the CSTR and SBR, respectively. However, the COD loss is not only due to oxygen leakage which promotes oxidation but also due to hydrogen and methane gases production, not measured in the COD analyses, and even reduction of sulfate to hydrogen sulfide.

SS were measured for the reactor and effluent samples. For both reactors, is observable that almost all of the TSS corresponds to VSS, with an average of 90% and 92% for the CSTR and SBR, respectively (Figure 4d). This means that most of the SS are biomass. However, the biomass concentration and evolution along the experiment are significantly different between the reactors. For the CSTR, the biomass growth was not significant in the first 38 days reaching just a concentration of 1.02gVSS/L. After the 38th day, a progressive slow increase was noticed, reaching a concentration of 4.00gVSS/L by the 73rd day. The VSS concentration of about 4.00gVSS/L is the highest value achieved for the CSTR. After the change of the ORL at the 80th day of operation, a decrease in biomass concentration was observed for a period of 7 days with a posterior recover to the same range of values around 4.00gVSS/L – that can traduce an adaption period to the new OLR. For the rest of the operation period, VSS values were stable until the 112th day of operation, after which a decline is observed. In contrast, after just 9 days of operation, the SBR presented greater biomass concentrations than the CSTR, achieving the highest concentration of around 6.00gVSS/L by the 24th day. At the 41st day of operation a significant decrease in VSS was registered – of an half to 3.00gVSS/L. This unexpected decrease was due to a problem in the influent pump, turned off during the weekend (around 8 cycles, perceived from the effluent volume withdrawn). After 7 days, the SBR biomass concentration was able to recover to previous concentrations. Thus, it was possible to conclude that a SBR configuration will promote a higher biomass concentration the STR, within the same time frame. During the period of time between the 73th and 80th day of operation,

the biomass concentration was controlled by purging manually part of the reactor volume. The volume purged was calculated daily by applying Equation 5, considering the biomass concentration measured and aiming a SRT of 3 days – this would allow the SBR to meet the same SRT and VSS values of the CSTR, enabling the comparison of performance just based in a different operation mode.

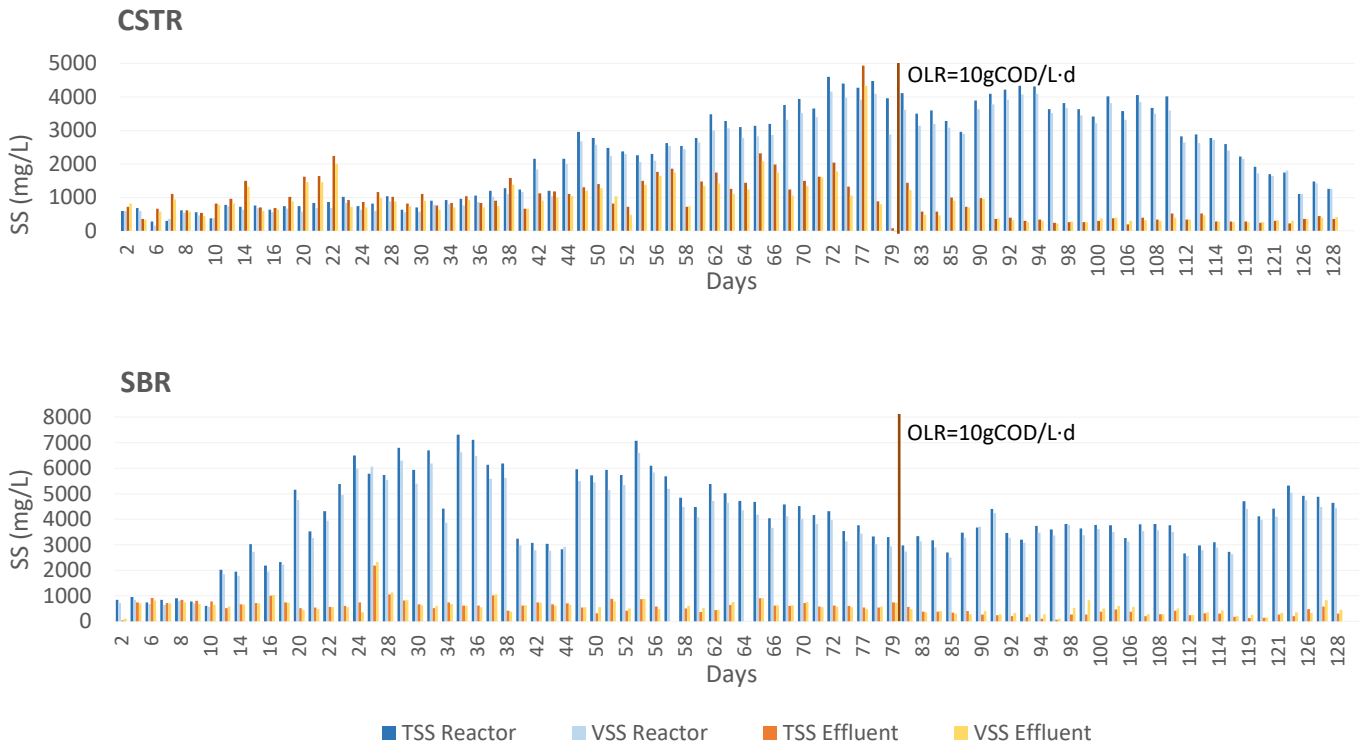


Figure 4d. SS of the reactor and effluent samples from the CSTR and SBR, during all the operation period.

Focusing on the effluent, a more constant pattern in the SBR is observed. VSS values of the SBR effluent under 0.10gVSS/L indicated that the effluent was clear and that the biomass presented good settling properties. During the last month of the experiment, some of the VSS values of the effluent were slightly higher than the TSS: this may result from the combination of improperly burned filters with very low SS concentrations and mass differences within a low order of magnitude. When looking at the CSTR, the effluent presented VSS concentrations higher than the VSS determined for the reactor. As stated above, this could derive from some mold growing in the surface of the settler and effluent tubes, which led to a change in the local of sampling. Between the 31st day and the change in OLR, the VSS values decrease to 57% of the VSS in the reactor. After

the change of the OLR, VSS of the effluent represented just 12.6% of the VSS in the CSTR, value very close to the 11.6% calculated for the same period in the SBR.

The biomass concentration can be given by pCOD as well as VSS. When plotting both variables against the operation days for each reactor (Figure 4e), a similar behavior was visible. This assured the reliability of the measurements. However, the CSTR presented a better correlation between both variables, shown by the higher correlation factor of the trend lines (Table 4b). As stated in section 3.1., the SS were used to monitor the biomass concentration instead of pCOD since the sample volume used was larger and the procedure is less prone to random errors.

Table 4b. Variables from the trend line equation calculated when pCOD from each reactor is plotted counter to the corresponding VSS. The slope is designated by m and r^2 is the correlation factor. Both graphics are included in Appendix D.

Variables	CSTR	SBR
m (gCOD/g)	3.206	2.837
r^2	5.130	5.512

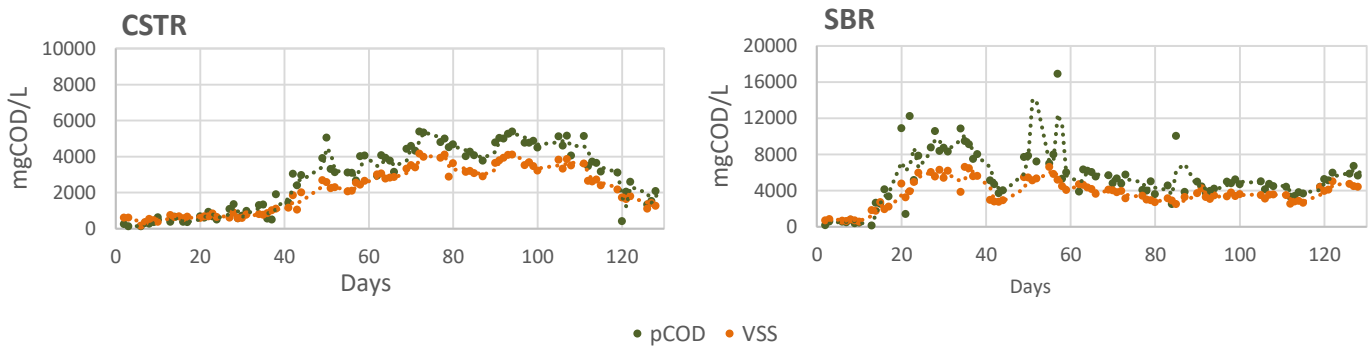


Figure 4e. Correlation between pCOD and VSS for each reactor, both parameters indicators of biomass concentration. Their similar evolution and proximity assured the reliability of the measurements.

Considering the COD and SS, is to highlight that both reactors presented a stable evolution under same operational parameters between days 73 and 80 for an OLR of 20gCOD/L·d and between days 85 and 113 for OLR of 10gCOD/L·d. During these time frames, a comparison of performance and fermentation profile based uniquely on a different operation mode is possible.

4.1.2 Biomass Settling Properties

The SVI was measured along the process in order to assess settling properties of the biomass from both reactors, with use of Equation 5. The reactors were inoculated at the beginning of the experiment with well-settling biomass and the SVI monitored along the 130 days (Figure 4f). For the CSTR, the SVI reached high values between days 12 and 37 of operation, in which no settled biomass could be observed in the graduated cylinder. However, for the majority of the process, the SVI was kept stable with low values. During the last 4 days of the experiment, the SVI oscillated due to a decrease in SS and some instability in the reactor. A stable behavior for the SBR was observed, with the biomass presenting low SVI values throughout the experiment, after just 12 days of culture stabilization. After adaption of the culture of both reactors, values of SVI under 50.0mL/g – an average of 34.3mL/g and 37.2mL/g for CSTR and SBR, respectively - is possible to conclude that both MMC presented good settling properties. Is to note that, after the 30min required in the SVI determination procedure, the supernatant of the SBR was always clearer than the one from the CSTR.

The granulated biomass with good settling properties obtain in this work, represents a major advantage over activated sludge. Activated sludge is often related with poor settling properties and floccular structures, requiring large reactor volumes to keep an appropriate biomass level to achieve the targeted removal efficiency. Moreover, constrains the range of applicable hydraulic loading that can be fed to the reactor and requires recirculation of settled sludge.[178] Thus, anaerobic processes can be suitable and preferred to perform some waste treatment.

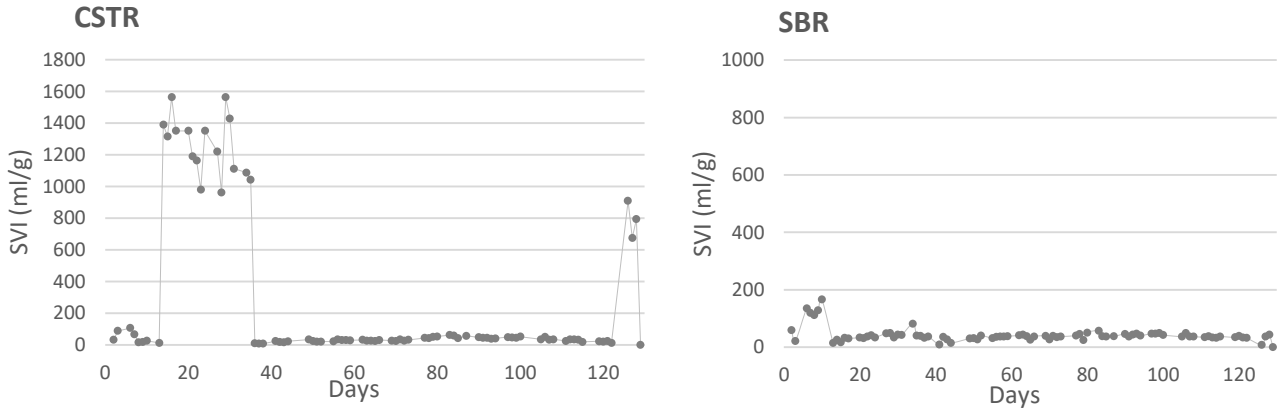


Figure 4f. SVI values measured along the entire experiment for the CSTR and SBR.

4.2 Fermentation Profile

4.2.1 Fermentation Products

Daily samples collected from the substrate and reactor were submitted to GC and HPLC analyzes in order to quantify the FP. In Figure 4h is possible to observe the concentration evolution of the main FP, namely EtOH, BuOH, HLa, HAc, HPr and HBu for both reactors, with addition of HiBu only detected in the CSTR. The concentration of the main FP is compared with the concentration of all FP detected and with the sCOD of the reactor samples, which include all FP plus the glucose that still available in the medium.

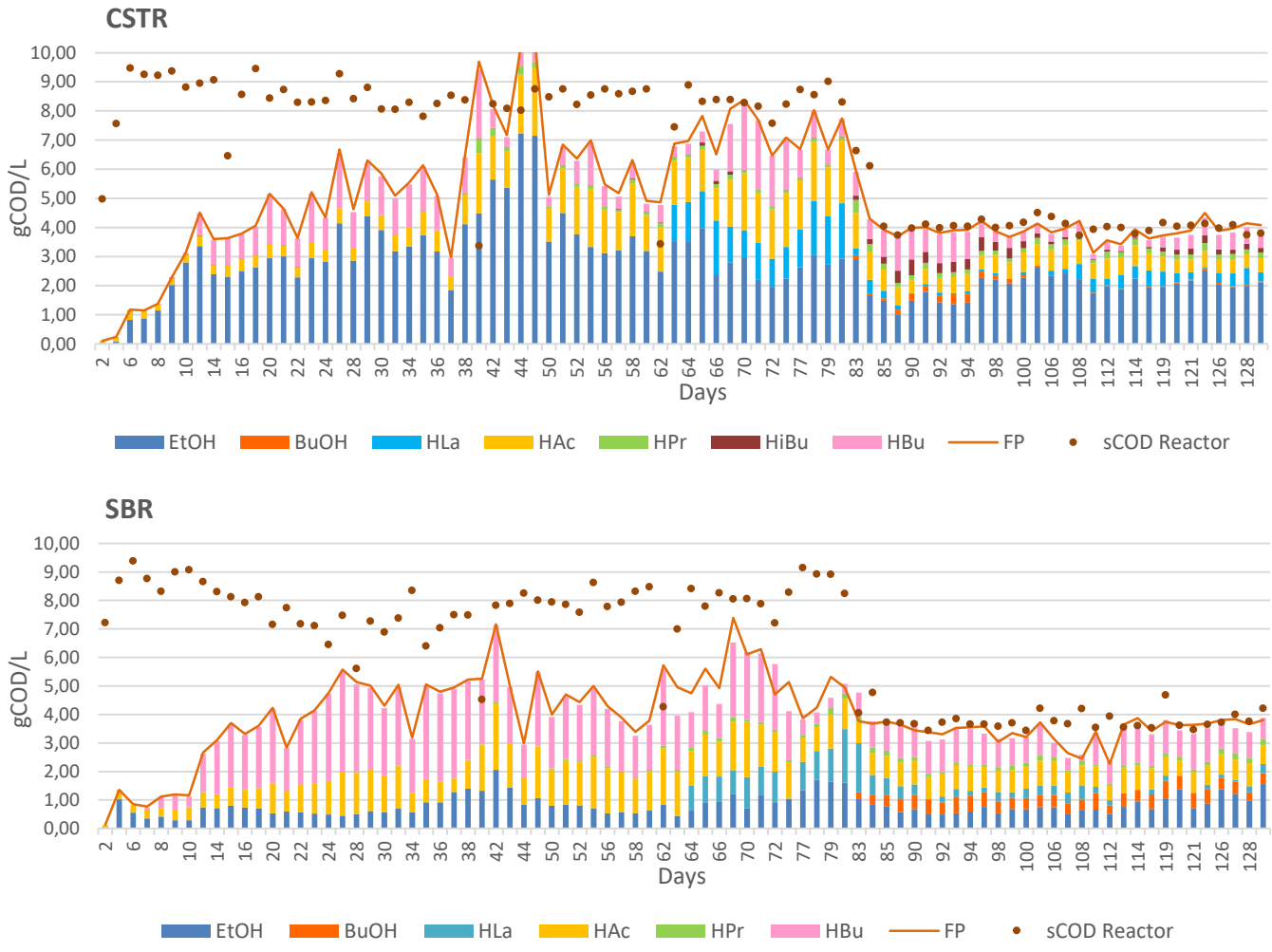


Figure 4g. Acidogenic fermentation profile, considering the main FP produced in each reactor, the concentration of all FP products detected and the sCOD available for conversion in the substrate.

