



Inês Miguel Troles Duarte do Carmo

Mestrado Integrado em Engenharia Química e Bioquímica

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production of polyhydroxyalkanoates by
mixed microbial cultures**

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Orientador: Professora Doutora Maria Ascensão C. F. Miranda Reis
Co-orientador: Doutora Catarina S. S. Oliveira
Doutora Anouk F. Duque



FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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"I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Isaac Newton

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É com orgulho que dou por terminada mais uma etapa da minha vida académica. Contudo, não posso deixar de agradecer a todos aqueles quem me incentivaram e cooperaram para a sua realização. A todos vós os meus sinceros agradecimentos.

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Resumo

Os Polihidroxialcanoatos (PHAs) são poliésteres constituídos por ácidos hidroxilos gordos, cuja acumulação se dá no interior das culturas microbianas e são utilizados como fonte de carbono/energia.

Os PHAs são uma promissora alternativa aos plásticos convencionais uma vez que, são polímeros de origem biológica, biodegradáveis e dispõem de uma elevada gama de propriedades termoplásticas.

Actualmente a sua produção industrial incide essencialmente na utilização de culturas microbianas puras. Contudo, apesar da elevada eficiência de produção em PHAs, a utilização de culturas puras apresenta elevados custos associados à esterilização de todo o processo, bem como a utilização de matérias-primas quimicamente definidas contendo ácido orgânicos (AOs) como fonte de carbono.

As culturas microbianas mistas (CMMs) surgem por isso como alternativa á utilização de subprodutos industriais como matérias-primas na produção destes polímeros, porém a maioria destes resíduos é produzida sazonalmente. Assim, é necessário estudar o comportamento das culturas mistas quando sujeitas a diferentes substratos. O principal objectivo deste trabalho foi então compreender em que medida o processo de produção de PHAs, através de culturas microbianas mistas, é afectado por uma mudança de substratos, nomeadamente soro de leite (SL) e melação de cana-de-açúcar (MCA), simulando a disponibilidade sazonal das matérias-primas. Contudo, a utilização de resíduos industriais por CMM requer a conversão do seu principal constituinte, os açúcares, em AOs, que é conseguida através da sua fermentação anaeróbia.

O processo de produção de PHA por culturas microbianas mistas realizado neste trabalho compreende as seguintes etapas: (1) fermentação anaeróbica realizada num bioreactor membranar (AnMBR) dos substratos (SL e MCA) em ácidos orgânicos; (2) selecção de culturas microbianas ricas em organismos acumuladores de PHAs nos substratos fermentados produzidos na primeira etapa e quando impostas condições de fome/fartura (F/F) ao reactor; e (3) a produção de biopolímero pelas culturas seleccionadas e as matérias-primas fermentadas produzidas nas etapas anteriores.

Numa primeira fase deste trabalho estudou-se o efeito das diferentes matérias-primas (SL e MCA) na fermentação acidogénica (etapa 1). Assim, o AnMBR foi operado com SL até estado estacionário. Posteriormente procedeu-se sua à substituição por MCA, tendo sido observado um período adaptação de 10 a 15 dias, por parte da cultura anaeróbia à nova matéria-prima. Contudo, quando este último foi novamente substituído por SL, a cultura respondeu rapidamente a esta mudança, sendo que apenas foram necessários, aproximadamente, 7 dias para a sua adaptação.

Em termos de perfis de ácidos orgânicos constatou-se que os mesmos foram similares em ambos os períodos cujo SL foi suplementado ao reactor anaeróbio (%g-COD HAA/g-COD S): 65% acetado, 10% propionato, 22% butirato, 2% valerato, e 1% lactato. Estes resultados demonstram assim a reprodutibilidade do sistema. Por sua vez, a fermentação acidogénica de outros substratos,

nomeadamente do MCA, resultou num perfil de ácidos orgânicos consideravelmente diferente (%g-COD HAA/g-COD S): 24% acetato, 38% propionato, 19% butirato, e 19% valerato.

Numa segunda fase deste trabalho, diferentes substratos fermentados foram utilizados na selecção de microorganismos acumuladores de PHAs, sob condições de fome/fartura num SBR (etapa 2). Inicialmente o SBR foi então alimentado com uma solução sintética de AOs; e seguidamente por MCA fermentado (fMCA) e SL fermentado (fSL) conforme foram produzidos no AnMBR. Após terem sido atingidos os estados estacionários com os diferentes substratos, realizaram-se ensaios de acumulação de polímero com as culturas mistas seleccionadas e as respectivas matérias-primas (etapa 3). Rendimentos em polímero de 0.74, 0.49, e 0.73 C-mol PHA/C-mol AOs e conteúdos celulares em polímero de 60%, 56% e 65 % g PHA/g VSS foram obtidos nos ensaios de acumulação com as culturas alimentadas com a solução sintética de AOs, o fMCA e o fSL, respectivamente. No que diz respeito á composição do polímero produzido constatou-se que a mesma está directamente relacionada com o perfil de ácidos orgânicos dos substratos fermentados.

Embora estudo do efeito da modificação sazonal de substratos, no processo de produção de PHAs por CMM, ainda se encontre numa fase embrionária, este trabalho demonstrou que é possível a selecção de culturas acumuladoras de PHAs capazes de adaptar o seu metabolismo a diferentes substratos. Consequentemente, inúmeras matérias-primas poderão ser utilizadas como fonte de carbono, bem como a melhorar estratégias tendo em vista à optimização das etapas fermentação acidogénica, selecção de culturas e produção de polímero.

Palavra-chave: Biopolímeros; Polihidroxicanoatos (PHA); Culturas microbianas mistas (CMM); Fermentação acidogénica; Melaço de cana-de-açúcar; Soro de leite; Fartura e fome; sazonal.

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyl fatty acids, which are accumulated in microbial cells as carbon/energy reserves.

PHAs are bio-based and biodegradable and display a wide range of thermoplastic properties, being a promising alternative to conventional plastics.

Presently, industrial PHA production was primarily based on pure microbial cultures. Although this process has high PHA production efficiency, it presents high costs associated with the use of chemically-defined feedstocks, and to the need for sterility.

An attractive feature of mixed microbial cultures (MMCs) PHAs production is the ability to use waste/surplus feedstocks. Many industrial wastes are seasonally produced making it necessary find the best method of utilization of this feedstock on PHA production process. Two different approaches might be taken account: (1) stock of industrial wastes during their production for their use throughout the year. However, the high fermentability of these agro-industrial wastes makes them susceptible to degradation during storage period; (2) the use of different feedstocks over the year according its availability. It is thus important to study MMC's response to different feedstocks. The aim of this work is study how MMC PHA production process is affected by a feedstock shift, using cheese whey (CW) and sugar cane molasses (SCM) as model feedstocks. The use of waste based feedstock by MMCs requires a previous conversion of sugars to organic acids (OAs), which is achieved through anaerobic fermentation.

In this study, a three-stage MMC PHA process was used, comprising: (1) anaerobic fermentation of surplus feedstocks to produce OAs in a membrane bioreactor (AnMBR); (2) PHA accumulating culture selection in a sequencing batch reactor (SBR) under feast and famine conditions using fermented feedstocks; and (3) PHA production using the selected cultures and the OAs produced in the earlier stages.

Initially the effect of both feedstocks (CW and, SCM) in the acidogenic fermentation (stage 1) was assessed. Firstly, the AnMBR was operated under steady state with CW. When the feedstock was changed to SCM an adaption period of about 10 to 15 days was observed. When SCM was replaced by CW a faster adaptation response, approximately 7 days, was observed.

The AnMBR reached similar OAs profiles in both phases when CW was fed (% g-COD HAA/g-COD OAs): 65% acetate, 10% propionate, 22% butyrate, 2% valerate, and 1% lactate. These results demonstrate that the system's performance is reproducible. On the other hand, the anaerobic fermentation of a different feedstock, SCM, resulted in a different OAs profile (%g-COD HAA/g-COD OAs): 24% acetate, 38% propionate, 19% butyrate, and 19% valerate.

In a second phase, different fermented feedstocks were used in the selection of PHA-storing organisms under a feast and famine regime in a SBR (stage 2). Initially the SBR was fed with a synthetic OAs solution; then fermented SCM (fSCM) and fermented CW (fCW) were subsequently fed as they were produced in the AnMBR. The adaption of the MMC to fSCM was faster than the adaptation to fCW. Whenever steady state was reached, PHA accumulation tests were performed using the enriched MMC fed with the corresponding feedstock (stage 3), namely synthetic OAs

solution, fSCM, and fCW. Storage yields of 0.74, 0.49, and 0.73 C-mol PHA/ C-mol OAs were obtained with synthetic OAs solution, fermented molasses, and fermented CW, respectively. The culture reached a maximum PHA content of 60%, 56% and 65%, when feedstock fed were synthetic OAs solution, fSCM and fCW, respectively. A direct relation between the used feedstock and the polymers composition was observed, which was related with the different OAs profile.

Even though, the shift of complex feedstock in three-stage MMC PHA process is still at a very early stage of development, this work illustrates the advantage of favoring the selection of cultures with the capacity to adapt its metabolism to different feedstocks. This will offer the possibility of using numerous substrates and improving strategies to optimize acidogenic fermentation, culture selection and polymer production.

Keywords: Biopolymers; Polyhydroxyalkanoates (PHA); Mixed Microbial Cultures (MMC); Acidogenic Fermentation; Sugar Cane Molasses; Cheese Whey; Feast and Famine; Feedstock Shift.