For the CSTR, EtOH was the main FP throughout all the experiment. This can be related with a significant presence of bacteria prone to produce EtOH in the experimental conditions imposed, with contribution from a large population of yeast, observed in microscopy images (section 4.4). After EtOH, HAc and HBu were the FP more significantly produced, alternating their dominance. At the beginning of the experiment, no HBu was produced but a low concentration of HAc could be detected. After the 9th day of operation, HBu became the dominant acid and its concentration increased progressively along with the HAc. All the samples submitted to HPLC analysis for HLa quantification had this SCOA as sample component. The HLa presented a significant average concentration of 1.38gCOD/L with a standard deviation of 0.11gCOD/L until the change of the OLR, followed by a decrease to an average concentration of 0.12gCOD/L with a standard deviation of 0.11gCOD/L until the 108th day of operation. Afterwards, the HLa concentration increased slightly to an average value of 0.39gCOD/L with a standard deviation of 0.14gCOD/L for the remaining days of the experiment. HPr was also detected from the 41st day of operation, although in a small concentration of average 0.09gCOD/L with a standard deviation of 0.04gCOD/L. The HPr was present during the remaining time of the experiment and became slightly more significant after reducing the OLR, to concentration of average 0.12gCOD/L with a standard deviation of 0.04gCOD/L. Also after the change of the OLR, the HiBu emerges as a FP, overcoming the HPr concentration and equaling the HAc concentration; HiBU was detected in small concentrations from the 100th day of operation until the end of the experiment. However, there is no record that HiBu has been previously detected in similar processes. A similar behavior was observed for the BuOH, detected after the change in the OLR and present in an average concentration of 0.18gCOD/L with a standard deviation of 0.08gCOD/L until the 105th day of operation. The BuOH was again detected after the 114th day of operation in a lower average concentration of 0.03gCOD/L with a standard deviation of 0.01gCOD/L for the remaining days of operation.

In contrast to what was observed in the CSTR, for the SBR it was perceptible a dominance of HBu during great part of the experiment. Although EtOH and HAc are the main FP in the first days, as in the CSTR, after the 8th day of operation, HBu was present in greater concentrations in the SBR. The increase in HBu was followed by an increase of HAc and some EtOH. It can be assumed that the EtOH presented a constant average concentration, though oscillations along the operation. At day 73, a steep decrease in HBu concentration is observed, as well for the HAc, and a temporary dominance by EtOH. However, after changing the OLR to a half at the 80th day, the previous relative concentrations are restored. The HLa was also present in a significant concentration from day 64, with an average concentration of 1.12gCOD/L and a standard deviation of 0.32gCOD/L until the OLR

was changed. Afterwards, the HLa concentration decreased progressively until the end of the experiment. At the 62nd day, the presence of HPr was noticeable, keeping an average concentration of 0.13gCOD/L with a standard deviation of 0.05gCOD/L throughout the experiment. Similar to what was observed for the CSTR, BuOH was detected in the SBR after the decrease in the OLR at day 80. The BuOH concentration did not present many variations, with an average concentration of 0.45gCOD/L with a standard deviation of 0.08gCOD/L. However, the BuOH was a dominant FP, overcoming the HLa and HPr concentrations.

The samples from both reactors were submitted to GC analysis in order to determine the concentration of some expected FP along the experiment, as described in section 3.2.4. However, when considering just EtOH, HAc, HPr, HiBU and HBu as FP, both reactors were far from achieving full fermentation, i.e. conversion of the majority substrate into FP. Graphically, this difference could be observed by a gap between the dots representing the sCOD available for conversion and the line representing the sum of the FP which were present in the reactor. A few exceptions could be identified in the graphic, but the very low values of sCOD or unexpected high concentrations of FP in this cases indicated errors in the measurement as main reason for the abnormality. Thus, it was considered that other FP not being identified by the GC method could be present in the reactor medium in significantly concentrations and their quantification would allow for a more accurate conclusion on the fermentation potential.

Considering that the HLa is one of the simplest SCOA and largely reported to be produced by MMC fermentation[179 180], reactor samples from the 64th day on of operation were submitted to HPLC analysis for HLa quantification, as described in section 3.2.5. All samples from both reactors had HLa in their composition, revealed to be one of the main FP in most samples, as showed above.

By the analysis of the GC chromatograms, it was possible to observe, after the 83rd day of operation, a peak with an area comparable to the peaks' area from other FP identified (Figure 4h). The peak presented a retention time of 2.459min not included in the quantification. Based on the low retention time, it was suggested that it could be an alcohol with low vaporization temperature. Thus, several alcohols were analyzed – methanol, 1-propanol, 1-butanol and 2-butanol – and the 1-butanol (BuOH) presented a retention time of 2.435min, similar to the targeted time. A calibration curve for BuOH was included in all GC runs posterior to the identification, allowing for the BuOH quantification. BuOH from samples previously analyzed was quantified based in the correlation factor determined dividing the slope of the calibration curve for EtOH by the slope of the calibration

curve for BuOH. The conversion factor calculated – 0.1482 – was multiplied by the area of the BuOH peaks measured previously.

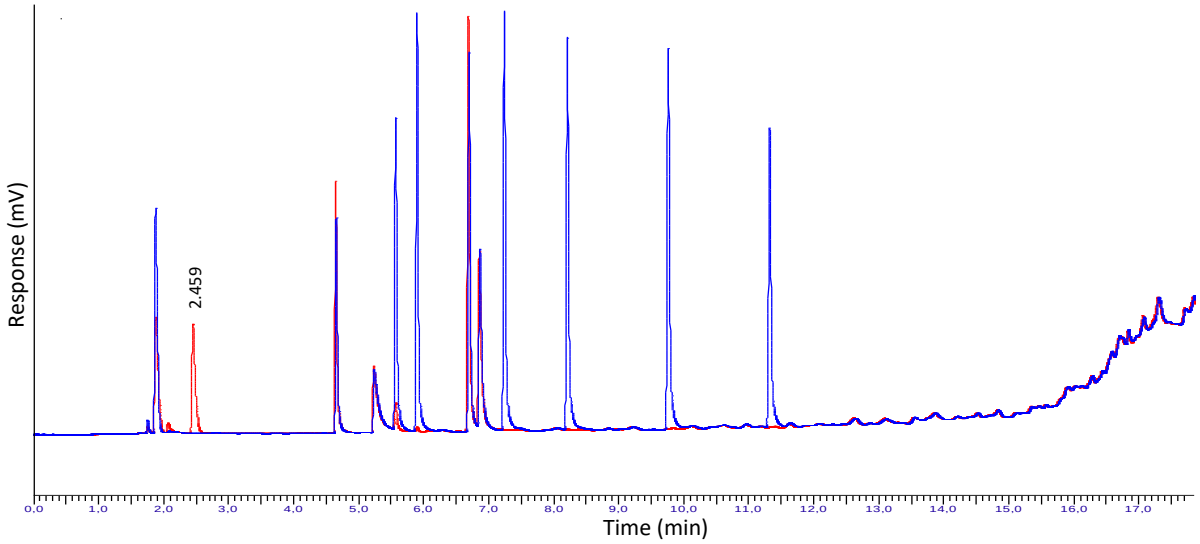


Figure 4h. Chromatogram from a reactor sample of the CSTR (red) plotted with the chromatogram from the standard solution (blue). A peak with retention time of 2.459min is detected in the sample, with no correspondent peak in the standard solution chromatogram.

After the quantification of HLa and BuOH and the inclusion of their concentration into the data analysis, it was possible to take different conclusions regarding the fermentation profile and potential. Considering that full fermentation is achieved when a conversion of substrate into FP is higher than 90% (Equation 12), both reactors reached a complete conversion of the influent COD. For the CSTR, a consistent full fermentation was achieved during the periods between days 85 and 98, from 100 to 108 and from 114 until the end of the experiment, with average conversion of 97%, 94% and 97%, respectively. The SBR also achieved full fermentation from day 87 to 97 and from day 113 on, with average conversions of 95% and 98%, respectively.

The differences between both reactors, considering the range of alcohols and SCOA identified and the concentration of the main FP, can be explained by a different composition of the MMC. The different types of microorganisms present in the culture will be responsible for the production of the several FP the organisms' relative abundance can be correlated with the relative concentrations of the FP. Since all the operational parameters are the same for both reactors, the selective pressure on the culture derives mainly from the reactor configuration and operation mode.

4.2.2 Consumption of NaOH

The NaOH solution consumption was also monitored during all the experiment. The concentration of the solution was progressively increased, from the initial concentration of 1M to 4M, as described in section 3.1.4. The increase of concentration was based in the volume of NaOH solution consumed and inference of the NaOH needed to maintain the pH value at 5 without adding a volume of solution that would interfere significantly with the reactor working volume.

For both reactors, it was possible to observe a correlation between the SCOA produced and the volume of NaOH solution consumed: the more SCOA are produced in mol per day, higher will be the volume of added base (Figure i). After the change of NaOH concentration to 4M, a period of instability was observed in both reactors. However, after 40 days, the SCOA production and NaOH solution consumption was kept constant for the CSTR while for the SBR a slight decrease was noticed. This point could also be connected to the change in the OLR at the 80th day of operation.

The base consumption is related with the fermentation evolution: if the MMC is performing a stable production of FP, namely acids that interfere with the pH value, the consumption of NaOH should also be stable. If a full fermentation is achieved with constant ratio of FP, the consumption of NaOH is also constant. The base consumption is further linked to the fermentation performance when comparing both reactors (section 4.2.3) and with the complementary cycle studies (section 4.3).

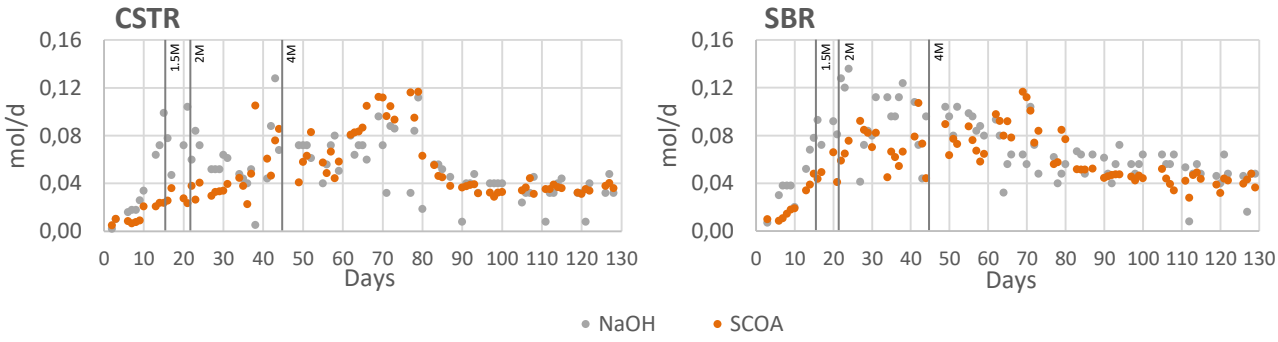


Figure 4i. Correlation between the consumption of NaOH and the SCOA produced daily, in the CSTR and the SBR. The vertical lines indicate the change in the concentration of the NaOH solution added.

4.2.3 Assessment of Fermentation Evolution and Potential

In order to answer the main question that leads this work, the reactors needed to present the same operational conditions, namely pH, temperature, HRT, OLR and SRT. As described in section 4.1.1, the former four parameters were kept constant in the predefined values along the entire experiment. Considering the SRT value, it was concluded that it was kept stable between days 64 and 80 for an OLR of 20gCOD/L·d and from day 100 to 114 with an OLR of 10gCOD/L·d. Thus, it was within these time frames that was possible to compare reactor performance based uniquely in operation mode.

As for the analysis of the fermentation profile during the entire experiment, when focusing on the time frames stated, it was also possible to identify differences in the range of FP synthetized and their relative concentrations between the two reactors configurations and even between the two different OLR (Figure 4j). For an OLR of 20gCOD/L·d, the CSTR presented EtOH as dominant FP while the SBR, although with a significant presence of EtOH, showed to promote the production of HAc and HBu. The HAc and HBu were the acids produced in higher concentrations in both reactors; however, the HAc overcomes the HBu concentration in the CSTR while the opposite was observed for the SBR. The presence of HLa and HPr was detected in both reactors, with similar average concentrations. During this period, a small concentration of HiBu was detected just in the CSTR, marking a difference in the range of FP produced in each reactor. After the change of the OLR to an half, none of the reactors presented differences in the relative concentrations of the FP. The EtOH still was the main FP product in the CSTR and the HAc and HBu the dominant products in the SBR. The HAc and HBu also presented similar relative concentrations for the OLR of 10gCOD/L·d with higher concentrations of HAc in the CSTR and of HBu in the SBR. The HLa was also synthesized in significant concentrations in both reactors, with similar relative concentrations to the ones registered for the OLR of 20gCOD/L·d. When focusing on the HPr, its concentration slightly increased for the lower OLR in the CSTR and slightly decrease in the case of the SBR. Thus, the HAc relative concentration increased with the decrease in the OLR. For the OLR of 10gCOD/L·d, the range of FP products was the same for both reactors, although different from the one observed in the first time frame. No HiBu was detected in the CSTR nor in the SBR. However, BuOH was detected in both reactors: the CSTR presented a low concentration of BuOH, while in the SBR the BuOH concentration overcame the concentration of HLa and HPr.

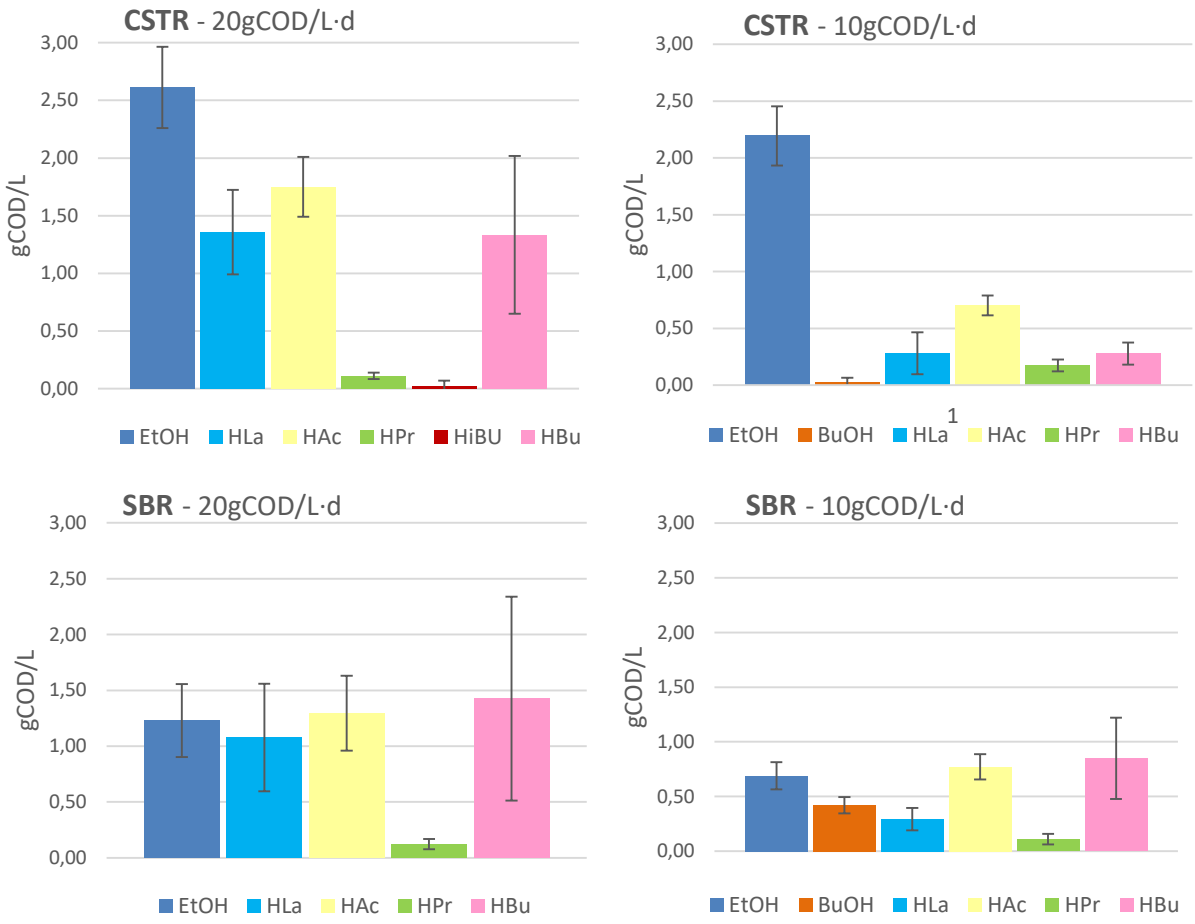


Figure 4j. Graphic representation of the main FP produced in the CSTR and SBR and for the two OLR studied, during the periods of time in which both reactors had the same SRT. The range and average concentration of the FP is represented as well as the respective standard deviations.

Besides the analysis of the FP, some parameters were calculated, namely Y_{FP}, Y_{SCOA}, $CONV_{FP}$ and $CONV_{SCOA}$ (Table 4c). These parameters allow to infer about the MMC capacity of converting the available substrate into all the FP or specifically into SCOA. The Y_{FP} calculated for the CSTR, as well as the $CONV_{FP}$, were higher than the same parameters calculated for the SBR in both of the OLR used in the experiment. The same could be observed for the Y_{SCOA}, higher for the CSTR than for the SBR. When focusing on the $CONV_{SCOA}$, a different relation is observed, depending on the OLR. For an OLR of 20gCOD/L·d, the CSTR presented a higher conversion of the substrate into SCOA, while for an OLR of 10gCOD/L·d the SBR presented a higher $CONV_{SCOA}$. Considering the effect of the change of the OLR in the parameters stated, Y_{FP} and Y_{SCOA} decreased in both reactors with the lower OLR, while the $CONV_{FP}$ and $CONV_{SCOA}$ increased with the decrease of the OLR, except for the

$CONV_{SCOA}$ of the CSTR. Thus, it was possible to conclude that the lower OLR was more suitable if the production of SCOA is targeted. For the lower OLR, a SBR configuration can be preferred since the conversion of the substrate into SCOA was higher.

Table 4c. Parameters calculated for each reactor, namely Y_{FP}, Y_{SCOA}, and conversion of the substrate into all FP or specifically into SCOA, which enable to infer about the potential and efficiency in production of FP of interest; standard deviation (σ) is also represented.

OLR gCOD/L·d	**CSTR** (gCOD/gCOD)								**SBR** (gCOD/gCOD)							
	Y_{FP}	σ	Conv $_{FP}$	σ	Y_{SCOA}	σ	Conv $_{SCOA}$	σ	Y_{FP}	σ	Conv $_{FP}$	σ	Y_{SCOA}	σ	Conv $_{SCOA}$	σ
20	0.877	0.07	0.875	0.09	0.826	0.11	0.567	0.08	0.788	0.07	0.637	0.14	0.660	0.07	0.365	0.17
10	0.834	0.04	0.916	0.07	0.671	0.05	0.380	0.05	0.732	0.05	0.844	0.16	0.604	0.08	0.486	0.12

It was concluded that, for the same operational conditions, a continuous reactor configuration results in a different range of FP with different relative concentrations than a batch reactor. The acidogenic fermentation was carried in both reactors, although an alcoholic fermentation was also performed in the SBR and more significantly in the CSTR. While the CSTR promotes the activity of HAc-producing bacteria, the SBR has an MMC enriched in HBu-producing bacteria.

Is important to highlight that other variables should be considered when a SCOA production process is designed and the reactor configuration defined, namely ease of process control, the type of FP aimed to produce and also properties of the MMC. A wider time frame where both reactors have the same SRT could complement the conclusions made and also enable more accurate predictions about the fermentation potential. More similar experiments should be performed in order to support and better understand the conclusions outlined.

4.3 Cycle Studies

4.3.1 Cycle Studies of the SBR

Since samples from the SBR were taken daily, right before the settling and withdraw phases, it was not possible to assess what happens during a cycle of 6h and which alterations are induced by the feeding phase. Due to that, 3 cycle studies were performed at the 43rd, 55th and 62nd days of

operation – named C1, C2 and C3, respectively - in which samples were taken periodically (as described in section 3.1.4) and analyzed for pH, sCOD, SS and FP composition determination.

The pH was kept constant at 5.5, value previously established. The evolution of sCOD was followed during the 6 hours of the cycle (Figure 4k, plot 1). All cycles showed the same sCOD value evolution: an increase during the first minutes of the cycle, followed by a slight decrease until reaching a constant sCOD concentration after approximately 3h. The initial increase was due to the feeding phase, corresponding to the first 15min of the cycle. The following short decrease resulted from some biomass growth and probably COD losses from oxygen leakage or reduction reactions. The last phase of sCOD consistency was assumed to result from the direct conversion of the sCOD available into FP, also counting for the sCOD in the reactor.

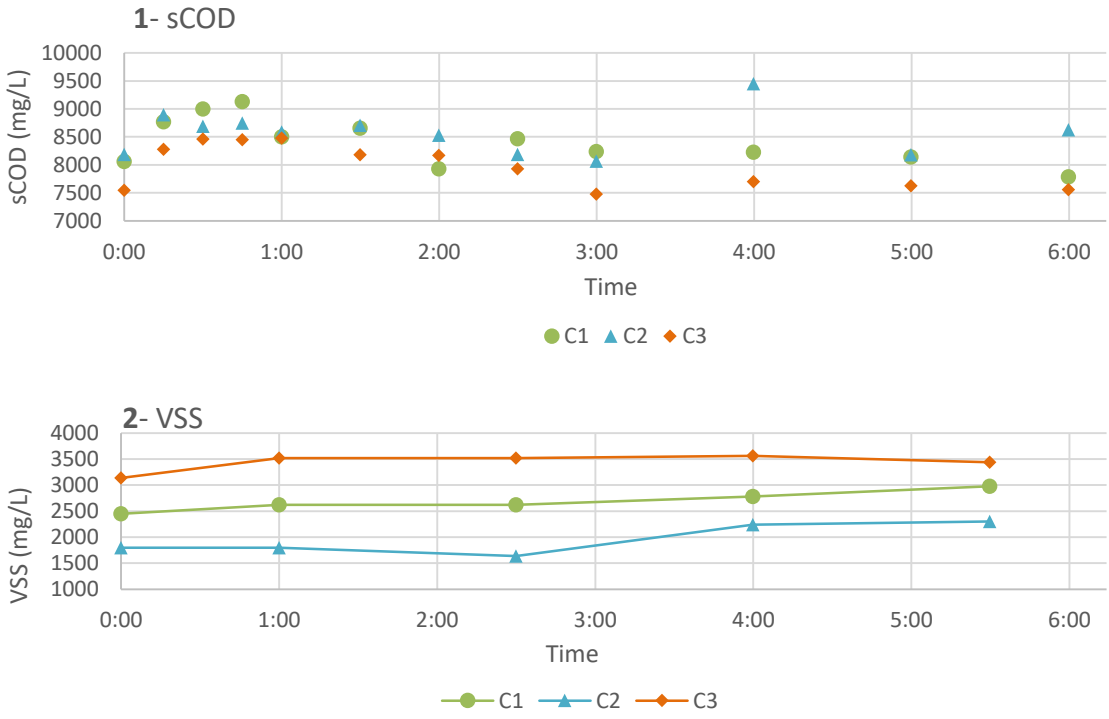


Figure 4k. Evolution of (1) sCOD and (2) VSS during a complete cycle of 6h for the three SBR cycle studies, C1, C2 and C3, performed at the 43rd, 55th and 62nd days of operation, respectively.

The measured VSS showed the same evolution along the cycle, although the biomass concentration differed between the cycles (Figure 4k, plot 2). All VSS values were normalized to a work volume of 1L. Although some differences in the VSS evolution along the 6h in each cycle study, all experiments presented a final VSS higher than the initial one, with an increase of approximately 500mg/L. Thus, it was possible to conclude that biomass growth happens at each cycle. Differences

in initial biomass concentration could be justified by oscillations in the settling properties of the biomass during the settling phase.

The main FP detected were EtOH, HAc, HPr and HBu and their concentration was followed along each cycle (Figure 4l).

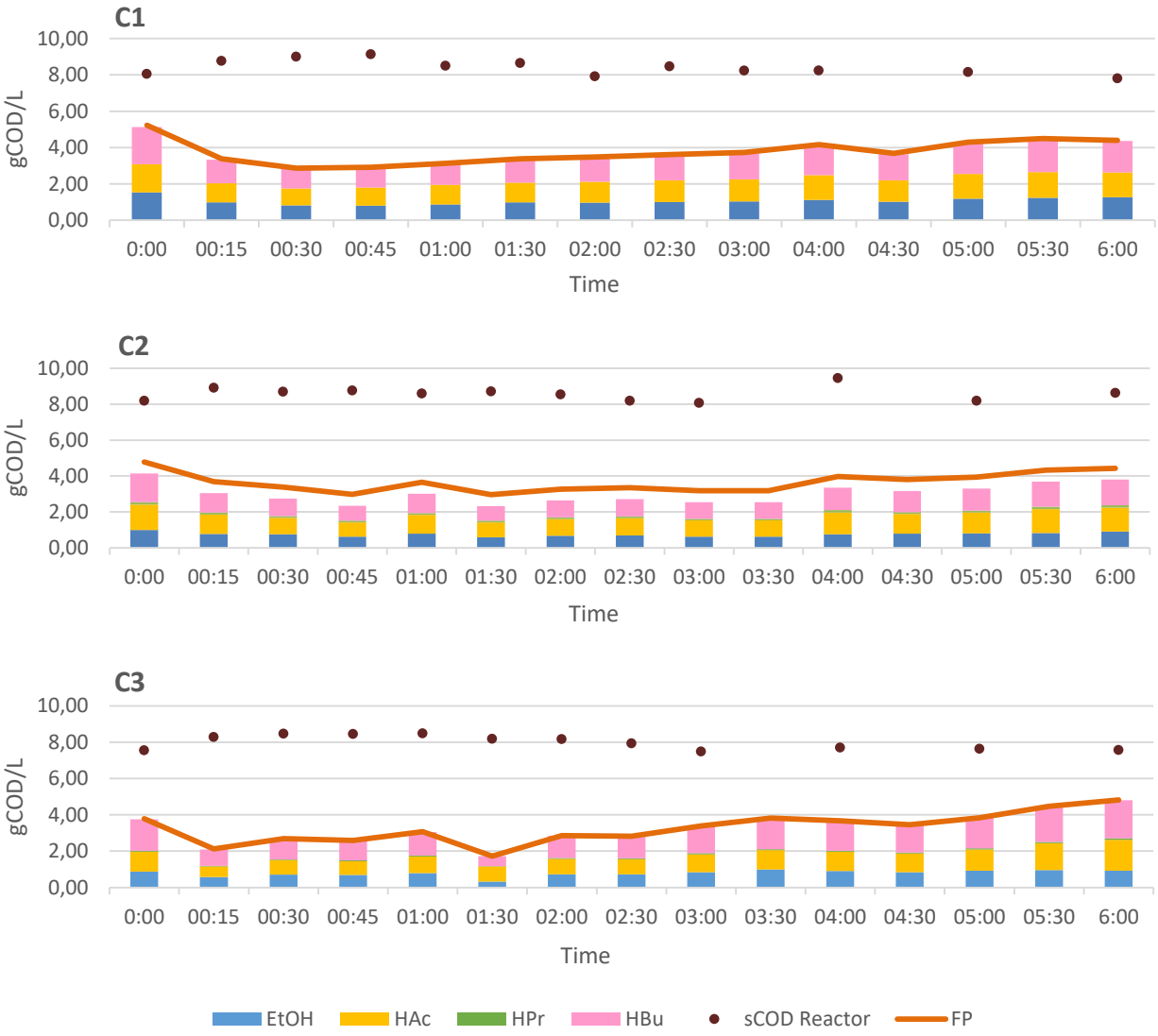


Figure 4l. Evolution of the FP concentration, with highlight for the main FP detected, namely EtOH, HAc, HPr and HBu, and correlation with the measured sCOD.

In the beginning of all cycles studied, a decrease was observed in the total FP concentration, due to the feeding phase and dilution of the reactor medium. After this initial phase, all cycles showed an increase in the FP concentration along the 6h. Some exceptions could be noticed, but is assumed they derive from random errors of the procedure or slips in the analytical measurements.

In all cycles, HBu was the dominant fermentation product, followed by the HAc, consistent with what was observed for the entire experiment, as described in section 4.2.3. HPr was detected in a very low concentration in C2 and C3, barely contributing for the overall SCOA production. EtOH was always detected in a significant concentration, although never surpassing the concentration of HBu or HAc. These four FP were the dominant FP in cycles C1 and C3, since the line representing the sum of all FP meets the stacked bars. However, in C2, a gap between the line and stacked columns showed that other FP, such as other SCOA or alcohols, might be present in a significant concentration. Is to note that no samples from the SBR cycles studies were submitted to HPLC analysis for HLa measurement. Another relevant observation was that none of the cycles achieved a full conversion of the substrate. The plot analysis revealed that the FP just corresponded to less than 50% of the sCOD, distant from a full fermentation. This conclusion is consistent with what was observed for the correspondent days in the overall fermentation profile (section 4.2.1). Thus, a reduction in the OLR to an half was considered suitable to promote the reactor to meet its full potential and achieve a conversion of the substrate into FP higher than 90%.

For each cycle, several parameters were calculated, namely the initial SCOA concentration before feeding (T_{SCOAin}), total SCOA at the end of the cycle ($T_{SCOAout}$), Y_{SCOA}, $CONV_{SCOA}$, the VSS in the effluent and the volume of 4M NaOH solution dosed into the reactor, in order to quantify the fermentation potential (Table 4d).

Table 4d. Parameters determined for the cycle studies C1, C2 and C3, namely Y_{SCOA} based in the initial and final SCOA concentrations, conversion of the sCOD, VSS in the effluent and NaOH consumed. The determination was performed as described in section 3.3.

	$T_{SCOA\ in}$ gCOD/L	$T_{SCOA\ out}$ gCOD/L	Y_{SCOA}	$CONV_{SCOA}$	VSS_E (g/L)	NaOH (mL)
C1	2.397	3.132	0.429	0.402	0.34	10.0
C2	2.915	3.529	0.69	0.409	1.90	6.00
C3	1.537	3.880	0.765	0.513	1.24	8.00

The Y_{SCOA} increased at each cycle, which can indicate an improvement along the experiment when considering the production of SCOA. However, the conversion of the sCOD is always near 50%. The volume of NaOH solution dosed into the reactor during the cycle is also related with the SCOA produced: the higher concentration of SCOA in the reactor, higher will be the NaOH volume

added in order to compensate the decrease of the pH value. Is also to highlight that the low VSS concentration in the effluent indicated the good settling properties of the biomass.

4.3.2 Study of 24h of Operation

At the 112th day of operation, a cycle study for both reactors was performed simultaneously. The feeding was suppressed for 24h in order to assess if full fermentation could be achieved and to have an overview on the fermentation profile. As for the SBR cycle studies, NaOH consumption, sCOD, SS and FP were determined. The pH value was kept constant for both reactors along the 24h.

The sCOD evolution was followed along the 24h (Figure 4m). For the CSTR, a decrease in sCOD was observed during the first 3h of the cycle, followed by an increasing of 0.76gCOD/L and kept constant from the 5h for the remaining time. With further regard on the FP, sCOD was expected to stay constant for the entire cycle. It was considered that the decrease in sCOD could be due to the storage of polymers inside the cells. With the synthesis of storage compounds, less sCOD is available in the medium. Posteriorly, these storage compounds could be consumed by the cells and the byproducts released in the medium, causing the increase of the reactor sCOD. A similar situation was observed by Tamis et al[132], who considered that a niche from the MMC could perform polyglucose storage as strategy for rapid glucose uptake. The anaerobic storage of glucose was also reported before by Shimada et al.[181]

When focusing on the SBR, an initial increase in sCOD resulted from the feeding phase. The same was observed for the shorter cycle studies: a slight decrease followed by a constant sCOD value. At the end of the 24h, the sCOD value meets the initial values after the feeding phase, indicating a conservation of COD entering the reactor and its full conversion into fermentation products, as described later. When looking at the VSS, both reactors showed the same biomass progress: an increase in biomass concentration for the first 4h, followed by a decrease until the end of the cycle. Since the feeding was suppressed for 24h, biomass will present some growth at the beginning of the study but the biomass concentration will eventually stabilize after no more substrate is available in the medium.

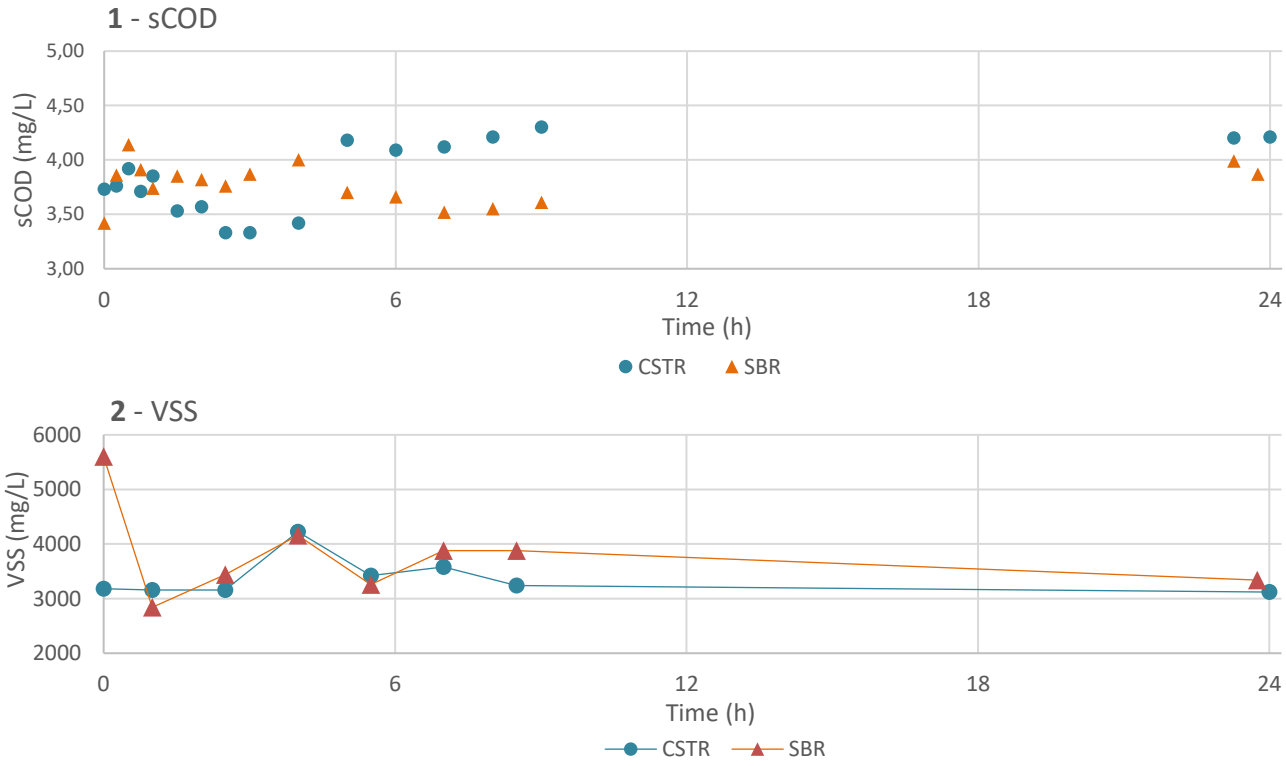


Figure 4m. Evolution of (1) sCOD and (2) VSS for the 24h cycle study for the CSTR and SBR, with feeding suppression, performed at the 112th day of operation.