Abbreviations

%PHA max – Maximum PHA content, in;

ADF – Aerobic Dynamic Feeding;

AnMBR – Anaerobic Membrane bioreactor;

C/N – Carbon to nitrogen ratio, in C-mol/N-mol;

C/N/P – Carbon to nitrogen to phosphorus ratio, in C-mol/N-mol/P-mol;

CSTR – Continuous stirred tank reactor;

CW – Cheese whey;

%DA – Degree of acidification, $\%(\text{g-COD OAs/g-COD S})$;

F/F – Feast to Famine ratio, in h/h;

fCW – Fermented cheese whey;

fSCM – Fermented sugar cane molasses;

HA – Hydroxyl acid;

HAc – Acetic acid;

HB – Hydroxybutyrate;

HBut – Butyric acid;

HHx – Hydroxyhexanoate;

HLac – Lactic acid;

HPLC- High Performance Liquid Chromatography;

HProp – Propionic acid;

HRT – Hydraulic retention time, in days;

HV – Hydroxyvalerate;

HVal – Valeric acid;

mcl-PHA- Medium chain length polyhydroxyalkanoates;

MMC- Mixed microbial cultures ;

OAs – Organic acids;

OLR – Organic loading rate, in C-mmol/L.d or g-CO g-COD/L.d;

PE – Polyethylene;

PHA – Polyhydroxyalkanoates;

qOAs – Organic acids production rate, in g-COD OAs/gVSS h;

-qOAs – Organic acids uptake rate, in C-mol OAs/C-mol X h;

qPHA – PHA production rate, in C-mol OAs/C-mol X h;

-qS – Substrate uptake rate, in g-COD S/gVSS h;

rOAs – Organic acids volumetric productivity, in g-COD OAs/L d;

SBR – Sequencing Batch Reactor;

SCM – Sugar cane molasses;

SRT – Sludge retention time, in days;

StSt – Steady-state;

Sug - Sugars;

TN_b – Total nitrogen;

TOAs – Total organic acids, in g-COD OAs/L or C-mmol/L;

VFA – Volatile fatty acids, in C-mmol VFA/L;

VSS – Volatile suspended solids, in gVSS/L;

X – Active biomass;

Y_{OAs} – Organic acids yield, in g-COD OAs/ g-COD S;

Y_{PHA} – PHA storage yield, in g-VSS/g-COD S;

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Chapter I – Introduction

In the past few years, it has become increasingly clear a dependency of the world from crude oil and its byproducts. The necessity to find a balance between Nature and Man has intensified the search for new renewable resources, as alternative to fossil fuels and, therefore, attain a sustainable industry, environmental efficient and green chemistry (Akaraonye et al., 2010).

Plastics are one of the largest sectors of application for crude oil, with over 200 million tons of plastic consumed per year and a growth of 5% per annum (EPA, 2000). However, in the eminence of “depletion” of crude oil as well as the constant fluctuation of its prices, it becomes necessary to find new alternatives (Albuquerque, 2009; Reis et al., 2011). Furthermore, synthetic-plastics are recalcitrant to biological degradation, which causes the accumulation of millions of tons of wastes every year in landfills and marine environments. In fact, only 20% of the synthetic-plastics are recycled or incinerated (Albuquerque, 2009).

Bioplastics appear as a possible response to the problematic of harmful effects of the plastics which are petroleum derivatives, since they can be biodegradable, biobased or both. Therefore, it may be possible to reduce greenhouse emissions and the use of nonrenewable resources (Salehizadeh H. and Van Loosdrecht M.C.M., 2003; Reis et al., 2011).

1.1. Polyhydroxyalkanoates: a family of bioplastics for the future.

Polyhydroxyalkanoates (PHAs) are one of the most well-known groups of biodegradable plastics. They are completely bio-based, as they can be produced from renewable carbon sources. Moreover, their thermoplastic and elastomeric properties are similar to those of conventional plastics such as polyethylene and polypropylene, with the advantage of being both biodegradable and biocompatible (Salehizadeh and Van Loosdrecht, 2003; Albuquerque et al., 2010)

PHAs are polyesters of hydroxyl fatty acids accumulated into microbial cells, in amorphous state, as granules, and are used as carbon/energy reserves (Salehizadeh and Van Loosdrecht, 2003; Johnson, 2010; Reis et al., 2011)

1.1.1. Polyhydroxyalkanoates: Chemical Structure, Properties and Applications.

➤ Chemical Structure

PHAs can be applied in several industry sectors. However, their applications are dependent on their structure, which can lead to different physical and chemical properties, ranging from stiff and brittle plastics to elastomers (Platt, 2006; Albuquerque, 2009)

Hydroxyalkanoic acids monomers are the mainly constituent of PHAs, and can be classified into two groups depending on the length of the carbon chains: short chain length (SCL) PHAs, which consist of 3 to 5 carbon monomers (C3-C5); and medium chain length (MCL) that have 6 to 14 carbon monomers (C6-C14) (Khanna and Srivastava, 2005; Platt, 2006; Albuquerque, 2009; Liu et al., 2009; Akaraonye et al., 2010). Each type of PHA generally consists of 100-30000 monomers and the polymer molar mass ranges from 1×10^4 to 3×10^6 Da (Sudesh, 2000; Albuquerque, 2009).

Poly(3hydroxybutyrate), P(3HB), was the first PHA to be identified on *Bacillus megaterium* by Lemoigne M., (1926) and is the most common type of PHA produced by various microorganisms in nature. Wallen and Rohwedder, (1974) also discovered different repeating units of HAs, such as hydroxyvalerate (HV) and, Hydroxyhexanoate (HHx). Subsequently more than 150 different types of HA monomers have been recognized, being synthesized by over 300 bacterial (Sudesh et al., 2000; Albuquerque, 2009). Depending on the kind of monomers present in their structure PHAs can be: homopolymers, containing one type of a repeating units or heteropolymers (copolymers), which have more than one type of HA (Sharma et al., 2011).

In Figure 1.1 the general chemical structure of PHA monomers is represented, with n indicating the number of carbons in the linear polyester structure and R_1 and R_2 the variable hydrocarbon side chains.

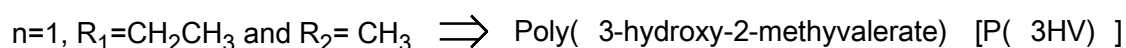
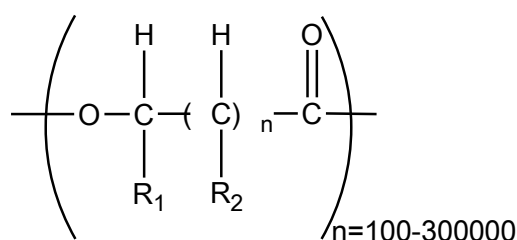


Figure 1.1 - General chemical structure of PHA monomers that constitutes polymer macromolecules (Adapted from Albuquerque, (2009)).

➤ Properties

The properties of PHAs can differ depending on the composition of the monomer units in the polymer backbone. P(3HB) is a typically thermoplastic polymer with a melting point (T_m) of 180 °C, a glass transition temperature (T_g) of 4 °C. It is highly crystalline (55-80%), brittle and stiff, limiting it to some applications (Volova, 2004; Albuquerque, 2009; Reis et al., 2011; Sharma et al., 2011). The incorporation of other HA units on the P(3HB) structure, such as 3-hydroxyvalerate (3HV), to form a PHA copolymer can improve its physical properties. These polymers exhibit lower crystallinity, and lower melting and glass temperature transitions, enabling their thermal processing without a thermal degradation at lower temperatures and reducing molecular weights losses. Furthermore, the presence of HV monomers also makes the polymer tougher, which means it is more resistant to impact strength, and decreases the Young's modulus, *i.e.* the flexibility increases (Khanna and Srivastava, 2005; Reis et al., 2011). Introduction of HA monomers, other than 3HV, on the P(3HB) structure also originates copolymers with different mechanical properties, as shown in Table 1.1.

Table 1.1 - Physical-chemical and mechanical properties of polyhydroxyalkanotes (PHAs) polymers (Adapted from (Khanna S. and Srivastava A. K., 2005; Liu X. et al., 2009).

Properties Polymer	Melting point, T_m (°C)	Glass transition temperature, T_g (°C)	Tensile strength (MPa)	Young's Modulus (GPa)	Elongation to break (%)	Crystallinity (%)
P(3HB)	180	4	43	3.5	5	55-80
P(3HB-co-3HV)						
3 mol% 3HV	170	8	38	2.9	-	-
14 mol% 3HV	150	4	35	1.5	-	-
25 mol% 3HV	137	-6	30	0.7	-	-
P(4HB)	53	-48	104	-	1000	34
P(3HB-co-4HB)						
16 mol% 3HV	130	-7	26	-	444	45
78 mol% 3HV	49	-37	17	-	1120	17
90 mol% 3HV	50	-42	65	-	1080	28
P(3HBco-3HHx)	52	-4	20	-	850	-
Polypropylene	170	45	34.5	1.7	400	-
Low density polyethylene (LDPE)	130	-30	10	0.2	620	-

➤ Applications

PHAs have a wide range of applications owing to their features, which enable their use in several sectors of industry. They can be used as cover paper or cardboard to make water-resistant surfaces, being better than aluminium which is utilized nowadays and is non-biodegradable (Philip et al., 2007).

In medicine biodegradable polymers are playing an important role. PHAs are frequently applied in tissue engineering, such as bone plates, osteosynthetic material and surgical sutures. They are also useful in the slow release of drugs and hormones (Philip et al., 2007).

One of the major contributions of PHAs to the medical field has been in the cardiovascular area. Tephra Inc., a USA company, has specialized in manufacturing pericardial patches, artery augments, cardiological stents, vascular grafts sutures, dressings, etc, (Williams and Martin , 2002).

In the agricultural sector, PHAs have been used as mulch films (Hocking P. J. and Marchessault et al., 1994). Recently, Procter & Gamble has been producing Nodax™ which is a copolymer containing mainly 3HB and small quantities of MCL monomers. This product can be used as a coating for urea fertilizers, which are used in rice fields, or for herbicides and insecticides (Philip et al., 2007).

1.2. Current mixed microbial culture PHA synthesis

Industrial PHA production is nowadays based on pure microbial cultures in their wild form or genetically modified strains. They may accumulate PHA up to 90% of the cell dry weight, but the high costs of substrate and sterilization have an economically negative impact on PHA production by pure cultures (Dias et al., 2006; Serafim et al., 2008; Jiang Y., 2011).

In recent years, attempting to reduce PHA production costs, low value substrates as waste feedstock, and microbial mixed cultures (MMC) have been investigated (Dias et al., 2006; Serafim et al., 2008).