For the fermentation profile analysis, a similar approach to the SBR 6h cycle studies was made, and the relative concentration of the main FP, the total concentration of the FP detected and the sCOD available were followed (Figure 4n). Focusing in the CSTR, it was possible to conclude that the main FP were EtOH, HLa, HAc, HPr and HBu, with dominance by EtOH and by HAc among the acids. By analysis of the plot referring to the CSTR, it was observed that a high percentage of sCOD is converted into FP and that full fermentation is achieved. Some outlying sCOD values could have resulted from procedure errors and might not indicate precisely what is occurring in the reactor. When looking at the SBR fermentation profile, it was possible to observe the same main FP plus the BuOH, however with the HAc concentration surpassing the EtOH concentration. In the SBR, it was also possible to observe that the other acids quantified by GC besides HAc, HPr and HBu contributed significantly to the overall FP, since stacked columns do not meet the FP line (plot referring to the SBR), opposite to what was observed for the CSTR. As for the CSTR, it was possible to conclude that the SBR achieved full fermentation, which was not observed for the shorter cycle studies with a higher OLR. For both reactors, the proportion between the concentrations of the main FP in each reactor was kept constant along the 24h. However, the range of FP was different

between the reactors since BuOH was detected for the SBR but not for the CSTR. The range of FP for each reactor and the relative concentrations of the main FP observed during the 24h cycle study was consistent with what was observed for the correspondent time frame of the overall experiment (section 4.2.1).

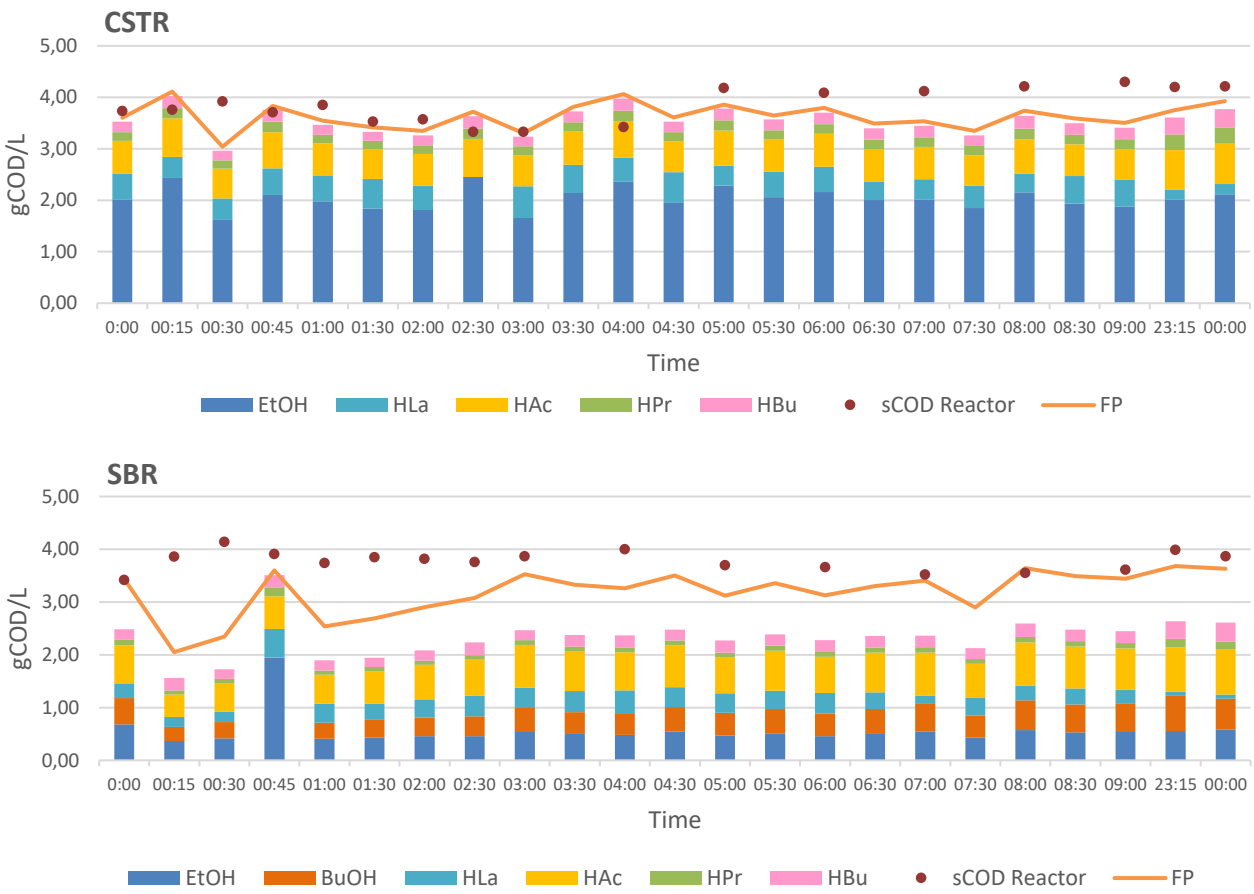


Figure 4n. Evolution of the concentration of the FP detected, with highlight for the dominant FP, and correlation with the measured sCOD.

The Y_{SCOA} and $CONV_{SCOA}$ were calculated and the VSS and 4M NaOH solution consumption monitored (Table 4e). The Y_{SCOA} determined for the CSTR was superior to the Y_{SCOA} calculated for the SBR. However, the $CONV_{SCOA}$ calculated for the SBR was higher than the one calculated for the CSTR. This correlation is consistent with what was observed by the calculations during the stable SRT for an OLR of 10gCOD/L·d: the CSTR presented a higher Y_{SCOA} but a lower $CONV_{SCOA}$. Moreover, the Y_{SCOA} and $CONV_{SCOA}$ calculated for the 24h cycle study are very close to the same parameters calculated for the overall time frame for the lower OLR (Table 4c). This correlation attests the

accuracy of the measurements and the stability of the fermentation during the time frames selected with a stable SRT.

Table 4e. Parameters calculated for the CSTR and SBR during the 24h cycle study with feeding suppression, namely Y_{SCOA} based in the initial and final SCOA concentrations, $CONV_{SCOA}$, VSS in the effluent and 4M NaOH solution consumed. The calculation followed the equations in section 3.3.

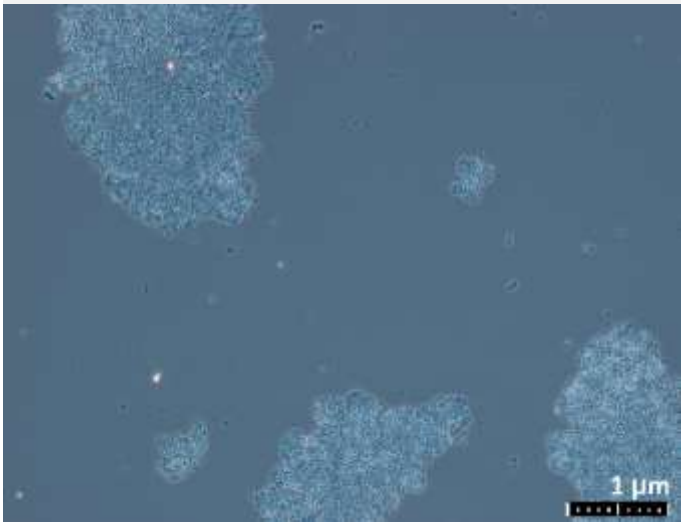
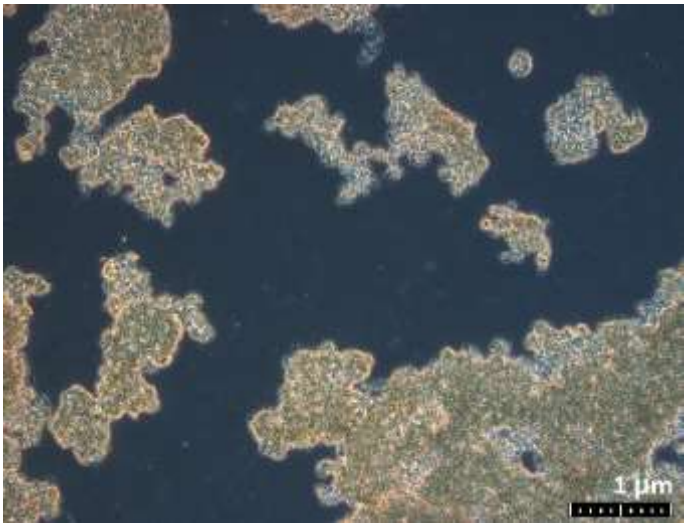
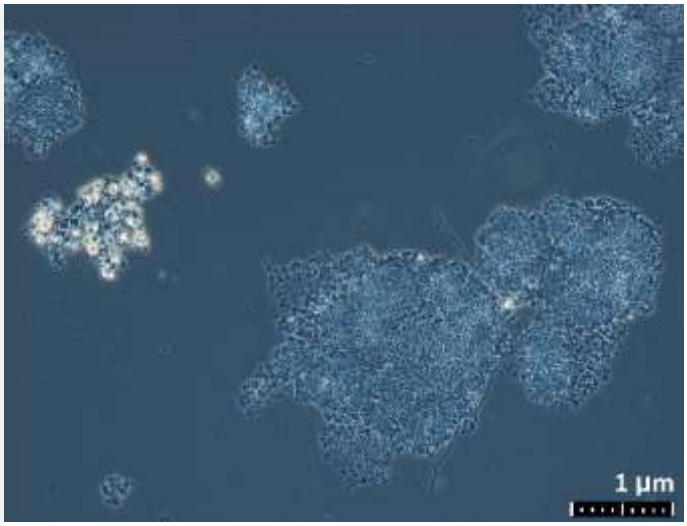
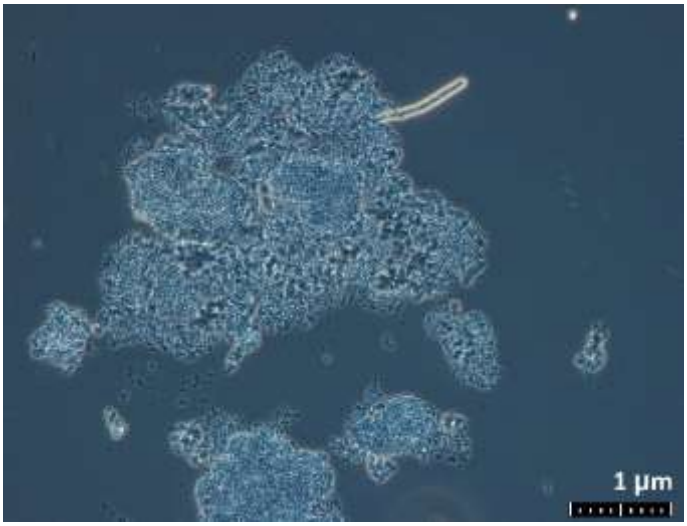
	$T_{SCOA\ in}$ gCOD/L	$T_{SCOA\ out}$ gCOD/L	Y_{SCOA}	$CONV_{SCOA}$	VSS_E (g/L)	NaOH (mL)
CSTR	0.460	1.811	0.734	0.385	0.40	4.00
SBR	0.069	2.462	0.700	0.507	0.20	4.00

From the 24h cycle study it was possible to conclude that the CSTR was running already in full fermentation. The SBR, after the initial feeding phase, performed fermentation during the first 3h; afterwards, full fermentation is achieved. Major differences between the reactors included the FP range and relative concentrations of each of the main FP. In the case of the SBR, other SCOA determined by GC also had a significant contribution for the overall FP concentration. Is also to highlight that conclusions and parameters calculated for the 24h cycle study are consistent with the observations from section 4.2.

4.4 Microscopic characterization of Biomass

A subjective microscopic characterization of the biomass was performed periodically, in order to characterize the cultures morphologically and correlate the observations with the conclusions made about the FP produced and settling properties of the biomass. The images from the selected samples for microscopic observation (as described in section 3.1.5), were captured and posteriorly analyzed (Table 4f). The characterization was made based on a scale ranging from 0 to 5 (Table 4h). Table 4g gathers the main parameters considered for the culture characterization during the experiment, allowing for an overview on the structures and differences observed as well as the evolution of the considered parameters.

Table 4f. Microscopy images from biomass of both reactors operated, obtain as described in section 3.1.5. All images selected have a magnification of 10x200.

	CSTR	SBR
28th day		
69th day		
87th day		

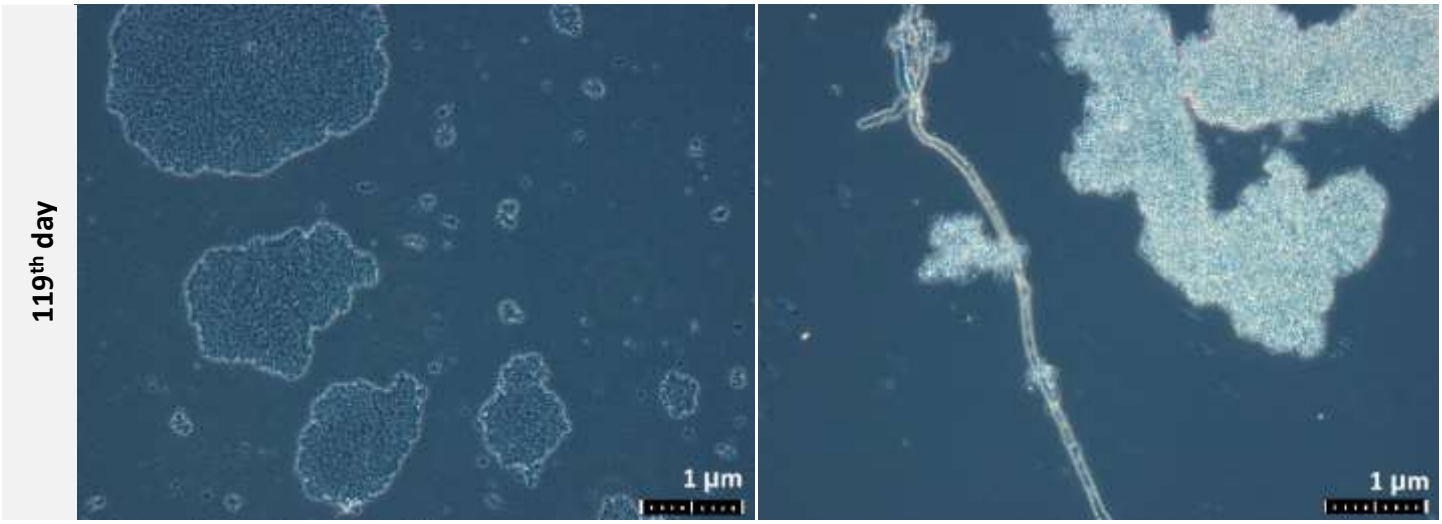


Table 4h. Characterization of the biomass observed at the microscope, from the CSTR and SBR, considering flocks morphology and presence of bacteria and fungi.

	CSTR										
	Flocks						Bacteria		Fungi		SVI
	Abund.	Round	Irregular	Amorph	Compact.	Size (µm)	Free	Filam.	Yeast	Mold	
27	1		x		1	5,0	5	3	0	1	961,5
68	3		x		3	20,0	4	0	4	2	26,6
70	3		x		2	15,0	4	0	3	0	35,5
72	4			x	4	20,0	4	0	2	0	31,8
76	4		x		4	25,0	4	0	2	0	44,4
78	3		x		2	20,0	4	0	2	0	50,5
82	3	x			2	50,0	3	0	3	0	62,9
84	4	x			3	30,0	3	1	3	0	42,7
86	4	x			3	5,0	2	3	3	1	57,4
89	4	x			3	25,0	3	2	2	0	48,7
91	4	x			3	40,0	3	2	2	2	45,0
93	4		x		3	20,0	4	3	3	2	41,7
96	3		x		2	20,0	3	3	2	2	49,5
98	3		x		3	20,0	4	2	2	2	44,0
104	4		x		3	15,0	4	1	3	1	34,8
107	3	x			4	35,0	4	2	1	2	35,3
110	4		x		4	30,0	4	3	3	3	24,9
112	4	x			3	15,0	3	0	2	1	34,7
114	3		x		3	10,0	4	0	1	3	19,2
118	4		x		3	20,0	4	0	0	2	22,5
121	3	x			3	15,0	4	1	0	0	11,5

	SBR										
	Flocks						Bacteria		Fungi		SVI
	Abund.	Round	Irregular	Amorph	Compact.	Size (μm)	Free	Filam.	Yeast	Mold	
27	4		x		3	30,0	4	0	0	1	48,8
68	4			x	5	50,0	4	0	0	1	39,3
76	3		x		4	20,0	3	0	0	2	39,9
78	4	x			3	35,0	3	0	0	3	24,2
82	4		x		3	100,0	3	0	0	3	56,9
84	4		x		4	25,0	4	0	0	3	37,0
86	4		x		4	45,0	3	0	0	3	37,4
89	4	x			4	40,0	3	0	0	1	46,2
91	4	x			5	80,0	2	0	0	2	43,4
93	4			x	4	40,0	2	0	0	3	40,1
96	4			x	4	40,0	3	0	0	3	47,2
98	4			x	4	30,0	3	0	0	2	44,0
104	4			x	4	30,0	4	0	0	4	37,2
107	4			x	4	15,0	4	1	1	1	36,6
110	3			x	4	30,0	3	0	0	3	34,6
112	3		x		4	35,0	2	0	0	3	33,6
114	3		x		4	25,0	3	0	0	1	58,8
118	4		x		4	35,0	2	0	0	2	34,0
121	4			x	4	40,0	2	0	0	3	7,5

Table 4g. Scale established for semi-quantitative characterization of the cultures from each reactor for the parameters stated, ranging from 0 to 5, based on Jenkins et al.[176]

Scale	0	1	2	3	4	5
	None	Few	Some	Common	Abundant	Dominant
	Negligible	Very Low	Low	Medium	High	Very High

The main differences between the reactors feature the properties of the flocks and the presence of different types of organisms in each reactor. The biomass flocks from the SBR were consistently bigger, more compact and also more abundant. This agrees with the lower SVI values for the SBR, since flocks of greater dimension and compactness will present better settling properties. This can also be verified by a clearer supernatant in the cylinder during SVI procedure and the fewer free bacteria observed. However, and as discussed in section 4.1.3, it can be considered that the MMC from both reactors present good settling properties. In the CSTR, bacteria were observed not only in flocks and free in the medium but also in filaments (Table 4g). No bacterial filaments were observed in the SBR.

When considering fungi, major differences were observed between the two reactors. During a large period of the operation time, it was possible to observe the presence of yeast, morphologically very similar to *Saccharomyces cerevisiae* (Appendix G). In some of the observations, the yeast population was dominant over the bacterial one; however, its presence decreased progressively and by the 116th day of operation no yeasts were observed. As discussed above, the CSTR presented a high concentration of EtOH during the overall operation time. It was suggested that the presence of yeast could be related with the measured concentrations of EtOH. However, when plotting yeast abundance along with the evolution of EtOH concentration, no direct correlation could be observed (Figure 4o). Although the yeast population could have contributed for the overall EtOH concentration, other organisms such as bacteria may have a more significant role in the EtOH synthesis. In some occasions, a few filamentous fungi were also observed in the CSTR. In reverse, no yeasts were ever observed in the SBR while filamentous fungus were detected throughout all observations. These filaments were never dominant over bacteria and appeared frequently associated with the flocks and bigger aggregates.

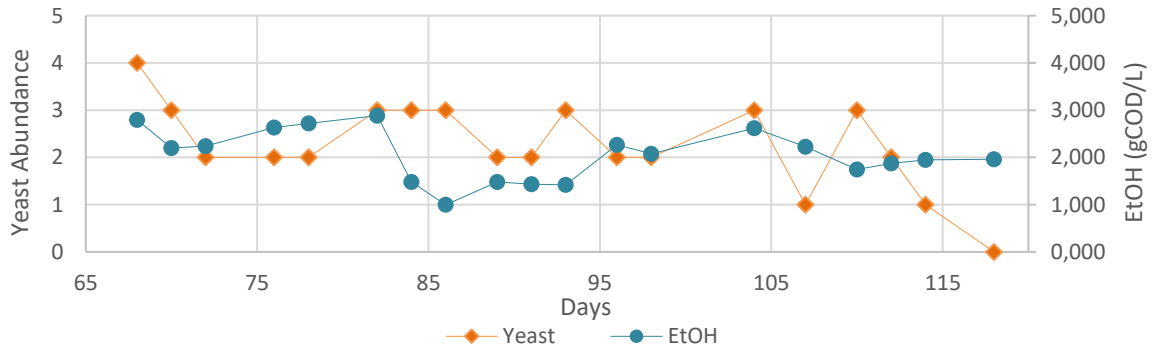


Figure 4o. Plot of the EtOH concentration measured by GC and the abundance of yeast determined by microscopic analysis. No direct correlation between the two variables was observed.

Differences between operation modes were also attested by the microscopic observations: different types of organisms were present in each reactor and respective biomasses were morphologically dissimilar. Since the MMC is directly related with the FP produced, the microscopic observations are consistent with the different FP range and relative abundance observed for each of the studied reactors. Different MMC will perform acidogenesis with different efficiency and the cultures will develop differently.

4.5 Extraction of Polyhydroxyalkanoates

4.5.1 Accumulation of Polyhydroxyalkanoates

As pure culture, the *C. necator* DSM 545 was selected since it is well known by its capacity to produce and accumulate PHA and well described in literature for this purpose.[182] [183] For the accumulation of PHA by *C. necator*, a pre-inoculum in LB medium was used. Since the LB medium is a rich medium, the *C. necator* culture was enabled to rapidly grow and achieve high cell density. The culture was monitored by taking samples each hour and measuring the absorbance of the cell suspension. By plotting the absorbance along time, it was possible to follow the culture growth. When a steep slope was observed, it indicated that the culture was in the exponential phase, in which the bacterial growth has the highest rate and the culture is better adapted to the medium. At this point, a certain volume of the LB culture (Equation 14) is used to inoculate the defined medium. In the defined medium, the culture presented a longer lag phase due to the need of adaption to the new environment composition. The defined medium presented a nutrient limitation, namely in nitrogen, in order to subject the cell to an adverse environment and promote the PHA synthesis and accumulation. Similar to the LB culture, the culture growth of the inoculated defined medium was monitored by spectrophotometry. However, in this case it was intended that the culture achieved the stationary phase, when the PHA storage was maximum. At this point, the culture was stopped by centrifugation of the medium, washing the remaining medium, freezing at -80°C and posterior lyophilization of the biomass, as described in section 3.4.1.

Several cultures were grown for PHA accumulation along the experiment. Although the initial cultures accumulated the biopolymer, confirmed by standard chloroform extraction and by GC analysis, at some point of the experimental work, the cultures did not store any PHA. It was suggested that a strain degeneration had occurred and, more likely, that some components of the trace elements solution or the defined medium suffered any alteration during storage of the mediums due to pH change, light incidence or procedure systematic errors or even in their package.

Thus, biomass from eight cultures were used for further extraction experiments, since PHA storage was confirmed initially by chloroform extraction and then quantified by GC analysis (Table 4i).

Table 4i. Percentage of PHB stored by the *C. necator* cultures under nutrient limitation conditions, determined by GC analysis.

***C. necator* culture**	**A**	**B**	**C**	**D**	**F**	**G**	**H**	**I**
% PHB	25	34	23	38	40	28	32	17

The MMC samples used had been already submitted to conditions favoring the polymer accumulation. Thus, it was just needed to collect them, centrifuge and properly wash the biomass since hardwood sulphite spent liquor is a dark and complex waste containing several inhibitory and toxic compounds. Afterwards, the culture was frozen at -80°C and lyophilized. Three different MMC samples were used for application of the extraction procedure – however, the polymer content quantification was not made by GC analysis and just by the standard chloroform procedure, as described in section 3.4.6 (Table 4j).

Table 4j. Percentage of PHA stored by the *MMC*, determined by chloroform extraction.

MMC	**A**	**B**	**C**
% PHA	47	41	62

4.5.2 Enzymatic Solution from *P. variotii*

P. variotii is a filamentous fungus extensively reported in literature due to its ability to produce a wide range of enzymes, such as tannases, phytases, celullases and amylases, and its suitability for numerous biotechnological applications and potential to be explored as source of industrial products.[170] Due to its catalytic efficiency and diversity, it was selected to produce the enzymatic solution applied in the extraction work described later.

One culture of *P. variotii* was grown and submitted to hydrolysis by ultrasounds to release the intracellular enzymes, as described in section 3.4.4. Small periods of lysis were programmed in order to avoid high temperatures that could damage and disable the proteins. The final enzymatic solution also contained intracellular enzymes and some residues from the fungus and components of the buffer solution (from which SDS can be advantageous for the PHA extraction since it contributes to the cell disruption as surfactant). The Biuret Test was applied in order to quantify the protein content of the enzymatic solution. Based in the method described in section 3.4.4, the protein concentration was 3.14g/L. However, this value is merely indicative since it measures not

only the proteins with enzymatic activity – which are the significant ones for the extraction work – but all other constitutive proteins of the fungus. The enzymatic hydrolysate was stored at 4°C and the remaining fungus biomass stored at -4°C for possible use in future works.

4.5.3 Development of the Extraction Procedure

The work aimed the development of an extraction procedure since little information is available considering PHA extraction with the extractive agents used. Numerous preliminary assays were conducted in order to conceive an extraction procedure that could answer to the efficiency, reproducibility and sustainability aimed in this work.

First, it was considered the cell lysis when the phospholipidic layers that compose the cellular membranes are disrupted and allow for the release of the PHA granules to the medium, which was performed mixing 0.8g of lyophilized biomass with 5.0mL of the extraction agent. The time selected for further experimental work was 24h since it allows an interaction between biomass and extractive agent for a long period and is the time used in the conventional extraction procedures with chloroform and sodium hypochlorite. In preliminary essays performed, after 24h with stirring, it was possible to observe a homogeneous solution, attesting the proper mixture of the biomass and extractive agent. Afterwards, the extraction solution was centrifuged at 2000rpm for 1h at 25°C and washed two times with distilled water with the same centrifugation conditions, in order to remove cell debris and the surfactant or the fungus enzymatic hydrolysate.

As a **second** step, it was necessary to develop a strategy that, ideally, would dissolve the PHA rather than any other component of the extraction solution. From the solvents tried – acetone, hexane, propylene carbonate and dimethyl carbonate – only the last one able to dissolve the PHA. The acetone was unable to solubilize efficiently the PHA and, if temperature was increased, acetone was easily evaporated. Both hexane and propylene carbonate did not dissolve the PHA, even when the temperature was increased to 50°C. Dimethyl carbonate is an acyclic alkyl carbonate produced through a green process of catalytic oxidative carbonylation of methanol. In addition, it presents low toxicity, is fully biodegradable, has negligible reactivity in the formation of photochemicals and is non-irritating and has no mutagenic effects, being a preferred and versatile solvent in detriment of hazardous components. Extraction of PHA from *C. necator* using dimethyl carbonate was recently reported by Samori et al[167], achieving a recovery over 85% and a purity of 95% and emerged as a promising and more environmentally friendly alternative to PHA extraction with chlorinated solvents. In this article, the extraction was carried at 90°C, temperature at which the cell lysis is

observed, and the polymer recovered by evaporation of the solvent. In this work, 3.0mL of dimethyl carbonate was applied in the pellet obtained from the first step at 65°C with stirring, and let to react for 2h. At this temperature and during this period, as experimentally tested and reported by Samori et al[167], the dimethyl carbonate was able to selectively dissolve the PHA without causing significant cell disruption. After 2h, the mixture was left to settle and the formation of two different phases was observed: an organic bottom phase consisting of the dimethyl carbonate with the polymer dissolver and a top top phase with the remaining water and cell debris.

As **last** step, the bottom phase corresponding to the organic phase composed by dymethil carbonate and the solubilized PHA, is collected and left to evaporate, process which took up to 3 days without subjection to inert gas flow. After complete evaporation of the solvent, the polymer was in the form of a thin and viscous film that can posteriorly be weighted ant the extraction percentage calculated through Equation 15.

Different containers (in size, shape and material) and equipment were tried during the development of the extraction procedure in order to optimize the process, assure efficient stirring, minimize losses of extraction solution or final polymer and allow for a reproducible and practical method. Also the duration of each step was intensively studied in order to achieve the desired results as well as assuring the protocol was logistically possible to perform.

Thus, the final procedure defined for application of the different extractive agents comprised:

1. Addition of 5mL of surfactant or enzymatic solution, in a determined concentration, to 0.8g of lyophilized biomass;
2. 24h of reaction at 28°C with magnetic stirring;
3. One centrifugation cycle at 2000rpm and 25°C for 1h plus two more cycles with distilled water and under the same centrifugation conditions with discard of the supernatant;
4. Addition of 3.0mL of dimethyl carbonate to the pellet obtained and let react for 2h at 65°C with magnetic stirring;
5. Settling of the solution and formation of two distinguishable phases;
6. Collection of the organic bottom phase which is left to dry for 3 days;
7. Weight of the polymer film and calculate the respective extraction percentage by Equation 15.

The procedure described was posteriorly applied systematically for all extractive agents in pure and MMC containing PHA. Several temperatures as well as extractive agent concentrations were studied for the cell lysis (step 1.), as described in the next section.

4.5.4 Application of Extractive Agents

The procedure conceived and described in section 4.5.3 was systematically applied for PHA extraction using the surfactants – BRIJ 30, TWEEN 80, TRITON X-114 and TRITON X-110 – and the enzymatic hydrolysate from the *P. variotii*. Different concentrations – 2%, 5%, 7% and 10% (v/v)- and temperatures – 30°C, 40°C and 50°C - were used, in order to study which values combination would lead to a more efficient extraction. Figure 4p summarizes the assays performed and the percentages of polymer recovered in each one.

From the overall analysis, it was possible to conclude that the surfactant BRIJ 30 was the most promising compound for PHA extraction among the surfactants used. Not only it allowed for some of the highest recovery percentages, as it was the compound that presented better results for the PHA extraction from the MMC at different temperatures and concentrations. The Tween 80 presented high viscosity when used in concentrations of 7% and 10% as well when applied to the MMC, forming an agglomerate that could not be separated in its components. For the pure cultures, 30°C showed to be the most appropriate temperature for extraction with TWEEN 80, although percentage values are just slightly higher than the ones from 40°C and 50°C. TRITON X-110 and TRITON-114 presented similar behavior: the extraction was more efficient with a concentration of 5% of surfactant and the higher extraction results were obtained at 30°C (although the highest isolated result of all experiments was obtained for TRITON X-114 at 40°C). Just some of the assays with MMC were succeed, not allowing for any conclusions.

When considering the enzymatic solution, it was only possible to extract some polymer with a concentration of 10% and one assay with a concentration of 7% regarding the MMC. In this case, and opposite to what was observed for the surfactants for which a high concentration can lead to the formation of micelles and inhibition of the interaction with the biomass, the enzymatic concentration was too low. Thus, higher concentration of the enzymatic hydrolysate should be applied to achieve a significant PHA extraction.

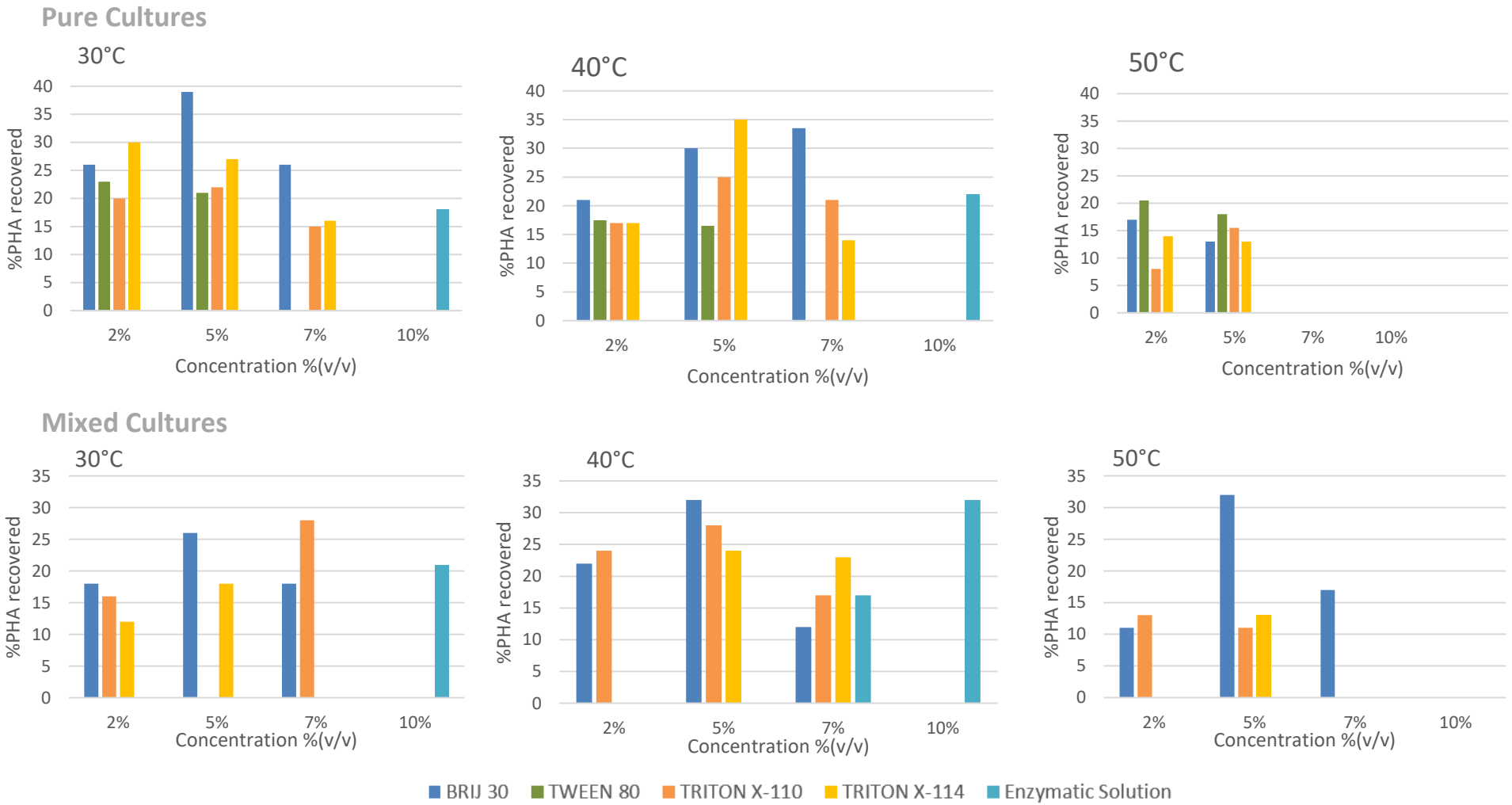


Figure 4p. Percentage of PHA extracted and recovered from pure cultures of *C. necator* and MMC by application of the extraction procedure developed. Each column represents the average percentage of PHA recovered for each temperature and concentration.