MMC are, usually, microbial populations of unknown composition, which are selected by the operational conditions imposed on the system (Dias et al., 2006). The success of PHA production by mixed cultures is due to the enrichment of an open culture with microorganisms with superior ability to accumulate PHAs in high amounts. This enrichment can be attained through two different strategies: (1) application of alternating anaerobic and aerobic conditions to the mixed culture; or (2) enrichment under alternating feast and famine conditions, which means the presence of carbon source followed by an absence of substrate. Both strategies apply continuous dynamic conditions, which favor PHA storage capacity in one phase, and the degradation of PHA in another phase (Johnson, 2010).

1.2.1. Feast and famine (FF) or Aerobic dynamic feeding (ADF): PHA enrichment strategy

The dominant PHA enrichment strategy for mixed culture is the fully aerobic feast and famine cycle (Figure 1.2), using a sequencing batch reactor (SBR). This process, involves a simultaneous growth and biopolymer storage when external carbon source is available (feast period). Hereupon, PHA accumulated in this phase can be used as carbon/energy reserve when an absence of substrate is verified. Therefore only the microorganisms that accumulated polymer in the feast phase can be survived to the starvation period (Johnson, 2010; Jiang, 2011). At the end of each feast-famine cycle, excess of biomass produced is withdrawal, favoring selective pressure of the PHA-accumulating organisms.

It is important to understand that the storage capacity appears when excess carbon source is available and growth limiting conditions are, simultaneously, imposed to the aerobic bacteria culture (Salehizadeh and Van Loosdrecht, 2003; Reis et al., 2011).

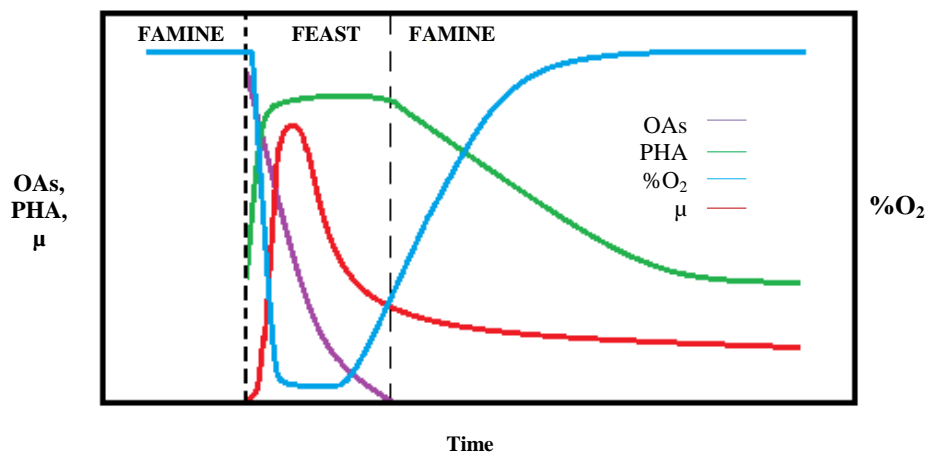


Figure 1.2 – Typical SBR cycle of an aerobic PHA storing culture. (Adapted from Albuquerque, (2009))

1.2.2. Three-step process for PHA production by mixed microbial cultures from waste streams

PHA production by MMC from complex feedstocks, such as surplus from different agro-industrial sectors, is based on a three-step process, comprising: (1) acidogenic fermentation of carbohydrate-rich feedstocks to organic acids (OAs); (2) PHA storing culture selection and biomass production with a high and stable PHA storage capacity, using the fermented feedstock produce in

the first stage; and (3) PHA production with the selected culture and the organic acids produced in the previously stages. The physical separation of the culture enrichment and PHA production stages allows the optimization of the process, since different optimal conditions were shown to be required in each step (Johnson, 2010). PHA produced in the accumulation step is then extracted and purified (Albuquerque, 2009).

Figure 1.3 presents a scheme of the three-step process for PHA production by mixed microbial.

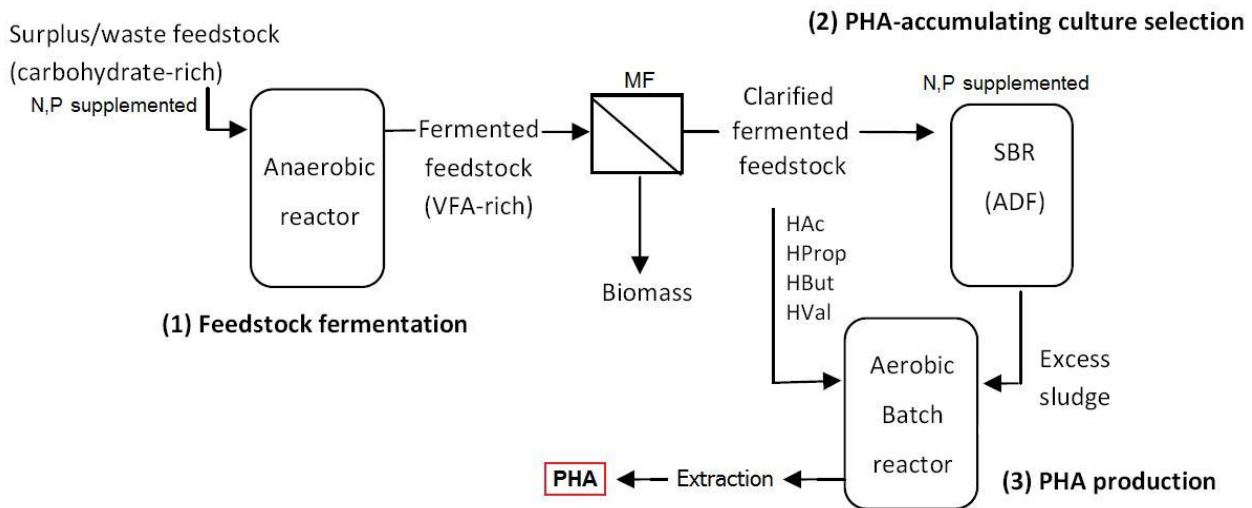


Figure 1.3 – Three-step process for PHA production by mixed microbial cultures from waste streams (adapted from Albuquerque, (2009)).

❖ Acidogenic Fermentation

Carbohydrates are the main constituents of waste feedstocks used in the three-step PHA production process by MMC. However, they cannot be directly process in PHAs. In fact, PHA-accumulating organisms do not store sugars as polymer but rather as glycogen (Carta et al., 2001). Therefore, a previous anaerobic phase is necessary to convert sugars into OAs, the precursors of polyhydroxyalkanoates. (Figure 1.3 - stage 1), (Bengtsson et al., 2008; Albuquerque, 2009).

In anaerobic digestion, the organic matter is converted into fatty acids (acidogenesis), and these into biogas (methane and carbon dioxide) if operating conditions allow it (Albuquerque, (2009)). As a consequence, acidogenic fermentation has to be designed to maximize the acidogenesis instead of methane production. Several authors have been studying the effect of operational conditions (such as pH, HRT, temperature, hydrogen partial pressure, etc.) on the resulting products of feedstock acidification (organic acids, alcohols, etc.) (Fang and Yu, 2001; Yu and Fang, 2003; Bengtsson et al., 2008; Yuan et al., 2011; Arroja et al., 2012). Moreover, different

organic acids profiles can be attained and, consequently, the composition of PHA produced in later stages (Albuquerque, 2009).

❖ PHA – Accumulating Culture Selection

The second stage of the three-step process for PHA production by MMCs is the culture enrichment on PHA-accumulating organisms using alternating presence and absence of the carbon source, feast/famine (F/F) periods, respectively, as selective pressure (Johnson, 2010; Jiang, 2011). Indeed, the effectiveness of this stage is determinant for the development of stable and highly adapted mixed culture to F/F regime, with a high PHA storage capacity. Therefore, the first aim of this stage is to sustain a high selective pressure for favoring a PHA-storing culture and avoiding non-storing organisms, which have a negative impact not only in PHA productivity, but also on the downstream processing, increasing polymer extraction costs (Albuquerque, 2009; Reis et al., 2011).

A second goal of the selection stage is to produce the enriched culture for the following PHA accumulation stage. Therefore, higher biomass volumetric productivities are desirable. Despite PHA production by MMCs have been increasing in terms of specific storage rates, PHA contents and storage yields, the volumetric productivities attained by MMCs still fall short when compared to those obtained with pure cultures. Indeed, the use of feast and famine conditions to carry out culture selection limits the culture's primary metabolism (cell growth). Therefore, lower cell densities and volumetric productivities are observed in PHA production stage (Reis et al., 2011).

The most common reactor type used for biomass enrichment step is the SBR, which is ideal to achieve the feast-famine conditions regime in a continuous mode (Johnson, 2010). Each SBR cycle consists, usually, of four discrete periods: (1) fill, in which reactor is filled up with fresh medium; (2) aerobiosis, when feast and famine regime occurs; (3) settling phase, when the reactor is not aerated, nor stirred; and (4) exhausted supernatant withdrawal (Johnson, 2010; Reis et al., 2010). The common values of operating parameters used for PHA-accumulating enrichment culture in MMC processes are: 1 - 20 days of sludge retention time (SRT), 1.8 - 31.25 g/L.d of organic loading rate (OLR), 0.18 - 2.7 g/L of substrate concentration, 10 - 140 C-mol/N-mol C/N ratio, pH between 7 and 9, and 2 to 12 h of SBR cycle (Albuquerque, 2009).

✓ Factors that affect mixed microbial culture selection under FF conditions

Several authors have been studying the operating conditions that influence the performance of the culture selection stage, such as feast to famine ratio (F/F ratio), OLR, SRT, pH, carbon to nitrogen ratio (C/N), hydraulic retention time (HRT), substrate, and nutrients concentration (Beccari et al., 2009; Albuquerque et al., 2010a; Albuquerque et al., 2010b; Johnson et al., 2010; Albuquerque et al., 2011; Arroja et al., 2012).

The amount of substrate supplied, and consequently the OLR, is a critical factor to the effectiveness of PHA-accumulating culture selection. Indeed, in order to select microbial cultures with high storage capacity, the SBR has to be operated at higher OLR. However, if OLR is too high, the F/F ratio is diminished, which suggests the needed of an optimal OLR to be found (Vilano et al., 2010). Albuquerque, et al. (2007) studied the effect of OLR on a SBR operated without pH control, 12h cycle, SRT of 10 day, 25°C, 1.4 and 2.5 N-mmol/L, and two OLR of 120 and 60 C-mmol/L.d. The authors demonstrated that PHA storage capacity was lost at higher OLR for similar cycle length and nutrient conditions resulting in inhibition by high substrate concentration (Serafim, et al. 2008; Albuquerque, 2009).

F/F ratio is directly related with the changes on OLR and influent substrate concentration. If the OLR or substrate concentrations are low (lower F/F ratios) the fast PHA-storing microorganism will be enough to quickly remove substrates and store it as PHA, and a feast/famine regime will be established. Consequently, these microorganisms will be better fit to survive the starvation phase (famine). On the other hand, if OLR or substrate concentrations are too high, resulting in high F/F ratios, PHA-storing microorganisms will not be able to quickly remove all available substrate (PHA saturation) allowing non-storing microorganisms to grow unconstrained during the feast periods. In this manner, the competition among these organisms will be based on culture growth, and not on the PHA storage capacity. Thus, the selective pressure favouring storing microorganisms will be partially lost (Dionisi, et al. 2006; Alkaya and Demirer, 2010; Albuquerque, et al. 2011; Reis, et al. 2011; Arroja, 2012) studied the effect of OLR (in the range of 8.5-31.25 g-COD/L.d, and F/F ratios range 0.10-1.15 h/h, respectively) on the performance of SBR culture selection, using a mixture of acetic, lactic, and propionic acids. The authors showed a growth response at higher OLR and, consequently, F/F ratios higher than 0.9 h/h. On the other hand, at lower OLR values (F/F ratios up to 0.26 h/h) a storage response was observed. Albuquerque, et al. (2011) studied the effect of influent substrate concentration (30 to 60 C-mmol VFA/L) on the selection PHA-storing culture, using fermented sugar cane molasses. A similar trend was showed and the predominant storage response was observed at lower influent substrate concentration (1.6 g-COD/L), which resulted in a low F/F ratio, 0.21 h/h.

The effective growth rate of a culture is given by the reciprocal of the SRT (Reis et al. 2011). Thus, a lower SRT can be representative of a higher growth rate, which means that a higher fraction of the substrate is being used for growth, instead of storage polymer. Beun J. et al., (2002) confirmed this assumption, and observed that, for SRTs lower than 2 days, a polymer storage yield and productivity decreased sharply with the decreasing SRT. On the other hand, a SRT higher than 2 days and for specific acetate uptake rates over 0.3 C-mol/C-molX.h, the PHA yield from acetate under excess nutrients was constant (reviewed by Albuquerque, 2009)

The C/N ratio used in the selection stage will not only affect the ratio of carbon driven toward PHA storage versus growth during feast but also affect the growth yield during the famine phase. Under excess of nutrient (throughout the full F/F cycle), the selected culture will be able to effectively use the intracellularly stored polymer to grow during the famine phase of the SBR cycle, thereby also obtaining a higher famine phase growth yield (Reis, et al. 2011). Moreover, PHA-storing organisms

will have two major competitive advantages: the faster rate of substrate uptake during feast enables them to rapidly deplete the limited available carbon substrate, while the presence of nutrients throughout the full cycle offers the possibility of growth at an almost constant rate (using the intracellular polymer) (Johnson, 2010; Reis et al., 2011). Consequently, volumetric productivity will increase with decreasing C/N ratio (Reis et al., 2011).

The majority of studies performed with MMC under feast and famine conditions have demonstrated an increase of pH during feast phase, as result of organic acids uptake, until a stabilized value (Albuquerque, 2009). Villano et al., (2010) investigated the effect of pH, over the 7.5 to 9.5 range on PHA production by mixed cultures enriched in a SBR using a mixture of acetate and propionate as substrates. Higher polymer production rates and yields were shown when the pH was controlled at 7.5, and those parameters have decreased as pH increased to 9.5. Chua et al., (2003) also studied the effect of pH on culture selection, using acetate as carbon source. It was observed that cultures acclimatized under pH 7 and 8 conditions in SBRs exhibited similar PHA storage capability. However, in PHA production assays, pH value influenced significantly the PHA accumulation behavior of the culture. The authors have shown that when pH of batch experiments was controlled at 6 or 7, a very low PHA production was observed. On the other hand, the production of PHA was stimulated when pH was kept at 8 or 9.

❖ PHA-accumulation stage

The PHA accumulation step is strongly dependent of good optimization of the previous PHA-accumulating selection stage since it is in this step that the performance of the stable enriched PHA-accumulating organisms will be evaluated. This stage is carried out in a fed-batch mode under external or internal limiting conditions, such as lack of nutrients or lower RNA enzyme levels, respectively. In this manner, if at the same time, an excess carbon source is available, maximization of PHA storage can be achieved (Albuquerque, 2009).

Attempting to improve the performance of the selected PHA storage culture and adjust the polymer composition to final application, lead several authors to study different strategies to maximize PHA production and its stability as polymer (Dionisi et al., 2005; Beccari et al., 2009; Bengtsson et al., 2010; Johnson K., 2010; Albuquerque et al., 2011).

Albuquerque et al., (2011) studied the effect of substrate composition and, feeding regime (continuous or pulses) on a PHA production stage, using sugar cane molasses as feedstock. The authors have shown that if a continuous feeding strategy was used in PHA production rather than a pulse feeding mode, higher substrate uptake rates and polymer storage yields were observed, mainly due to the constant residual substrate concentration in the reactor when a continuous feeding regime was applied. Moreover, the use of continuous feeding regime increased the percentage of HV monomer in the final polymer composition. Therefore, a variety of polymer structures were synthesized, which subsequently resulted in different polymer properties for different applications.

The effect of pH variation on the PHA production by a SBR enrichment culture selected on a mixture of acetic, lactic and propionic acids was also investigated by Dionisi D. et al., (2005). The

authors have shown that maximum productivities in PHA production stage were obtained at the same pH used for enrichment PHA accumulation culture selection stage or, at a slightly higher value (pH 7.5 and 8.5). Thus, it was demonstrated that each culture performs optimally close to its respective optimum pH value (Reis et al., 2011).

The PHA accumulation stage finishes with the polymer recovery. Several methods for PHAs extraction have been studied. The most common is the extraction of PHAs with organic solvents, such as chloroform, dichloroethane, propylene carbonate and methylene chloride. However, the large amount of solvent required and the toxic nature of the chemical compounds used in the extraction makes this method economically and ecologically unattractive (Albuquerque, 2009; Reis, et al. 2011).

1.3. Substrate selection: a critical factor of polyhydroxyalkanoates production

PHAs can be applied in several industrial sectors, namely, medicine, agriculture, pharmacology, food industry and engineering. However, the substrate selection is a critical factor for its production since the quantity to use, the type of substrate, the quality of the same and the cost, changes for each application. Furthermore, feedstocks constitute the major cost in PHA production and influence the bacterial performance and the final product. Thus, a good approach in the selection of the substrate must consider all these factors (Volova, 2004; Chee, et al. 2010b).

Microorganisms are capable of producing PHA from various carbon sources such as complex waste effluents of various industries (cheese whey, molasses, etc.) as well as simple compounds like alcohols, carbohydrates, acids, sugars and carbon dioxide. In this manner, the amount of industrial wastes that are discharged every year in landfills and marine environments can be utilized as carbon source, which contribute not only for a reduction of substrates costs and, consequently, the cost of PHA, but also for lowering the costs of waste disposal (Volova, 2004; Chee et al., 2010b).

In Table 1.2 the price of some substrates used in PHA production are presented.

Table 1.2 – Substrate cost (Volova T. G., (2004)).

Substrate	Aproximate price, US \$ kg ⁻¹
Sucrose	0.290
Glucose	0.493
Ethanol	0.440
Methanol	0.110
Acetate	0.595
Dextrose	0.360
Hydrogen	0.500
Cane sugar	0.200
Sugar Molasses	0.220
Cheese whey	0.071
Hemicellulise hydrolysate	0.069

PHA production from waste streams is confronted with other problems, *i.e.*, the availability of the feedstock during an entire production year. In fact, some waste streams are seasonal. In this manner, for the planning and design of the PHA production facilities it is crucial to consider the seasonal availability of the waste-based feedstocks.

○ Sugar Molasses

Sugar molasses is a liquid residue resultant of the condensation of sugar cane or sugar beets until crystal precipitate. The high sugar content, 48 to 50 %, is result of the presence of glucose, sucrose and fructose. Also water and polysaccharides (dextrin, pentosans, polyuronic acids) are constituents of sugar molasses, ranging between 17 to 25%, and 2 to 5%, respectively (Arroja L., (2012)).

About 145 million sugar tons are produced worldwide each year. In 2004, the global sugar production generated about 39 million tons of sugar cane molasses and 12 million tons of beet molasses, which generates environmental residues (<http://melasse.de/index.php?id=originsofmolasses>).

- **Cheese Whey**

Cheese whey is a major by-product of cheese-manufacturing or casein from bovine milk. It represents 80 to 90% of the total volume of milk transformed and retains 55% of milk nutrients. Lactose (4.5 – 5 (%w/w)), lipids (0.4 – 0.5 (%w/w)) soluble proteins (0.6 – 0.8 (%w/w)) and mineral salts (8 – 10 (%w/w)) are the main constituents of cheese whey. However, only lactose, lipids and soluble proteins are fermentable (Siso, 1986; Farinha, 2009). In 2000, in the European Union 6.385.000 tons of cheese were produced, resulting in 40.5 million tons of whey. Despite 67% of this whey is used for the production of animal feed, the remaining 33%, which represent 13.5 million tons of whey, constitute a serious disposal problem for the dairy industries (Koller, 2008; Farinha, 2009).

To overcome the environmental problem, and considering the high content of lactose and proteins on cheese whey, and fructose, glucose and sucrose on molasses these fermentable feedstock have been used for production of valuable products, such as polyhydroxyalkanoates (Farinha, 2009).

Chapter II – Thesis Motivation and Outline

2.1. Thesis Motivation

Plastics are one of the main sectors of application for crude oil. They have become an important commodity of quotidian life and in almost all industries. However, these petroleum based-products are the most contributors to the millions of tons of wastes accumulated every year in the environment. Bioplastics, such as PHAs, offer the best possible response to the problematic of conventional plastics harmful effects.