From the observation of Figure 4p is also possible to conclude that a surfactant concentration of 10% was too high for the extraction procedure. When this concentration was applied, the procedure was compromised at the first step since a dense extraction solution is form with no segregation of phases during centrifugation. The temperature of 50°C for the extraction is also unsuitable for the extraction procedure since no recovery of the polymer was possible in most of cases or presented a very low recovery percentage.

From the results obtained, it was not possible to establish a consistent correlation between the temperature, concentration and efficiency of the extraction. Thus, the improvement of the procedure as well as a calculated experimental planning are fundamental to improve the extraction. An important factor to consider as well should be the biomass used for the extraction procedures. In this study, several biomass samples were used, with different and relatively low PHA contents and probably with morphological differences due to the production in different batches. Ideally, a homogenous biomass sample from a pure culture with high PHA-storage capacity should be used. Previously to the extraction, MMC enriched in PHA-storing microorganisms can be subjected to an accumulation assay in order to promote the highest polymer content possible. The quantity of biomass used in each extraction can also affect the results: the higher the amount of biomass subjected to the extraction, the more polymer will be recovered and the easier and more precise will be the manipulation and measurement of the final polymer. This can explain that, although MMC are a more complex and heterogeneous biomass and more difficult to lyse it had a higher PHA content and it presented some high extraction percentages when compared to the pure cultures.

5. FINAL REMARKS

Research on SCOA production is often related with the production of PHA. PHA is a bioplastic that represents a promising alternative to common petroleum-based plastics due to its biodegradability, biocompatibility and versatile properties. SCOA has been found to be a preferred substrate by the PHA-synthetizing bacteria and therefore acidogenic fermentation is considered a suitable process for feedstock pre-treatment and conversion of carbon sources into SCOA for further conversion into PHA. This work aimed to study the influence of the reactors configuration in the acidogenic fermentation, namely the comparison between a CSTR and a SBR.

The operational conditions were kept stable at the predefined values: pH 5.0, 22°C, 1L of working volume and the OLR of 20gCOD/L·d for 80 days and then an OLR of 10gCOD/L·d until the end of the experiment. The monitoring of COD and SS allowed for the calculation of the SRT. Both reactors presented a similar and stable SRT value between days 64 and 80 for the lower OLR and between days 100 and 114 for the higher OLR. Thus, comparison of the fermentation profile based uniquely on reactors configuration was possible within the two time frames stated. Both MMC presented good settling properties. As discussed above, this is a major advantage over activated sludge, often related with poor settling properties.

The reactors configurations showed a different range of FP and especially different relative concentrations. For the OLR of 20gCOD/L·d, the CSTR presented, by descending order of concentration: EtOH, HAc, HLa, HBu and HPr and HiBu. For the same OLR, the SBR presented the following FP in descending order of concentration: HBu, HAc, EtOH, HLa and HPr. The HiBU was only detected in the CSTR and just for the higher OLR. Considering the OLR of 10gCOD/L·d, both reactors presented the same FP and same relative concentrations from the OLR of 20gCOD/L·d. However, BuOH was detected in both reactors for the lower OLR, with the lowest concentration among all measured FP in the CSTR and above HLa in the SBR.

For both OLR studied, the CSTR presented higher Y_{SCOA} than the SBR. However, for the OLR of 20gCOD/L·d, the $CONV_{SCOA}$ was higher for the CSTR than for SBR; 0.567 and 0.365, respectively. The opposite was observed for the OLR of 10gCOD/L·d, since the SBR presented a higher $CONV_{SCOA}$ than the CSTR; 0.486 and 0.380, respectively. Both reactors achieved full fermentation only after the decrease in the OLR.

The cycle studies performed complemented the observations from the overall experiment. When considering the cycle studies of the SBR, all three cycles were consistent when considering the evolution of the parameters monitored and the FP range and relative concentrations. It was

also possible to observe that the SBR did not achieve full fermentation, fact that influenced the decision to decrease the OLR. From cycle C1 to C3, an increase in Y_{SCOA} and $CONV_{SCOA}$ was noticed, indicating an improvement along the fermentation. The 24h cycle study, performed during the period of the experiment with an OLR of 10gCOD/L·d, showed that both reactors achieved full fermentation, with a FP range and relative concentrations consistent with what was observed for the overall process for the same OLR. The same conclusion was taken considering the Y_{SCOA} and $CONV_{SCOA}$ calculated, similar to the values referring to the fermentation period.

Differences between operation modes were also attested by the microscopic observations: different types of organisms were present in each reactor and respective biomasses were morphologically dissimilar. Since the MMC is directly related with the FP produced, the microscopic observations are consistent with the different FP range and relative abundance observed for each of the studied reactors. However, it was not possible to stablish a direct correlation between the type of organisms identified and the FP.

This work enforces that operation mode and reactor configuration have a direct influence in the fermentation profile and in the efficiency of the conversion of the substrate into SCOA. However, a complex synergy between operational conditions and reactor set up will determine the fermentation profile and a transversal model has not yet been established. The conclusions indicated that a SBR may be a more suitable configuration for the production of SCOA, considering the conversion of the substrate, the faster bacterial growth and higher biomass concentration as well as presenting SCOA as main FP. However, more studies with the same objective of this work must be performed in order to endorse the conclusions stated. In addition, other variables should be considered for the reactor and process design, such as FP intended to obtain, what purpose or further application is envisioned for the SCOA produced and also ease and convenience of the process control.

In order to improve the overall PHA production process and to contribute for a more sustainable method that can be applied to large scale processes, research about extraction procedures is also required. Literature does not cover this topic extensively and most PHA extraction works reported are not related with other studies. Moreover, many extraction procedures are protected by patents, hindering their dissemination.

This work aimed to develop an extraction procedure that allows for a high PHA recovery, without damaging the polymer, and that is environmentally friendly. One of extraction agents selected were surfactants, namely BRIJ 30, TWEEN 80, TRITON X-110 and TRITON X-114, which are

non-anionic organic substances with no toxicity associated. Moreover, surfactants applied for PHA extraction might be recovered at the end of the process with potentiality to be reused. The other extractive agent studied was enzymes, namely an undefined mixture of enzymes. Opposite to the expensive commercially available enzymes, the enzymatic solution applied in the experimental work was obtained by hydrolysis of the fungus *P. variotii*. The knowledge about the enzymes present in the mixture is irrelevant since synergy of hydrolytic enzymes is desired for a better disruption of cell membranes.

A procedure comprising extraction reaction, separation of the PHA and recovery of the final polymer was developed for the application of the stated extractive compounds. Moreover, equipment and all materials used were carefully selected in order to minimize polymer or biomass losses and to make the protocol as easy as possible. The developed procedure was applied in pure cultures of *C. necator* and MMC containing PHA, combining different temperatures and concentrations of the extractive agent.

Among the surfactants applied, BRIJ 30 resulted in the highest number of successful extractions and also in the greatest average recoveries percentage. It was also the component that better performed in the PHA extraction from MMC. Tween 80 presented poor results mainly due to its viscosity – the negative impact of the viscosity was bigger for higher concentrations of the surfactant applied in the pure culture. None of the extraction of PHA from MMC using Tween 80 were successful. TRITON X-110 and TRITON X-114 presented similar behavior in the extraction subjected to the same conditions, allowing for successful extractions from both the pure and MMC. The impossibility to take consistent conclusions about the efficiency of each surfactant and how its behavior is influenced by the temperature and concentration, leads to necessity of further work. Extraction using the enzymatic mixture was possible for concentrations of 10%, indicating that assays with higher concentrations – or even the application of the enzymatic hydrolysate with no dilution – might result in higher recoveries. The low recoveries obtained can be related with the low efficiency of the extractive agent or with the low PHA content in the biomass – however, the major cause cannot be determined. Moreover, it can also indicates that the procedure developed still needs to be improved.

Despite the low recoveries for some essays, this work is a promising initial for further studies on PHA extraction with surfactants and with an undifferentiated enzymatic mixture. First steps towards the development of a sustainable and efficient PHA extraction procedure with these extractive agents are taken and the practical knowledge acquired is also a valuable tool for further

improvements. This work will lead to further studies in extraction, aiming that literature covering this topic is enlarged and more related research is performed.

6. FUTURE PROSPECTS

Further research is needed in order to understand and complement the scarce literature covering the topics that lead this work. First of all, not many articles describe the comparison of acidogenic fermentation performed in a CSTR, SBR or other reactor configuration submitted to the same operational conditions. Moreover, research about systems usually used for SCOA production often focus on the operational parameters over the reactor configuration, proved to affect the process as well.

In this work, a synthetic feeding with glucose as carbon source was used. However, and as discussed above, renewable feedstocks have been used for SCOA production due to the environmental and economic sustainability related with their usage. Thus, it would be pertinent to perform the experiment described in this work with a renewable feedstock.

Considering all the information available about the influence of the operational parameters in the SCOA production process, it would also be relevant to perform a similar study submitting the reactors to a different pH value, temperature or even OLR in order to assess if similar conclusions would be taken. This would contribute to attest the results obtained.

The cycle studies carried out allowed for a better understanding of the fermentation potential and profile. However, more 24h extended cycle studies could have been performed at different times of the experiment – for instance, for the OLR of 20gCOD/L·d - to complement the information about the reactors performance.

The microscopic examinations also revealed to be a useful way to characterize the culture and identify differences between the biomass of each reactor and evolution of the culture from the same inoculum into a different community. More frequent observations and examination by microscopy from the beginning of the experiment could have allowed for a more detailed understanding and correlation with the acidogenic fermentation. Phenotypic characterization and further genetic analysis would allow to identify the organisms present in the culture and which ones are responsible for the production of the main FP measured in each case. Thus, differences between the MMC could be specified and more detailed.

Concerning the extraction of PHA, further work is still needed in order to improve the efficiency of the extraction procedure and also to make it logistically easier. As stated above, more assays combining different temperatures and extractive agents concentrations are required in

order to stablish solid conclusions about the hydrolytic power of the surfactants and enzymatic mixture used. Moreover, experimental design can be applied to optimize the process conditions.

It was relevant to extract PHA from both pure and MMC, since pure cultures are responsible for the industrial and large scale PHA production and MMC are becoming gradually more promising and suitable for large biopolymer production. Further work should be performed in larger quantities of biomass. Ideally, the biomass samples used should be homogenous and well characterized, with a high PHA content.

Although all biomass samples used were lyophilized, it would be interesting to apply the extraction method developed in fresh slurry. If the extraction was successful, the lyophilization step could be removed and the costs reduced.

Also envisioning the sustainability of the process, alternative sources of the extractive agents could be explored. For instance, the extraction potential of surfactants produced biologically could also be assessed. The obtainment of the enzymes mixture from hydrolysis of a fungus already represents an inexpensive method – however, *P. variotii* is known for its ability to grow in several complex wastes. Thus, instead of growing the *P. variotii* in defined medium, costs could be reduce by using wastes or industrial by-products as carbon source.

Finally, the characterization of the recovered PHA could also be relevant to draw conclusions about the extraction effect on the polymer structure and thermochemical properties. This characterization represents valuable data if further application of the extracted PHA is intended.

7. REFERENCES

1. Chen, Y. J. Protecting Environmental Sustainability by Use of Energy Materials. in *5th International Conference on Information Engineering for Mechanics and Materials* 1048–1053 (2015).
2. Aeschelmann, F. & Carus, M. Bio-based Building Blocks and Polymers in the World Capacities , Production and Applications : Status Quo and Trends towards 2020. (2015).
3. United Nations. Transforming our world: the 2030 Agenda for Sustainable Development. (2015).
4. Prieto, M. A. From Oil to Bioplastics, a Dream Come True? *J. Bacteriol.* **189,** 289–290 (2007).
5. Chanprateep, S. Current trends in biodegradable polyhydroxyalkanoates. *J. Biosci. Bioeng.* **110,** 621–632 (2010).
6. Groot, J., Bing, X., Bos-Brouwers, H. & Bloemhof-Ruwaard, J. A comprehensive waste collection cost model applied to post-consumer plastic packaging waste. *Resour. Conserv. Recycl.* **85,** 79–87 (2014).
7. Brockhaus, S., Petersen, M. & Kersten, W. A crossroads for bioplastics : exploring product developers ' challenges to move beyond petroleum-based plastics. *J. Clean. Prod.* **127,** 84–95 (2016).
8. Jain, R. & Tiwari, A. Biosynthesis of planet friendly bioplastics using renewable carbon source. *J. Environ. Heal. Sci. Eng.* **13,** 11 (2015).
9. Avérous, L. & Pollet, E. in *Environmental Silicate Nano-Biocomposites* 13–39 (2012). doi:10.1007/978-1-4471-4108-2
10. Packaging World. Biodegradable polymers market to grow at 13% through 2014. (2010). at <http://www.packworld.com/sustainability/strategy/biodegradable-polymers-market-grow-13-through-2014>
11. Koller, M., Salerno, A., Muhr, A., Reiterer, A. & Braunegg, G. Polyhydroxyalkanoates: Biodgradable Polymers and Plastics from Renewable Resources. *Mater. Technol.* **46,** 23–30 (2012).
12. Valappil, S. P., Misra, S. K., Boccaccini, A. R. & Roy, I. Biomedical applications of polyhydroxyalkanoates , an overview of animal testing and in vivo responses. 853–868 (2006).
13. Kılıçay, E. *et al.* European Journal of Pharmaceutical Sciences Preparation andcharacterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHX) based nanoparticles for targeted cancer therapy. *Eur. J. Pharm. Sci.* **44,** 310–320 (2011).
14. Grillo, R. *et al.* Controlled release system for ametryn using polymer microspheres: Preparation, characterization and release kinetics in water. *J. Hazard. Mater.* **186,** 1645–1651 (2011).
15. Bourbonnais, R. & Marchessault, R. H. Application of Polyhydroxyalkanoate Granules for Sizing of Paper. 989–993 (2010).
16. Gumel, a. M., Annuar, M. S. M. & Chisti, Y. Recent Advances in the Production, Recovery and Applications of Polyhydroxyalkanoates. *J. Polym. Environ.* **21,** 580–605 (2013).
17. Wang, Y., Yin, J. & Chen, G.-Q. Polyhydroxyalkanoates, challenges and opportunities. *Curr. Opin. Biotechnol.* **30,** 59–65 (2014).
18. Reis, M. A. M., Serafim, L. S., Lemos, P. C. & Ramos, A. M. Production of polyhydroxyalkanoates by mixed microbial cultures. **25,** 377–385 (2003).
19. Serafim, L. S., Lemos, P. C., Albuquerque, M. G. E. & Reis, M. A. M. Strategies for PHA production by mixed cultures and renewable waste materials. *Appl. Microbiol. Biotechnol.* **81,** 615–628 (2008).
20. Kunasundari, B. & Sudesh, K. Isolation and recovery of microbial polyhydroxyalkanoates. *Express Polym. Lett.* **5,** 620–634 (2011).
21. Lemoigne, M. Products of dehydration and of polymerization of ß-hydroxybutyric acid. *Chem. Biol. Soc.*

8, 770–782 (1926).