In the last decade, alternative pure cultures PHAs production processes, aiming to substantially decrease their cost, have been developed. Such alternative processes include the use MMCs which do not required sterile conditions.

One big advantage of MMC PHA production processes is that cultures can use a range of low cost complex feedstocks, such as agricultural and industrial residues or by products, namely starch, tapioca hydrolysate, cheese whey, sugar cane molasses, malt, and soy (Dias et al., 2006). Furthermore, from their acidogenic fermentation can result different OAs profiles. Consequently, a variety of polymer compositions can be achieved and their utilization in different applications sectors will be possible. However, many of these industrial wastes are seasonally produced creating serious difficulties concerning to the sustainability of production of this polymer. In fact, the quality and the polymer composition cannot be guarantee under these conditions. It is thus important to study MMC's response to different feedstocks according its availability during the year.

In this work, a three-stage PHA production process by MMC from two different surplus feedstocks, SCM and CW, was studied in order to understand the MMC's response to a feedstock shift, mimicking a seasonal feedstock scenario.

2.2. Dissertation Objectives

In the last few years, more cost-effective mixed microbial PHA production processes have been studied by several authors. Nevertheless the considerable effort and knowledge gathered on PHA storage by mixed cultures, a question remain without an answer, namely if the MMC will be capable to adapt its metabolism to a feedstock shift.

By mimicking a seasonally feedstock modification the major goals of this thesis were:

- (1) to study the individual effects on each of the three-stage processes. For that purpose, two waste-based feedstocks, cheese whey and sugar cane molasses, were used as model feedstocks with different composition.
- (2) to understand how a feedstock shift affects agro-industrial waste acidogenic fermentation. Cheese whey and sugar cane molasses were used as model feedstock on OAs production.
- (3) To assess the effect of clarified fermented substrates (fCW and fSCM), produced in acidogenic fermentation, or a synthetic OAs solution shift on PHA-accumulating culture selection reactor performance;
- (4) to evaluate the PHA production performance of the cultures selected on synthetic OAs solution, fSCM, and fCW.

2.3. Thesis Outline

This thesis is composed of four chapters, including an overview of the state of the art on PHA production (**Chapter I**) and the motivation and outline of the work developed (**Chapter II**). The following chapters include the description of the work developed in order to accomplish the objectives of this thesis (**Chapter III and IV**).

The state of the art (**Chapter I**) starts by mirroring the dependency of the world on petroleum-based byproducts, their environmental impact, and the importance of bioplastics development as a possible solution. It then focuses particularly on PHAs, describing their mechanical, physical and thermal properties, as well as their application sectors. In the second section, the current biotechnology industrial approach to PHA production by MMC, and an overview of PHA production mechanisms by open culture systems, enriched in PHA-accumulating organisms solely by operational conditions imposed on the reactor are described. Finally, in the third section the importance of using low cost carbon sources is described.

Chapter III presents the impact of a feedstock shift, using cheese whey and sugar cane molasses, on anaerobic fermentation, the first step of the 3-stage MMC PHA production process.

Chapter IV focuses on the feasibility of using the clarified fermented feedstocks produced in the anaerobic fermentation stage for the selection of PHA-accumulating organisms. The effect of substrate shift on the sequencing batch reactor (SBR) performance was also evaluated, as well as PHA production using the selected cultures and the fermented feedstocks previously produced.

Chapter III – Acidogenic fermentation of cheese whey and sugar cane molasses: feedstock shift impact on organic acids production

3.1. Introduction

Several industrial wastes (e.g., effluent paper mill, cheese whey, sugar cane molasses, etc.) represent a significant loss of resources and a serious pollution problem (Saddoud et al., 2007). However, due to their high organic content, numerous microbial species, through their fermentative metabolic processes (hydrolysis, acidification, acetogenesis and methanogenesis) and under anaerobic conditions, can convert them into organic acids (OAs) (propionic, butyric, acetic, valeric and lactic acid) and other fermented products such as alcohols, carbon dioxide, and methane (Saddoud et al., 2007; Bengtsson et al., 2008). This process commonly designated as anaerobic digestion, consists: primarily in the conversion of carbohydrates to organic acids by acidogens; further OAs are converted by acetogens to acetate and H_2/CO_2 ; finally methanogens convert acetate and H_2/CO_2 into methane (Saddoud et al., 2007; Bengtsson et al., 2008; Yu et al., 2008). In anaerobic digestion, acid forming and the methane forming organisms differ widely in terms of physiology, nutritional needs, growth kinetic, and sensitivity to environmental conditions (Chen et al., 2008; Arroja et al., 2012). Therefore, in order to maintain an anaerobic sludge with a high metabolic activity to favor either OAs or biogas production, it is necessary to apply favorable environmental conditions. Among these factors are temperature, pH, the absence of toxic materials, hydraulic retention time (HRT), availability of nutrients and substrate type (Fang and Yu, 2001; Rajagopal and Béline, 2011). Fang H. and Yu H., (2001) have investigated the individual effects of pH and temperature on the acidification of a wastewater containing lactose as sole carbon source. It was concluded an optimum acidification of lactose at pH 5.5 and 55 °C, which could indicate inhibition of methanogenic fermentation. In fact, methanogenesis requires a pH near the neutral value ($6.5 < pH < 7.5$), therefore a decrease in pH might lead to a reduction of the methane production rate and further accumulation of acids (Fang and Yu, 2001). Bengtsson et al., (2008) have studied the influence of HRT in acidogenic fermentation performance of whey and paper mill effluents. The authors have shown that the produced volatile fatty acids (VFA) composition profile was strongly affected with the increasing of HRT above 10h, since butyrate production decreased and propionate production increased. Rajagopal R. and Béline F., (2011) have developed a biochemical test to characterize the hydrolytic potential of various organic wastes (secondary sludge, pre-treated sludge, pig and cattle slurries). It was observed that the performance of hydrolysis and acidification steps is considerable dependent of the nature of substrate. Secondary sludge was the substrate which produced higher amount of OAs.

Organic acids have been used as substrate for microbial production of polyhydroxyalkanoates (PHAs) (Bengtsson et al., 2008). Substrate cost is still the major contributor

to PHA final cost. Low value feedstocks utilization (wastes and agro-industrial effluents) effectively reduces the negative economic impact on PHA production. However, some waste streams are seasonally available which is crucial to consider on organic acids production.

The aim of this work was to study how OAs production process was affected by feedstocks shift, mimicking a seasonal availability of substrates. Two agro-industrial waste/surplus feedstocks, cheese whey and sugar cane molasses, were used as feedstock.

3.2. Materials and Methods

3.2.1. Feed preparation

In this study, sugar cane molasses (SCM) and cheese whey powder (CW) were used as substrate for organic acids (OAs) production. These wastes/surplus based feedstocks were obtained from Refinaria de Açúcares Reunida (RAR, Porto), and from Lactogal (Porto), respectively.

SCM has a very high sugar content, 54 % (w/w M), consisting mainly of sucrose (62%) and fructose (38%) (Albuquerque et al., 2007). On the other hand, cheese whey powder is composed by 78 % (w/w CW) of lactose, 13.62 % (w/w CW) of protein and 1.21 % (w/w CW) of lipids (source: Lactogal).

Both carbon substrates were diluted with tap water in order to obtain a final sugar concentration of ca. 15 g/L, and were kept at 4 °C in a continuously stirred vessel. The SCM medium was supplemented with a micronutrients solution which is necessary for biomass growth. Therefore, NH_4Cl (0.10 g/L), and KH_2PO_4 (0.08 g/L) were supplied as source of ammonia and phosphate, respectively. Due to their high protein content, CW was not supplemented with nutrients.

3.2.2. Acidogenic fermentation reactor set up and operation

Acidogenic fermentation was performed in a laboratory scale anaerobic membrane bioreactor (AnMBR) (Figure 3.1) which consisted on a reactor with a working volume of 1250 ml coupled to an ultrafiltration hollow fiber membrane module (5×10^5 MW cut-off). Stirring was kept on 200 rpm being performed with magnetic stirrers and feeding with a peristaltic pump. CW and SCM feed solutions flow rates were adjusted to keep the reactor HRT at 1 day and an OLR of 15 g Sug/L.d.

The anaerobic bioreactor operated in continuous mode, under controlled pH 6, by addition of 2M NaOH, and temperature controlled at 37 °C with a water jacket and a hot water bath. Oxidation-reduction potential was monitored. The reactor biomass was purged by overflow in order to obtain a solids retention time (SRT) of 3 days. The effluents (fCW and fSCM) were clarified through ultrafiltration hollow fiber membrane module and kept at 4°C prior to its use as substrate for the

culture selection SBR (stage 2) or in PHA batch accumulation assays (stage 3). Sampling was taken daily from the reactor, in order to monitor sugars, organic acids (and other possible fermentation products), proteins, ammonia, phosphate, and biomass from volatile suspended solids (VSS). In the latter it was considered that all VSS were biomass and the volatile compounds contributor was despicable.

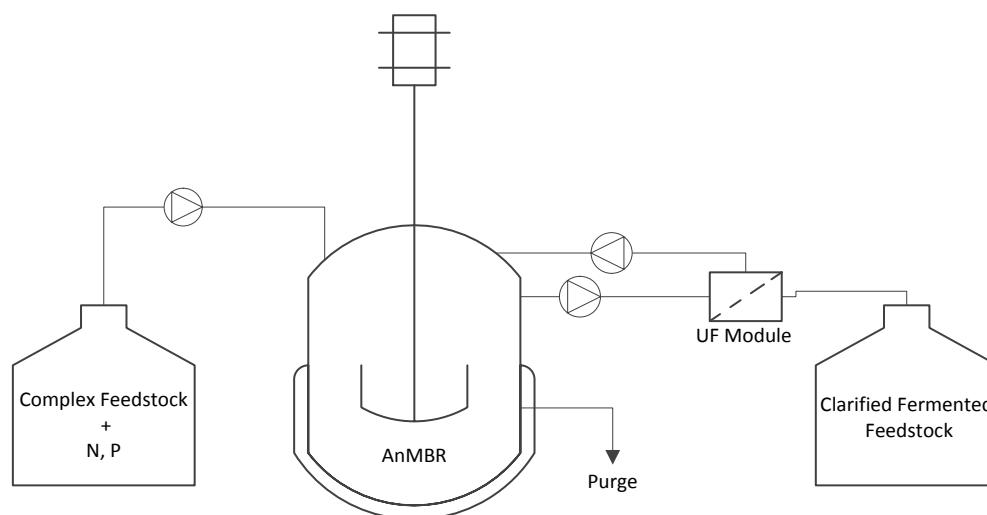


Figure 3.1- Acidogenic Fermentation set up.

3.2.3. Analytical Procedures

Ammonium concentration was measured in filtered samples (0.45 μm) with IDS3000 DIONEX ion chromatograph, equipped with a conductivity detector and a CSRS 300 (4 mm) DIONEX self-regenerating suppressor, and a IonPac CS16 column and a IonPac CG16 pre column (column temperature 30 $^{\circ}\text{C}$, methanesulfonic acid 48 mM eluent, flow rate 1 mL/min). A calibration curve was obtained with NH_4Cl standards (0.6-6 Nmmol/L).

Biomass concentration was measured as volatile suspended solids (VSS) concentration according to Standard Methods (APHA 1995).

Organic acids (acetate, propionate, butyrate, valerate, and lactate), and lactose were quantified by high performance liquid chromatography (HPLC) using a MerckHitachi chromatographer equipped with a RI detector and Aminex HPX-87H pre-column and column (BioRad, USA). Sulphuric acid 0.01N solution was used as eluent at a flow rate of 0.6 ml/min and 50 $^{\circ}\text{C}$ operating temperature. The organic acids concentrations were calculated through calibration curves in the range of 25 to 1000 mg/L. Samples were previously filtered through a 0.2 μm filter (VWR, spin filter 0.2 μm)

Protein content was determined spectrophotometrically at 750 nm as described by Lowry H. et al., (1951). Bovine serum albumin was used as calibration standard (20-200 mg/l).

Chemical oxygen demand (COD) and total nitrogen (TN_b) were analyzed using cuvette test kits (Hach-Lange, Germany).

Total sugars were determined by two different spectrophotometric methods due to the different characteristic of feedstock, namely, the color. In SCM were used the method as described Morris, (1948) with modifications by Koehler, (1952), Bailey, (1958) and Gaudy, (1962). Sucrose standards (0-100 mg/l) were used to determine total sugars. Absorbance was measured at 625 nm. On the other hand, sugars in CW feed were determined as described Dubois M. et a., (1956), using D-Lactose monohydrated as calibration standard (0-200 mg/L), instead of D-glucose monohydrated. Absorbance was measured at 490 nm.

Phosphate was analyzed by segmented flow analysis as described by (Carvalho et al., 2007).

3.2.4. Calculations

The degree of acidification (DA in %(g-COD OAs/g-COD S)) was calculated by the total organic acids (TOAs) produced divided by the waste surplus feedstock influent soluble COD (SCOD).

$$\%DA = \frac{TOAs}{SCOD} \times 100 \quad [1]$$

The OAs yield (Y_{OAs} in g-COD OAs/g-COD S) was calculated as amount of the OAs produced divided by the amount of substrate consumed.

$$Y_{OAs} = \frac{\Delta OAs}{\Delta S} \times 100 \quad [2]$$

The specific substrate uptake rate ($-q_s$ in g-COD S/gVSS h) and OAs production rate (q_{OAs} in g-COD OAs/gVSS h) were determined by applying individual mass balances to the reactor:

$$-q_s = \frac{\Delta S}{\Delta X} \times D \quad [3]$$

$$q_{OAs} = \frac{\Delta OAs}{\Delta X} \times D \quad [4]$$

where D is the dilution rate (h⁻¹); ΔS and ΔOAs are the variation of substrate consumed and organic acids produced over the time respectively; and ΔX the variation of active biomass over the time.

Organic acids volumetric productivity (, in g-COD OAs/L.d) was calculated by the total OAs produced multiple by dilution rate.

$$r_{OAs} = \sum TOAs \times D \quad [5]$$

The quantity of protein (Prot) removed for culture growth (in %(g/g)) was calculated by dividing the variation of protein inlet (TPro_{IN}) and protein outlet of the reactor (TPro_R) by the total protein inlet:

$$\text{Biological Protein removed} = \frac{TPro_{IN} - TPro_R}{TPro_{IN}} \times 100 \quad [6]$$

Total protein removal (in %(g/g)) was calculated by dividing the variation of protein inlet (TPro_{IN}) and the protein in the permeate (TPro_P) by the total protein inlet:

$$\text{Total Protein removed} = \frac{TPro_{IN} - TPro_P}{TPro_{IN}} \times 100 \quad [7]$$

Organic acids profile (in %(g-COD HA/g-COD OAs)) was obtained by fraction of each hydroxyl acid produced in relation to total OAs concentration (TOAs):

$$\%HA = \frac{HA}{TOAs} \times 100 \quad [8]$$

During AnMBR operation, average values were calculated for each operating condition.

3.3. Results and Discussion

Initially, the anaerobic MBR was operated under steady state with CW (StSt1). Subsequently, the feedstock was changed to sugar molasses, and the anaerobic culture adaptation was assessed until steady state was reached (StSt2). Finally, molasses was replaced by CW, and physiological adaptation of anaerobic culture was again evaluated until stability (StSt3). In this manner, the performance of anaerobic mixed culture to a substrate shift was monitored over the time in terms of OAs production rate, cell growth, sugars uptake rate, and organic acids profile.

3.3.1. Anaerobic fermentation response to a waste/surplus based feedstock shift

Figure 3.2 shows the organic acids production performance for the different feedstock periods. It was observed that, when CW was replaced by sugar cane molasses there was an adaptation period of about 10 to 15 days until the system reached steady state. This behavior is quite notorious between 17 and 30 days when total organic acids produced range from 8.99 to 14.53 g-COD OAs/l, respectively. In the steady state a total OAs produced of 14.8 g-COD OAs/l was obtained. On the other hand when molasses was replaced by CW, a faster adaptation response was observed. Seven days were necessary for steady state to be reached (between days 91 and 98). TOAs of 9.1 g-COD OAs/l was produced which was similar to the first period of CW (StSt1), 8.5 g-COD OAs/l. This response can be explained by the fact that AnMBR was initially operated with CW, which may have caused a selective pressure favoring organisms that convert the main carbohydrate (lactose) into OAs. Arroja et al., (2012) have studied acidogenic valorisation of high strength wastes products, such as molasses, and cheese whey, in anaerobic moving bed biofilm reactors. The effect of inoculums type was studied, *i.e.*, distinct anaerobic biomass was used, namely, acidogenic sludge previously adapted to glucose for 20 days for PHA production and conventional anaerobic biomass from full-scale municipal digester working on sludge digestion with methane recovery. The authors have shown that the biomass pre-adapted to glucose for PHA production had a better adaption to a change of substrate (glucose to sugar molasses) than the other inoculum type. Indeed, an adaptation of biomass is a feasible strategy for the efficient treatment of industrial wastes and recovery of bioproducts.

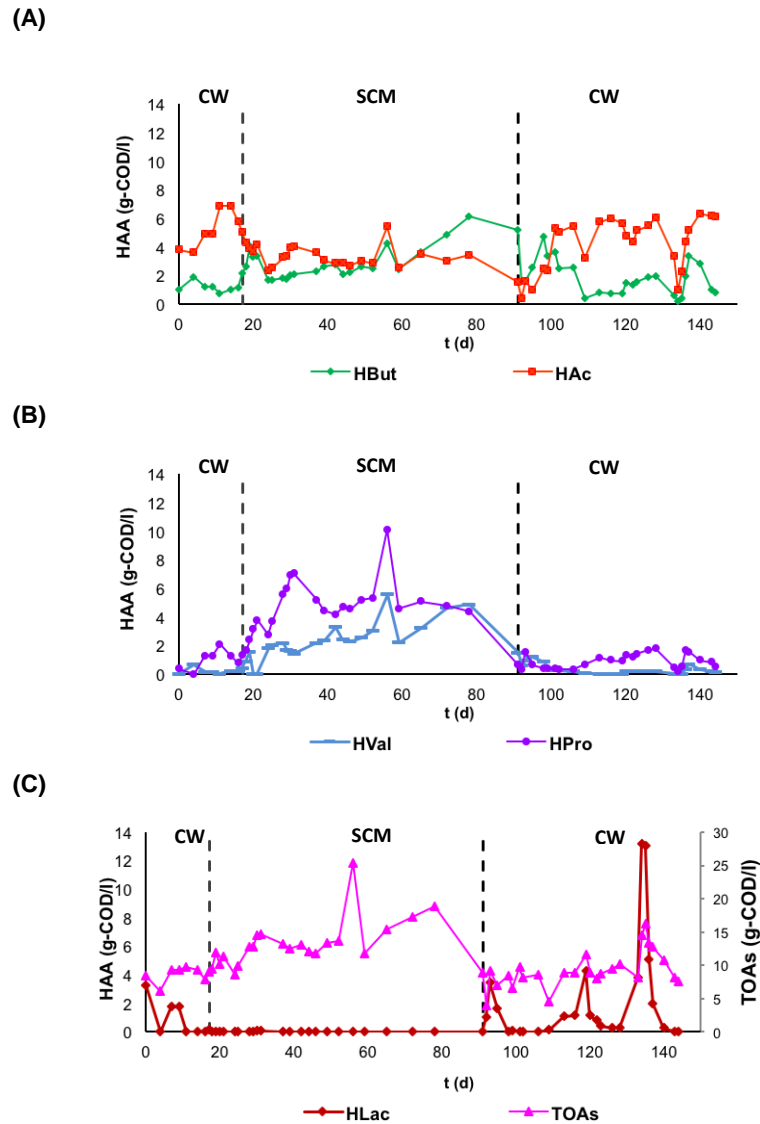


Figure 3.2 – Organic acids concentration profile during the AnMBR operation, (A) Acetic acid (HAc), and Butyric acid (HBut); (B) Valeric acid (HVal), and Propionic acid (HProp); (C) Lactic acid (HLac), and Total organic acids (TOAs).