22. Akaraonye, E., Keshavarz, T. & Roy, I. Production of polyhydroxyalkanoates: the future green materials of choice. *J. Chem. Technol. Biotechnol.* **85,** 732–743 (2010).
23. Philp, J. C., Bartsev, A., Ritchie, R. J., Baucher, M. & Guy, K. Bioplastics science from a policy vantage point. *N. Biotechnol.* **30,** 635–646 (2013).
24. Kynadi, A. Polyhydroxyalkanoates : Biodegradable Plastics for Environmental Conservation Industrial & Environmental Biotechnology. *Ind. Environ. Biotechnol.* (2014). doi:10.13140/RG.2.1.4642.5682
25. Fernández-Dacosta, C., Posada, J. A., Kleerebezem, R., Cuellar, M. C. & Ramirez, A. Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment. *Bioresour. Technol.* **185,** 368–377 (2015).
26. Gurieff, N. & Lant, P. Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. **98,** 3393–3403 (2007).
27. Gholami, A., Mohkam, M., Rasoul-Amini, S. & Ghasemi, Y. Industrial production of polyhydroxyalkanoates by. *Minerva Biotecnol.* **28,** 59–74 (2016).
28. Mo, J. & Kiewisz, R. Bacterial polyhydroxyalkanoates : Still fabulous ? **192,** 271–282 (2016).
29. Bugnicourt, E., Cinelli, P., Lazzeri, A. & Alvarez, V. Polyhydroxyalkanoate (PHA): Review of synthesis , characteristics , processing and potential applications in packaging. **8,** 791–808 (2014).
30. Yoshie, N. & Inoue, Y. Polyhydroxyalkanoates (PHAs), Structure, Composition and Solution Properties of Polyesters. *Biopolym. Online* (2005). doi:10.1002/3527600035.bpol3b06
31. Afzal, M. & Hameed, S. Green Chemistry Letters and Reviews Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic : Production , biocompatibility , biodegradation , physical properties and applications. **8253,** (2015).
32. Vastano, M., Casillo, A., Corsaro, M. M., Sannia, G. & Pezzella, C. Production of medium chain length polyhydroxyalkanoates from waste oils by recombinant *Escherichia coli*. *Eng. Life Sci.* **15,** 700–709 (2015).
33. Doi, Y., Kawaguchi, Y., Koyama, N., Nakamura, S. & Hiramitsu, M. Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus. **103,** 103–108 (1992).
34. Doi, Y. & Fukui, T. Eficient production of polyhydroxyalkanoates from plant oils by Alcaligenes eutrophus and its recombinant strain. **16,** 333–336 (1998).
35. Rai, R., Keshavarz, T., Roether, J. A., Boccaccini, A. R. & Roy, I. Medium chain length polyhydroxyalkanoates , promising new biomedical materials for the future. *Mater. Sci. Eng. R* **72,** 29–47 (2011).
36. Laycock, B., Halley, P., Pratt, S., Werker, A. & Lant, P. The chemomechanical properties of microbial polyhydroxyalkanoates. *Prog. Polym. Sci.* **39,** 397–442 (2014).
37. Khosravi-darani, K. & Mokhtari, Z. Microbial production of poly (hydroxybutyrate) from C 1 carbon sources. 1407–1424 (2013). doi:10.1007/s00253-012-4649-0
38. Ng, K. S., Wong, Y. M., Tsuge, T. & Sudesh, K. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3- hydroxyvalerate) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) copolymers using jatropha oil as the main carbon source. *Process Biochem.* **46,** 1572–1578 (2011).
39. Mamtesh, S., Kumar, P., Ray, S. & Kalia, V. C. Challenges and Opportunities for Customizing Polyhydroxyalkanoates. *Indian J. Microbiol.* **55,** 235–249 (2015).
40. Marchessault, R. & Yu, G.-E. Crystallization and Material Properties of Polyhydroxyalkanoates PHAs. *Biopolym. Online* (2005). doi:10.1002/3527600035.bpol3b07

41. Corre, Y. M., Bruzaud, S., Audic, J. L. & Grohens, Y. Morphology and functional properties of commercial polyhydroxyalkanoates: A comprehensive and comparative study. *Polym. Test.* **31,** 226–235 (2012).
42. Patel, M., Gapes, D. J., Newman, R. H. & Dare, P. H. Physico-chemical properties of polyhydroxyalkanoate produced by mixed-culture nitrogen-fixing bacteria. *Appl. Microbiol. Biotechnol.* **82,** 545–555 (2009).
43. Álvarez-Chávez, C. R., Edwards, S., Moure-Eraso, R. & Geiser, K. Sustainability of bio-based plastics: general comparative analysis and recommendations for improvement. *J. Clean. Prod.* **23,** 47–56 (2012).
44. Philip, S., Roy, I. & Keshavarz, T. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *J. Chem. Technol. Biotechnol.* **82,** 233–247 (2007).
45. Chen, G.-Q. Sustainable bioplastics for future applications. *Biotechnol. J.* **5,** 1117 (2010).
46. Chen, G.-Q. & Wu, Q. The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* **26,** 6565–78 (2005).
47. Shrivastav, A., Kim, H. & Kim, Y. Advances in the Applications of Polyhydroxyalkanoate Nanoparticles for Novel Drug Delivery System. **2013,** (2013).
48. Jain, R. & Tiwari, A. Bioplastics for use in medical industry. *Asian J. Pharm.* **8,** 139 (2014).
49. Saharan, B. S., Grewal, A. & Kumar, P. Biotechnological Production of Polyhydroxyalkanoates : A Review on Trends and Latest Developments. **2014,** (2014).
50. James, B. W., Mauchline, W. S., Dennis, P. J., Keevil, C. W. & Wait, R. Poly-3-hydroxybutyrate in Legionella pneumophila, an energy source for survival in low-nutrient environments. *Appl. Environ. Microbiol.* **65,** 822–827 (1999).
51. Tai, Y. T., Foong, C. P., Najimudin, N. & Sudesh, K. Discovery of a new polyhydroxyalkanoate synthase from limestone soil through metagenomic approach. *J. Biosci. Bioeng.* **xx,** (2015).
52. Braunegg, G., Bona, R. & Koller, M. Sustainable Polymer Production. *Polym. Plast. Technol. Eng.* **43,** 1779–1793 (2004).
53. Association, E. B. *Bioplastics - Furthering efficient waste management*. (2015). at <www.european-bioplastics.org>
54. Madison, L. L. & Huisman, G. W. Metabolic Engineering of Poly (3-Hydroxyalkanoates): From DNA to Plastic. *Microbiol. Mol. Biol. Rev.* **63,** 21–53 (1999).
55. Reis, M., Albuquerque, M., Universidade, F. C. T. & Lisboa, N. De. Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus / Wastes as Feedstocks. *Compr. Biotechnol.* **6,** 669–683 (2011).
56. Villano, M. *et al.* Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. *N. Biotechnol.* 1–8 (2013).
57. Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D. & Piotrowska-seget, Z. Production of polyhydroxyalkanoates from waste frying oil by Cupriavidus necator. *AMB Express* **1,** 11 (2011).
58. Linton, E., Rahman, A., Viamajala, S., Sims, R. C. & Miller, C. D. Polyhydroxyalkanoate quanti fi cation in organic wastes and pure cultures using a single-step extraction and H NMR analysis. 1000–1006 (2012). doi:10.2166/wst.2012.273
59. Ciesielski, S., Możejko, J. & Pisutpaisal, N. Plant oils as promising substrates for polyhydroxyalkanoates production. *J. Clean. Prod.* **106,** 408–421 (2015).
60. Choi, M. J. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. 13–21 (1999).

61. Yan, Q. *et al.* Biosynthesis of short-chain-length-polyhydroxyalkanoates during the dual-nutrient-limited zone by Ralstonia eutropha. 17–21 (2005). doi:10.1007/s11274-004-0877-5
62. Merugu, R., Girisham, S. & Reddy, S. M. Production of PHB (polyhydroxybutyrate) by Rhodopseudomonas palustris KU003 under nitrogen limitation. *Int. J. Appl. Biol. Pharm. Technol.* **1,** 676–678 (2010).
63. Zuñiga, C., Morales, M. & Revah, S. Bioresource Technology Polyhydroxyalkanoates accumulation by Methylobacterium organophilum CZ-2 during methane degradation using citrate or propionate as cosubstrates. **129,** 686–689 (2013).
64. Journal, B. Production and characterization of pha from recombinant. 1109–1118 (2011).
65. Sangkharak, K. & Prasertsan, P. Optimization of polyhydroxybutyrate production from a wild type and two mutant strains of Rhodobacter sphaeroides using statistical method. **132,** 331–340 (2007).
66. Wang, Q., Tappel, R. C., Zhu, C. & Nomura, C. T. Development of a New Strategy for Production of Medium-Chain-Length Polyhydroxyalkanoates by Recombinant Escherichia coli via Inexpensive Non-Fatty Acid Feedstocks. *Appl. Environ. Microbiol.* **78,** 519–527 (2012).
67. Anjum, A. *et al.* International Journal of Biological Macromolecules Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers : A review of recent advancements. *Int. J. Biol. Macromol.* **89,** 161–174 (2016).
68. Castilho, L. R., Mitchell, D. A. & Freire, D. M. G. Bioresource Technology Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresour. Technol.* **100,** 5996–6009 (2009).
69. Wallen, L. L. & Rohwedder, W. K. Poly-B-hydroxyalkanoate from Activated Sludge. *Environ. Sci. Technol.* **8,** 576–579 (1974).
70. Villano, M. *et al.* Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. *N. Biotechnol.* **31,** 289–296 (2014).
71. Dias, J. M. L. *et al.* Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: From the substrate to the final product. *Macromol. Biosci.* **6,** 885–906 (2006).
72. Arcos-hernández, M. V *et al.* Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). *Eur. Polym. J.* **49,** 904–913 (2013).
73. Temudo, M. F., Muyzer, G., Kleerebezem, R. & Loosdrecht, M. C. M. Van. Diversity of microbial communities in open mixed culture fermentations : impact of the pH and carbon source. 1121–1130 (2008). doi:10.1007/s00253-008-1669-x
74. Bengtsson, S., Werker, A., Christensson, M. & Welander, T. Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. *Bioresour. Technol.* **99,** 509–16 (2008).
75. Diba, H. & Seifi-kashani, R. Polyhydroxyalkanoates (PHAs), Intracellular Pathways and Properties. **10,** 644–649 (2015).
76. Chen, G.-Q. *Plastics from Bacteria: Natural Functions and Applications*. (Springer, 2010).
77. Naik, S., Gopal, Æ. S. K. V. & Somal, Æ. P. Bioproduction of polyhydroxyalkanoates from bacteria : a metabolic approach. 2307–2314 (2008). doi:10.1007/s11274-008-9745-z
78. Takabatake, H., Satoh, H., Mino, T. & Matsuo, T. Recovery of biodegradable plastics from activated sludge process. *Water Sci. Technol.* **42,** (2000).
79. Karahan, O., Orhon, D. & Loosdrecht, M. C. M. Van. Simultaneous storage and utilization of polyhydroxyalkanoates and glycogen under aerobic conditions. (2008). doi:10.2166/wst.2008.403
80. Albuquerque, M. G. E., Eiroa, M., Torres, C., Nunes, B. R. & Reis, M. A. M. Strategies for the

development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. **130,** 411–421 (2007).

81. Chen, Z. *et al.* Modeling polyhydroxyalkanoate (PHA) production in a newly developed aerobic dynamic discharge (ADD) culture enrichment process. *Chem. Eng. J.* **298,** 36–43 (2016).
82. Yaoping, L. Ü. Advance on the production of polyhydroxyalkanoates by mixed cultures. **2,** 21–25 (2007).
83. Ciesielski, S. & Pokoj, Æ. T. Molecular insight into activated sludge producing polyhydroxyalkanoates under aerobic – anaerobic conditions. 805–814 (2008). doi:10.1007/s10295-008-0352-7
84. Valentino, F., Brusca, A., Beccari, M., Nuzzo, A. & Majone, M. Start up of biological sequencing batch reactor (SBR) and short-term biomass acclimation for polyhydroxyalkanoates production. 261–270 (2013). doi:10.1002/jctb.3824
85. Serafim, L. S., Lemos, P. C., Oliveira, R. & Reis, M. a M. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. *Biotechnol. Bioeng.* **87,** 145–60 (2004).
86. Albuquerque, M. G. E., Torres, C. A. V & Reis, M. A. M. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses : Effect of the influent substrate concentration on culture selection. *Water Res.* **44,** 3419–3433 (2010).
87. Dionisi, D. *et al.* Olive oil mill effluents as a feedstock for production of biodegradable polymers. **39,** 2076–2084 (2005).
88. Chen, Z. *et al.* Modeling polyhydroxyalkanoate (PHA) production in a newly developed aerobic dynamic discharge (ADD) culture enrichment process. **298,** 36–38 (2016).
89. Chen, Z. *et al.* A new method for polyhydroxyalkanoate (PHA) accumulating bacteria selection under physical selective pressure. *Int. J. Biol. Macromol.* **72,** 1329–1334 (2015).
90. Salehizadeh, H. & Van Loosdrecht, M. C. M. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnol. Adv.* **22,** 261–279 (2004).
91. Smolders, G. J. F., Meij, J. Van Der, Loosdrecht, M. C. M. Van & Heijnen, J. J. Model of the Anaerobic Metabolism of the Biological Phosphorus Removal Process: Stoichiometry and pH Influence. **43,** 461–470 (1994).
92. Pisco, A. R., Bengtsson, S., Werker, A., Reis, M. A. M. & Lemos, P. C. Enrichment of glycogen-accumulating organisms producing polyhydroxyalkanoates (PHA) from fermented molasses: evolution in community structure and PHA 3 production. *AEM* (2009). doi:10.1128/AEM.02486-08
93. Zeng, R. J., Loosdrecht, M. C. M. Van, Yuan, Z. & Keller, J. Metabolic Model for Glycogen-Accumulating Organisms in Anaerobic / Aerobic Activated Sludge Systems. (2003). doi:10.1002/bit.10455
94. Madkour, M. H., Heinrich, D., Alghamdi, M. a., Shabbaj, I. I. & Steinbüchel, A. PHA recovery from biomass. *Biomacromolecules* **14,** 2963–2972 (2013).
95. Samorì, C. *et al.* Extraction of polyhydroxyalkanoates from mixed microbial cultures: Impact on polymer quality and recovery. *Bioresour. Technol.* **189,** 195–202 (2015).
96. Duque, A. F. *et al.* Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer composition. *N. Biotechnol.* **31,** 276–288 (2014).
97. Guo, Z. *et al.* Enhanced short chain fatty acids production from waste activated sludge conditioning with typical agricultural residues : carbon source composition regulates community functions. *Biotechnol. Biofuels* 1–14 (2015). doi:10.1186/s13068-015-0369-x
98. Jiang, Y. *et al.* Waste to resource: Converting paper mill wastewater to bioplastic. *Water Res.* **46,** 5517–5530 (2012).

99. Queirós, D., Fonseca, A., Lemos, C. & Serafim, L. S. Long-term operation of a two-stage polyhydroxyalkanoates production process from hardwood sulphite spent liquor. (2016). doi:10.1002/jctb.4841
100. Microbiol, A., Ferreira, A. M., Queirós, D., Gagliano, M. C. & Serafim, L. S. Polyhydroxyalkanoates-accumulating bacteria isolated from activated sludge acclimatized to hardwood sulphite spent liquor. (2015). doi:10.1007/s13213-015-1169-z
101. Moita, R., Freches, A. & Lemos, P. C. Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures. *Water Res.* **58,** 9–20 (2014).
102. Albuquerque, M. G. E., Concas, S., Bengtsson, S. & Reis, M. A. M. Mixed culture polyhydroxyalkanoates production from sugar molasses: The use of a 2-stage CSTR system for culture selection. *Bioresour. Technol.* **101,** 7112–7122 (2010).
103. Waller, J. L., Green, P. G. & Loge, F. J. Bioresource Technology Mixed-culture polyhydroxyalkanoate production from olive oil mill pomace. *Bioresour. Technol.* **120,** 285–289 (2012).
104. Esposito, G. *et al.* Anaerobic co-digestion of organic wastes. *Rev. Environ. Sci. Biotechnol.* **11,** 325–341 (2012).
105. Wastewater Engineering - Treatment and Reuse [Metcalf & Eddy].pdf.
106. Silva, F. C., Serafim, L. S., Nadais, H., Arroja, L. & Capela, I. Acidogenic fermentation towards valorisation of organic waste streams into volatile fatty acids. *Chem. Biochem. Eng. Q.* **27,** 467–476 (2013).
107. Angenent, L. T., Karim, K., Al-dahhan, M. H., Wrenn, B. A. & Domı, R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. **22,** (2004).
108. Dahiya, S., Sarkar, O., Swamy, Y. V. & Venkata Mohan, S. Acidogenic fermentation of food waste for volatile fatty acid production with co-generation of biohydrogen. *Bioresour. Technol.* **182,** 103–113 (2015).
109. Dircks, K., Beun, J. J., Loosdrecht, M. Van, Heijnen, J. J. & Henze, M. Glycogen Metabolism in Aerobic Mixed Cultures. (2001).
110. Lee, W. S., Seak, A., Chua, M., Yeoh, H. K. & Ngoh, G. C. A review of the production and applications of waste-derived volatile fatty acids. *Chem. Eng. J.* **235,** 83–99 (2014).
111. Albuquerque, M. G. E., Martino, V., Pollet, E., Avérous, L. & Reis, M. A. M. Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. *J. Biotechnol.* **151,** 66–76 (2011).
112. Chang, H. N., Kim, N., Kang, J. & Jeong, C. M. Biomass-derived Volatile Fatty Acid Platform for Fuels and Chemicals. 1–10 (2010). doi:10.1007/s12257-009-3070-8
113. Antonio, E. *et al.* Biogas from anaerobic digestion of fruit and vegetable wastes : Experimental results on pilot-scale and preliminary performance evaluation of a full-scale power plant. *Energy Convers. Manag.* **77,** 22–30 (2014).
114. Komemoto, K. *et al.* Effect of temperature on VFA ' s and biogas production in anaerobic solubilization of food waste. *Waste Manag.* **29,** 2950–2955 (2009).
115. Infantes, D., Gonz??lez Del Campo, A., Villase??or, J. & Fern??ndez, F. J. Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation. *Int. J. Hydrogen Energy* **36,** 15595–15601 (2011).
116. Fei, Q. *et al.* Bioresource Technology The effect of volatile fatty acids as a sole carbon source on lipid

accumulation by Cryptococcus albidus for biodiesel production. *Bioresour. Technol.* **102,** 2695–2701 (2011).