In both periods of acidogenic fermentation of CW (StSt1 and 3) outliers of Lactic acid were observed (Figure 3.2 (C)). These peaks were resulted of system disturbances which can be explained by electrical outages, temperature (T) and pH upsets. In fact, whenever culture was perturbed in terms of T or pH, it produces during a shorter period of time lactate. Therefore, these results were not considered on parameters calculations.

Even though the anaerobic culture showed the need of a period of acclimatization, when subjected to a feedstock shift, the capacity to adapt its metabolism to this modification was clear.

Average substrate uptake ($-q_S$) and organic acids production (q_{OAs}) rates did not vary significantly with the feedstock shift (Table 3.1). Nevertheless, when CW replaced SCM (StSt3) slightly higher specific rates were observed compared to the first period of CW feeding (StSt1);

substrate uptake rate and organic acids production rate increased from 0.20 to 0.27 g-COD S/g VSS.h and from 0.13 to 0.18 g-COD OAs/g VSS.h, respectively. These specific substrate conversion rates are consistent to that reported by Bengtsson et al., (2008), 0.31 g-COD S/g VSS.h, in the acidogenic fermentation of whey at pH 6.0 and a HRT of 50 hours (2.1 days). However, these rates are lower than the maximum value reported by Kissalita et al., (1989) and Yu and Pinder, (1993) for acidification of lactose, 1.5 and 3.0 g-COD/g VSS.h, respectively.

In terms of organic acids conversion yields (Y_{OAs}) the AnMBR reached 0.52 to 0.59 g-COD OAs/g-COD S for the acidogenesis of CW, and 0.69 g-COD OAs/g-COD S (or 0.58 C-mmol OAs/C-mmol S) for anaerobic digestion of CW and SCM (Table 3.2). The increase on OAs yield from anaerobic digestion of molasses compared with fermentation of CW can be explained by the differences between substrates composition. In fact, wastes from food industry are rich in organic matter and ideal for acidogenic fermentation. However, the anaerobic fermentation of these substrates may be hindered by the presence of various inhibitors such as proteins, mineral salts (Na, K, Mg, C, and Al), etc (Chen, 2008). The obtained sugars conversion yield of acidogenic fermentation of sugar cane molasses is similar to which has been reported by Albuquerque et al., (2007). The authors have developed a three-stage process to produce PHAs from sugar cane molasses. In this process the effect of pH on molasses acidogenic fermentation step operated in a CSTR at different pH values (7, 6, and 5) and a HRT of 10 hours were studied. Organic acids conversion yields of 0.63 – 0.75 C-mol VFA/C-mol S were observed. Bengtsson S. et al., (2008) have reported organic acids conversion yields of 0.74 - 0.87 g-COD OAs/g-COD S as result of acidogenic fermentation of CW (without protein and mainly composed by lactose (86%)). These results are considerably higher compared to which was obtained in this work, mostly due to differences of cheese whey.

Table 3.1 – Effect of feedstock shift on the MBR performance in steady state.

StSt	$-q_s$ (g-COD S/g VSS h)	q_{OAs} (g-COD OAs/g VSS h)	OAs volumetric productivity (g-COD OAs/l d)	Biological Protein removed (% (g /g))	Total Protein removed (% (g/g))	DA % (g COD OAs/g COD in)
CW	0.20	0.13	8.8	27	45	38
StSt1	(0.05)	(0.03)	(1.99)	(7.8)	(11.4)	(6.22)
M	0.13	0.07	13.3	nd ^a	nd ^a	47
StSt2	(0.08)	(0.02)	(5.23)			(7.97)
CW	0.27	0.18	10.6	47	53	41
StSt3	(0.07)	(0.04)	(3.20)	(13.2)	(15.7)	(5.83)

Standard deviation); ^and: not detected

Unlike organic acids productivity and organic acids yield, organic acids distribution, total OAs concentrations (Table 3.2), as well as volumetric productivities (Table 3.1) were strongly affected by the feedstock shift. When CW was replaced by molasses, and the system reached steady state (StSt2), total organic acid concentration (acetate, propionate, butyrate, valerate, and lactate) and volumetric productivities increased from 254 to 391 Cmol OAs/l (or 8.5 to 14.8 g COD/l) and from 8.8

to 13.3 g-COD OAs/l d, respectively. These results were mainly due to the increase on propionic acid concentration (from 1.2 to 4.6 g COD/l). Acetate (HAc) was the main concentrated organic acid obtained from CW acidogenesis, making up 65 % to 69 % of the TOAs produced, StSt3 and StSt1, respectively. In fact, in the both periods of acidogenic fermentation of CW similar organic acids profiles were obtained which demonstrate that the system performance is reproducible. When molasses was fed to the AnMBR, a great change on the organic acids profile was observed. Propionate (HProp) was predominant, constituting 38% of the TOAs concentration. Further, butyrate (HBut) increased from 0.16 to 0.19 g-COD HA/g-COD OAs, while valerate (HVal) from 0.04 to 0.19 g-COD HA/g-COD OAs. The differences observed were most likely due to the different composition in organic matter of both feedstocks. The high fermentability observed for molasses was expected since the organic fraction is dominated by sugars (fructose and sucrose) (Albuquerque, 2009).

Table 3.2 – MBR operating conditions and general performance in the steady state (StSt).

StSt	VSS (g/l)	ORL (g-COD/l h)	HRT SRT		Y _{OAs} (g-COD OAs/ g-COD S)	Total OAs (g-COD/l)	Organic acids concentration profiles (g-COD HA/g-COD OAs)				
			(d)	(d)			HAc	HProp	HBut	HVal	HLac
CW	3.6	16.42	1.0	2.7	0.52	8.5	0.69	0.11	0.16	0.04	nd ^a
StSt1	(0.50)	(2.70)	(0.10)	(0.6)	(0.05)	(1.20)	(0.07)	(0.09)	(0.10)	(0.05)	
M	6.5	16.66	1.0	3.3	0.69	14.8	0.24	0.38	0.19	0.19	nd ^a
StSt2	(1.80)	(4.96)	(0.10)	(1.6)	(0.14)	(3.50)	(0.03)	(0.07)	(0.05)	(0.05)	
CW	2.6	15.20	0.9	2.7	0.59	9.1	0.65	0.10	0.22	0.02	0.01
StSt3	(0.80)	(1.64)	(0.10)	(0.9)	(0.15)	(2.00)	(0.09)	(0.06)	(0.10)	(0.01)	(0.01)

*values in the brackets are *standard deviation*; ^and: not detected

One of the most important parameters of acidogenic fermentation, which is acidification degree (DA), compares the proportions of substrates converted into OAs (Arroja et al., 2012) This parameter was calculated for each period of substrate fermentation in order to express the acidification efficiency of the anaerobic cell culture when subjected to a feedstock shift (Table 3.1). Acidification degrees of both substrates did not vary significantly and values between 38 and 47 % g-COD OAs/g-COD S were observed. These results are consistent with those obtained by Arroja et al., (2012) for acidogenic fermentation of high strength waste products including CW and molasses (DA between 30 and 65% g-COD OAs/g-COD sug).

Figure 3.3 presents the AnMBR volatile suspended solids variation during the feedstock shifts. An increase on biomass concentration was observed between day 18th and 45th when molasses replaced CW (3.6 to 6.5 g VSS/l) (Table 3.2). However, a sharp drop in biomass concentration was observed (2.5 g VSS/l) after the shift from SCM to CW. These results can be explained by the fact that the only source of nitrogen, which is necessary for biomass growth, in CW results from the whey protein hydrolysis. It was observed that only 27% to 47% (g/g) (data not

shownt) of protein was consumed. Therefore, the anaerobic mixed culture might be nitrogen deficient which limits the culture's growth (Albuquerque, 2009; Reis et al., 2011; Rajeshwari, 2000).

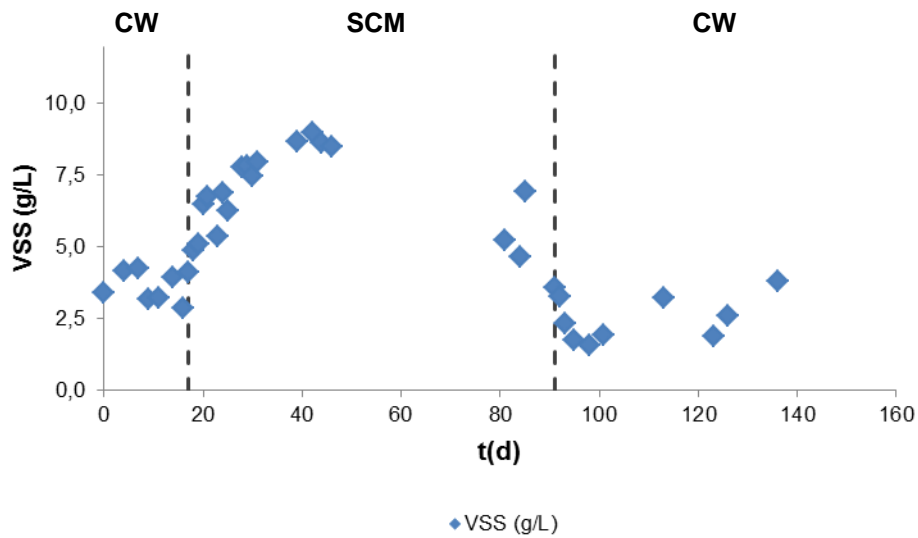


Figure 3.3 – Effect of feedstock shift on volatile suspended solids during the MBR operation.

Anaerobic fermentation appears to be suitable for valorization of wastewater streams of agro-industry as substrates for production of polyhydroxyalkanoates. Monomer composition of PHAs from a known OAs profile concentration can be anticipated (Dias et al., 2006; Bengtsson et al., 2008; Serafim et al., 2008; Arroja et al., 2012; Albuquerque, 2009). In this manner, considering propionate and valerate are the organic acids precursors of the HV monomer, it is expected that the PHA produced from the fermented molasses obtained have a high percentage of this monomer in its composition. Similarly, acetate and butyrate (HB precursors) were the main organic acids produced of CW fermentation. Thus, it is expected HB as principal constituent of the PHA produced from clarified fermented CW.