117. Baumann, I. & Westermann, P. Microbial Production of Short Chain Fatty Acids from Lignocellulosic Biomass : Current Processes and Market. **2016,** (2016).
118. Chen, H., Meng, H., Nie, Z. & Zhang, M. Bioresource Technology Polyhydroxyalkanoate production from fermented volatile fatty acids : Effect of pH and feeding regimes. *Bioresour. Technol.* **128,** 533–538 (2013).
119. Pittmann, T. & Steinmetz, H. Bioresource Technology Influence of operating conditions for volatile fatty acids enrichment as a first step for polyhydroxyalkanoate production on a municipal waste water treatment plant. *Bioresour. Technol.* **148,** 270–276 (2013).
120. Madigan, M., Martinko, J. & Brock, T. *Biology of Microorganisms*. (2006).
121. Temudo, M. F., Kleerebezem, R. & van Loosdrecht, M. Influence of the pH on (Open) Mixed Culture Fermentation of Glucose: A Chemostat Study. *Biotechnol. Bioeng.* **98,** 69–79 (2007).
122. Lee, W. S., Seak, A., Chua, M., Yeoh, H. K. & Ngoh, G. C. A review of the production and applications of waste-derived volatile fatty acids. *Chem. Eng. J.* (2013). doi:10.1016/j.cej.2013.09.002
123. Lemos, P. C., Serafim, L. S. & Reis, M. A. M. Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. *J. Biotechnol.* **122,** 226–238 (2006).
124. Pardelha, F., Albuquerque, M. G. E., Reis, M. A. M., Oliveira, R. & Dias, J. M. L. Dynamic metabolic modelling of volatile fatty acids conversion to polyhydroxyalkanoates by a mixed microbial culture. *N. Biotechnol.* **31,** 335–344 (2014).
125. Amani, T., Nosrati, M. & Kermanshahi, S. M. M. R. K. Analysis of the Syntrophic Anaerobic Digestion of Volatile Fatty Acids Using Enriched Cultures in a Fixed-Bed Reactor. **84,** (2008).
126. Bertin, L. *et al.* Anaerobic acidogenic digestion of olive mill wastewaters in biofilm reactors packed with ceramic filters or granular activated carbon. *Water Res.* **44,** 4537–4549 (2010).
127. Momoe, G. *et al.* Long-term stability of hydrogen and organic acids production in an anaerobic fluidized-bed reactor using heat treated anaerobic sludge inoculum. **34,** 3679–3688 (2009).
128. Taylor, P. Inhibition of Volatile Fatty Acid Production in Granular Sludge from a UASB Reactor. 37–41 doi:10.1081/ESE-200046616
129. Yang, X. *et al.* Bioresource Technology Continuous volatile fatty acid production from waste activated sludge hydrolyzed at pH 12. *Bioresour. Technol.* 4–10 (2014). doi:10.1016/j.biortech.2014.02.066
130. Valentino, F., Beccari, M., Fraraccio, S., Zanaroli, G. & Majone, M. Feed frequency in a Sequencing Batch Reactor strongly affects the production of polyhydroxyalkanoates (PHAs) from volatile fatty acids. *N. Biotechnol.* **00,** (2013).
131. Jiang, J. *et al.* Volatile fatty acids production from food waste: Effects of pH, temperature, and organic loading rate. *Bioresour. Technol.* **143,** 525–530 (2013).
132. Tamis, J., Joosse, B. M., Loosdrecht, M. C. M. va. & Kleerebezem, R. High-rate volatile fatty acid (VFA) production by a granular sludge process at low pH. *Biotechnol. Bioeng.* **112,** 2248–2255 (2015).
133. Bengtsson, S., Hallquist, J., Werker, A. & Welander, T. Acidogenic fermentation of industrial wastewaters: Effects of chemostat retention time and pH on volatile fatty acids production. *Biochem. Eng. J.* **40,** 492–499 (2008).
134. Liu, H. *et al.* Acidogenic fermentation of proteinaceous sewage sludge: Effect of pH. *Water Res.* **46,** 799–807 (2012).

135. Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S. N. & Jie, R. Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. *Bioresour. Technol.* **100,** 1399–1405 (2009).
136. Yuan, Q., Sparling, R. & Oleszkiewicz, J. A. VFA generation from waste activated sludge: Effect of temperature and mixing. *Chemosphere* **82,** 603–607 (2011).
137. Zhang, P., Chen, Y., Huang, T. & Zhou, Q. Waste activated sludge hydrolysis and short-chain fatty acids accumulation in the presence of SDBS in semi-continuous flow reactors : Effect of solids retention time and temperature. **148,** 348–353 (2009).
138. Xiong, H., Chen, J., Wang, H. & Shi, H. Influences of volatile solid concentration , temperature and solid retention time for the hydrolysis of waste activated sludge to recover volatile fatty acids. *Bioresour. Technol.* **119,** 285–292 (2012).
139. Kim, W., Gu, S. & Juntaek, S. Effect of temperature and hydraulic retention time on volatile fatty acid production based on bacterial community structure in anaerobic acidogenesis using swine wastewater. 791–798 (2013). doi:10.1007/s00449-013-0905-7
140. Demirel, B. & Yenigun, O. Anaerobic acidogenesis of dairy wastewater : the effects of variations in hydraulic retention. **760,** 755–760 (2004).
141. Fang, H. & Yu, H. EFFECT OF HRT ON MESOPHILIC ACIDOGENESIS OF DAIRY WASTEWATER By. 1145–1148 (2000).
142. Ferrer, I., Vázquez, F. & Font, X. Bioresource Technology Long term operation of a thermophilic anaerobic reactor : Process stability and efficiency at decreasing sludge retention time. *Bioresour. Technol.* **101,** 2972–2980 (2010).
143. Zhuo, G., Yan, Y., Tan, X., Dai, X. & Zhou, Q. Ultrasonic-pretreated waste activated sludge hydrolysis and volatile fatty acid accumulation under alkaline conditions : Effect of temperature. *J. Biotechnol.* **159,** 27–31 (2012).
144. Vanwonterghem, I., Jensen, P. D., Rabaey, K. & Tyson, G. W. Temperature and solids retention time control microbial population dynamics and volatile fatty acid production in replicated anaerobic digesters. *Sci. Rep.* **5,** 8496 (2015).
145. Yu, J. Production of PHA from starchy wastewater via organic acids. **86,** 105–112 (2001).
146. Rao, P. M. Effect of additives on volatile fatty acid production in anaerobic digestion. (1998).
147. Liu, H., Wang, J., Wang, A. & Chen, J. Chemical inhibitors of methanogenesis and putative applications. 1333–1340 (2011). doi:10.1007/s00253-010-3066-5
148. Jiang, S., Chen, Y. & Zhou, Q. Effect of sodium dodecyl sulfate on waste activated sludge hydrolysis and acidification. **132,** 311–317 (2007).
149. Jiang, S., Ã, Y. C., Zhou, Q. & Gu, G. Biological short-chain fatty acids (SCFAs) production from waste-activated sludge affected by surfactant. **41,** 3112–3120 (2007).
150. Sudesh, K., Maehara, A., Gan, Z., Iwata, T. & Doi, Y. Direct observation of polyhydroxyalkanoate granule-associated-proteins on native granules and on poly(3-hydroxybutyrate) single crystals by atomic force microscopy. *Polym. Degrad. Stab.* **83,** 281–287 (2004).
151. Sudesh, K., Gan, Z., Maehara, A. & Doi, Y. Surface structure, morphology and stability of polyhydroxyalkanoate inclusions characterised by atomic force microscopy. *Polym. Degrad. Stab.* **77,** 77–85 (2002).
152. Jacquel, N., Lo, C.-W., Wei, Y.-H., Wu, H.-S. & Wang, S. S. Isolation and purification of bacterial poly(3-hydroxyalkanoates). *Biochem. Eng. J.* **39,** 15–27 (2008).

153. Villas-Bôas, S. G., Roessner, U., Hansen, M. A. E., Smedsgaard, J. & Nielsen, J. *Metabolome analysis: an introduction*. (2007).
154. Streptomyces, P.--hydroxybutyrate Æ. Polyhydroxyalkanoates in Gram-positive bacteria : insights from the genera Bacillus and Streptomyces. 1–17 (2007). doi:10.1007/s10482-006-9095-5
155. Saegusa, H., Shiraki, M. & Kanai, C. Cloning of an Intracellular Poly [D(-)-3-Hydroxybutyrate] Depolymerase Gene from Ralstonia eutropha H16 and Characterization of the Gene Product. *J. Bacteriol.* **183,** 94–100 (2001).
156. Kung, S. S., Chuang, Y. C., Chen, C. H. & Chien, C. C. Isolation of polyhydroxyalkanoates-producing bacteria using a combination of phenotypic and genotypic approach. *Lett. Appl. Microbiol.* **44,** 364–371 (2007).
157. Tamer, I. M., Moo-Young, M. & Chisti, Y. Optimization of poly(beta-hydroxybutyric acid) recovery from Alcaligenes latus: Combined mechanical and chemical treatments. *Bioprocess Eng.* **19,** 459–468 (1998).
158. Mohammadi, M. *et al.* sp. EB172 by Simple Digestion with Sodium Hydroxide. *Sep. Sci. Technol.* **47,** 534–541 (2012).
159. Fiorese, M. L. *et al.* Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1,2-propylene carbonate. *Eng. Life Sci.* **9,** 454–461 (2009).
160. Ghatnekar, M. S., Pai, J. S. & Ganesh, M. Production and recovery of poly-3-hydroxy-butyrate from Methylobacterium sp V49. *J. Chem. Technol. Biotechnol.* **77,** 444–448 (2002).
161. Koller, M., Niebelschütz, H. & Braunegg, G. Strategies for recovery and purification of poly[(R)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. *Eng. Life Sci.* **13,** 549–562 (2013).
162. Hwang, K.-J., You, S.-F. & Don, R.-M. Disruption Kinetics of Bacterial Cells during Purification of Poly-β-Hydroxyalkanoate Using Ultrasonication. *J. Chinese Inst. Chem. Eng.* **37,** 209–216 (2006).
163. Hahn, S. K., Chang, Y. K., Kim, B. S. & Chang, H. N. Optimization of Microbial Poly (3-hydroxybutyrate) Recovery Using Dispersions of Sodium Hypochlorite Solution and Chloroform. **44,** 256–261 (1994).
164. Pursell, M. R., Mendes, M. A. & Stuckey, D. C. Interactions between surfactants and biomass during liquid-liquid extraction. *Biotechnol. Prog.* **25,** 1686–94 (2009).
165. Yang, Y. H., Brigham, C., Willis, L., Rha, C. & Sinskey, A. Improved detergent-based recovery of polyhydroxyalkanoates (PHAs). *Biotechnol. Lett.* **33,** 937–942 (2011).
166. Kim, M., Cho, K., Ryu, H. W., Lee, E. G. & Chang, Y. K. Recovery of poly (3-hydroxybutyrate) from high cell density culture of Ralstonia eutropha by direct addition of sodium dodecyl sulfate. *Biotechnol. Lett.* **25,** 55–59 (2003).
167. Samorì, C. *et al.* Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. *Green Chem.* **17,** 1047–1056 (2015).
168. Geys, R., Soetaert, W. & Van Bogaert, I. Biotechnological opportunities in biosurfactant production. *Curr. Opin. Biotechnol.* **30,** 66–72 (2014).
169. Yasotha, K., Aroua, M. K., Ramachandran, K. B. & Tan, I. K. P. Recovery of medium-chain-length enzymatic digestion treatments and polyhydroxyalkanoates (PHAs) through ultrafiltration. *Biochem. Eng. J.* **30,** 260–268 (2006).
170. Herrera Bravo de Laguna, I., Toledo Marante, F. J. & Mioso, R. Enzymes and bioproducts produced by the ascomycete fungus *Paecilomyces variotii*. *J. Appl. Microbiol.* n/a–n/a (2015). doi:10.1111/jam.12934
171. Hejazi, P., Vasheghani-Farahani, E. & Yamini, Y. Supercritical fluid disruption of Ralstonia eutropha for poly(beta-hydroxybutyrate) recovery. *Biotechnol. Prog.* **19,** 1519–1523 (2003).

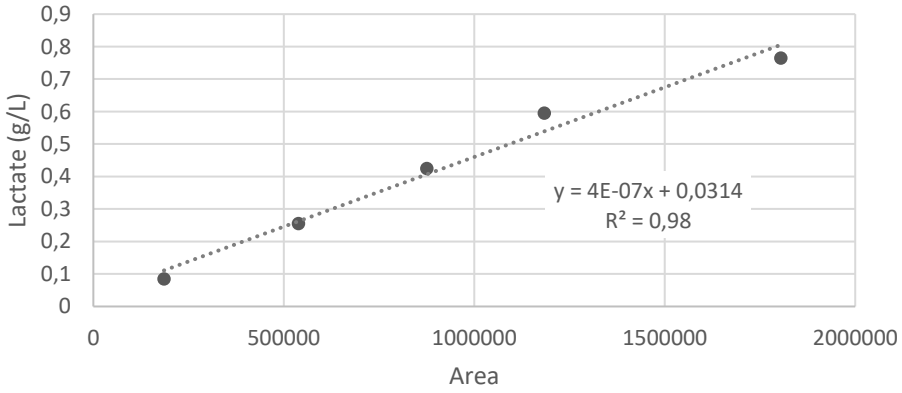
172. Khosravi-darani, K. & Vasheghani-farahani, E. Effect of Process Variables on Supercritical Fluid Disruption of Ralstonia eutropha Cells for Poly (R -hydroxybutyrate) Recovery. **20,** 1757–1765 (2004).
173. Page, W. J. & Cornish, A. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of poly-3-hydroxybutyrate. *Appl. Environ. Microbiol.* **59,** 4236–4244 (1993).
174. Fidler, S. & Dennis, D. Polyhydroxyalkanoate production in recombinant Escherichia coli. **103,** 231–235 (1992).
175. Schroll, G., Resch, S., Gruber, K., Wanner, G. & Lubitz, W. Heterologous phi X174 gene E-expression in Ralstonia eutropha: E-mediated lysis is not restricted to gamma-subclass of proteobacteria. *J. Biotechnol.* 211–217 (1998).
176. Jenkins, D., Richard, M. G. & Daigger, G. T. *Manual on the causes and control of activated sludge bulking and foaming*. (Lewis Publishers, 1993).
177. Zheng, M., Zheng, M., Wu, Y., Ma, H. & Wang, K. Effect of pH on types of acidogenic fermentation of fruit and vegetable wastes. *Biotechnol. Bioprocess Eng.* **20,** 298–303 (2015).
178. Janczukowicz, W., Szewczyk, M., Krzemieniewski, M. & Pesta, J. Settling Properties of Activated Sludge from a Sequencing Batch Reactor (SBR). **10,** 15–20 (2001).
179. Trontel, A., Batu, A., Gusi, I. & Slavica, A. Production of D - and L -Lactic Acid by Mono- and Mixed Cultures of Lactobacillus sp . **49,** 75–82 (2011).
180. Ghaffar, T. *et al.* ScienceDirect Recent trends in lactic acid biotechnology : A brief review on production to purification. *J. Radiat. Res. Appl. Sci.* **7,** 222–229 (2014).
181. Shimada, T., Zilles, J., Raskin, L. & Morgenroth, E. Carbohydrate storage in anaerobic sequencing batch reactors. **41,** 4721–4729 (2007).
182. Lopar, M. *et al.* Study of metabolic network of Cupriavidus necator DSM 545 growing on glycerol by applying elementary flux modes and yield space analysis. *J. Ind. Microbiol. Biotechnol.* **41,** 913–930 (2014).
183. Berezina, N. Novel approach for productivity enhancement of polyhydroxyalkanoates (PHA) production by Cupriavidus necator DSM 545. *N. Biotechnol.* **30,** 192–5 (2013).

8. APPENDICES

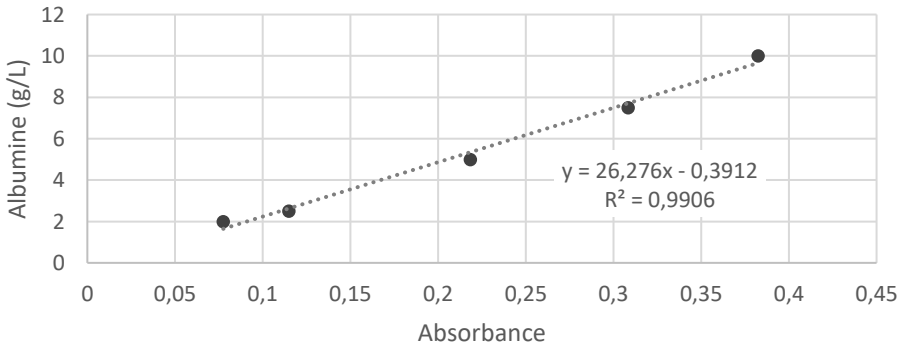
8.1 Appendix A: Standard solution for GC samples preparation

	mg/L	g/L
EtOH	656	0,656
HAc	1056,1	1,0561
HPr	993,3	0,9933
HiBu	948	0,948
HBu	970,3	0,9703
HiVa	921,1	0,9211
HVa	948,6	0,9486
HCa	931,2	0,9312
HHe	776,7	0,7767

8.2 Appendix B: Calibration curve for Lactate determination by HPLC



8.3 Appendix C: Calibration curve for the Biuret Test



8.4 Appendix D: Correlation between pCOD and VSS