3.4. Conclusion

The effect of feedstock shifts in the acidogenic fermentation performance of an anaerobic mixed microbial culture was investigated. Two different agro-industrial waste/surplus feedstocks, cheese whey, and sugar cane molasses, were used in organic acids production.

The main conclusion observed in this study was the capacity of anaerobic mixed cultures to adapt their metabolism to a waste/surplus based feedstock shift. However the need for biomass acclimatization to each substrate was demonstrated.

CW and sugar cane molasses were simultaneously converted to organic acids in acidogenic fermentation reactor with considerable acidification degrees (38-47%).

In terms of TOAs concentration and volumetric productivities, an increase of both parameters was observed when SCM replaced CW.

The composition of fermented feedstocks, regarding organic acids, was significantly affected by the substrate shifts. For whey, the main fermentation products were acetate and butyrate. On the other hand, when molasses was fed, a drastic modification in organic acids composition was observed. Propionate was the main concentrate organic acid (0.35 C-mol HA/C-mol OAs) followed by acetate and butyrate (0.20-0.25 C-mol HA/C-mol OAs).

These results indicate that, even though the anaerobic culture shows the need of a period of acclimatisation, the feedstock shift can be considered an interesting and possible approach to seasonably of agro industrial wastes. However, of the acidification of different substrates results different OAs profile which is a factor to be taken account on PHA production.

Chapter IV – Feedstock shift impact on PHA-accumulating culture selection and PHA production

4.1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters constituted of hydroxyl fatty acids. These polymers are accumulated into the microbial cell, in its amorphous state as granules, being used as carbon/energy reserves (Salehizadeh and Van Loosdrecht, 2003; Johnson, 2010; Reis et al., 2011). The storage capability of mixed cultures is induced by imposing growth-limiting conditions (such as lack of nutrients or an alteration on enzyme levels) and, at the same time, an excess of carbon source.

Even though, in the last decade, research has focused on the development of alternative production processes aiming to decrease PHA production cost, petrochemicalplastic still has the most cost-effective production. Nowadays, PHAs are industrially produced using pure microbial cultures and expensive pure feedstocks (Albuquerque, (2007)). Substrate cost is the major contributor for the increasing price of PHAs. It has been estimated to be about 40% of the total PHA production costs (Choi and Lee, 1997). Moreover, PHA production by pure cultures has an economically negative impact, since it is necessary to sterile all the equipment (Dias et al., 2006; Serafim et al., 2008; Jiang, 2011). Therefore a more cost-effective PHA production process should include: (1) the use of MMC; and (2) the selection of cheap substrates which can be effectively used by microorganisms to synthesized PHA at high productivities.

MMC are microbial populations, generally of unknown composition, which are selected by the operational conditions imposed on the system (Dias et al., 2006). The success of the PHA production by mixed cultures is due to the enrichment of a mixed culture with microorganisms with superior ability to accumulate PHAs in high amounts.

One biggest advantage of MMC processes, when compared with pure cultures, is that these cultures can use a range of variety of low cost complex feedstock, such as agricultural and industrial residues or by products(starch, tapioca hydrolysate, cheese whey, sugar molasses, malt, soy waste, etc) (Dias et al., 2006). However, many of these industrial wastes are seasonally produced therefore different scenarios might be taken account: (1) stock of industrial wastes during their production for their use throughout the year. However, the high fermentability of these agro-industrial wastes makes them susceptible to degradation during storage period; (2) the use of different feedstocks over the year according its availability. It is thus important to study MMC's response to different feedstocks.

In this work stage 2 and 3 of a three-step process for PHA production by mixed microbial cultures under FF conditions was used, which consist in a PHA-accumulating culture selection, using organic acids produce in earlier stage, and the PHA accumulation with the selected culture (stage 2) and fermented feedstocks produced on acidogenic fermentation (Chapter III).

The aim of the current work was studied the feedstock shift impact on the performance of an enrichment PHA-accumulating culture selected on SBR under feast and famine conditions, and in the PHA batch accumulation of the cultures selected. A synthetic OAs solution, clarified fermented cheese whey, and fermented sugar cane molasses were used as substrate.

4.2. Materials and Methods

4.2.1. Feed preparation

In this study three different feedstocks were administrated to the bioreactor, namely, a synthetic OAs solution (Syn), fermented sugar cane molasses (fSCM) and fermented cheese whey (fCW). On culture selection was used a SBR which had been operated with synthetic mixture of OAs (relative to another job) and it was subjected to a feedstock shifts: (1) from synthetic OAs solution to fSCM (produced during steady state of AnMBR operated with SCM); and (2) from fSCM to fCW being coincident with the feedstock shift in the AnMBR from SCM to CW. Each substrate was used as feed for the selection of a PHA-accumulating culture and was kept in a refrigerated vessel at 4°. Simultaneous mineral nutrients medium, at room temperature and containing ammonia and a phosphate source (as NH_4Cl and KH_2PO_4), was used to further dilute the substrates (Syn, fSCM,

The mineral nutrients solution ammonia and phosphate (5 N-mmol/L and 0.5 Pmmol/L) were adjust to keep the C:N:P ratio at 45:5:1 Cmol/Nmol/Pmol (Table 4.1).

4.2.2. Experimental Setup

The experimental work was conducted in two bench-scale reactors, a sequencing batch reactor (SBR), where the selection of PHA accumulating organisms under feast and famine conditions occurred, and a batch reactor for the PHA accumulation assays with the stable cultures selected in a SBR on Syn, fSCM, and fCW and the feedstock where they had been selected, in order to monitor them in terms of PHA production (Figure 4.1).

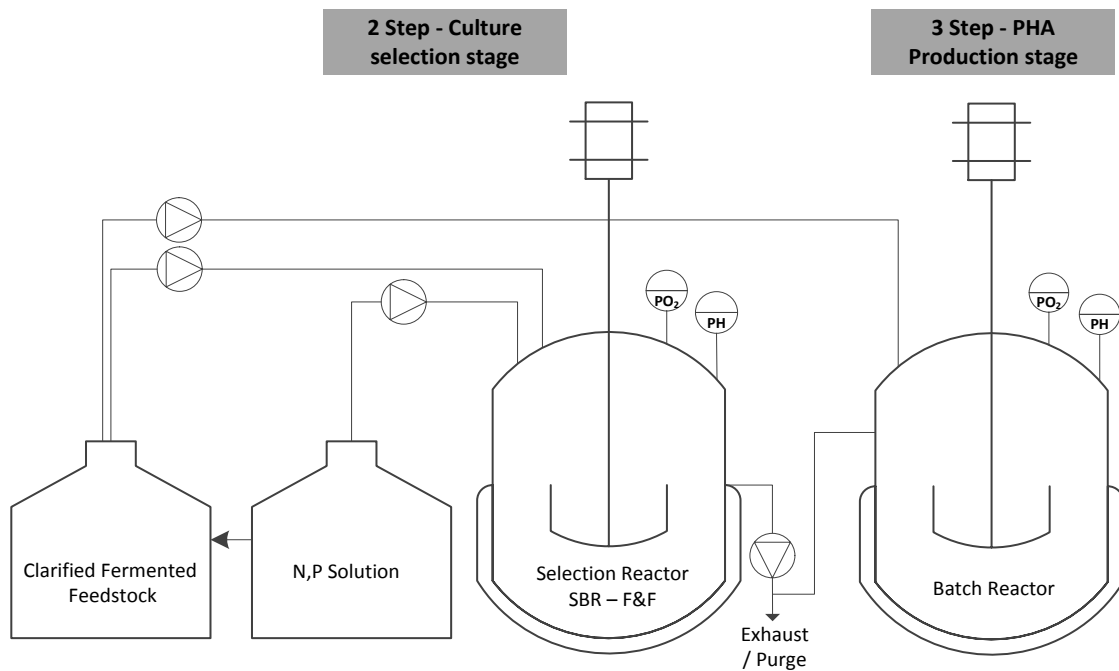


Figure 4.1 – Stage 2, and stage 3 of a three-step process for PHA production by mixed microbial cultures from, synthetic OAs solution, fermented sugar cane molasses, and fermented cheese whey.

4.2.3. Enrichment of PHA accumulating organisms reactor set up and operation

A SBR, with a working volume of 800 mL, was used for the culture selection. The operating conditions were similar to those reported by Albuquerque et al., (2010a). Therefore, independently on the substrate administered, the SBR was operate with a 1 day HRT, 12 hours cycle length, and a SRT of 4 days was kept by imposing a purge of 100 mL of mixed liquor at the end of reaction phase.

The SBR 12 hour cycles consisted of four discrete periods: fill (5 min); aerobiosis (feast and famine) (11 h); settling (45 min); and supernatant withdrawal (10 min).

The SBR was firstly fed with 100 mL/cycle of synthetic OAs solution (days 0 to 29), then with 88 mL/cycle of fermented molasses (days 29 to 71), and at last with 80 mL/cycle of fermented CW (days 71 to 117), imposing two feedstocks shifts in the SBR. Simultaneously, an ammonium and phosphate solution was supplemented to the SBR, 300, 308, and 320 mL/cycle, for the synthetic OAs solution, fSCM, and fCW as feedstock, respectively. In the entire experimental period a C/N/P molar ratio of 45/5/1 Cmol/Nmol/Pmol was kept (Table 4.1).

Air was supplied through a ceramic diffuser. Stirring was kept at 300 rpm. Reactor's operation was carried out with pH control to pH 8, through the addition of 2M HCl solution, and at room controlled-temperature (23-25°C). Pumping (fill and withdrawal), aeration, and mixing were

automatically controlled by a software program. In addition, the software also allowed online pH and dissolved oxygen data acquisition.

Table 4.1 – Operating conditions and feed composition for culture selection reactor under feast and famine conditions.

		Synthetic OAs Solution	fSCM	fCW
Operating conditions	HRT (days)		1	
	SRT (days)		4	
	OLR (C-mmol/L.d)		90	
	Volume Reactor (L)		800	
Influent Concentrations	OAs (C-mmol/L)		45	
	NH ₄ Cl (N-mmol/L)	50.00	15.43	3.61
	KH ₂ PO ₄ (P-mmol/L)	1.28	1.60	1.61
	C/N/P (C-mol/N-mol/-Pmol)		45/5/1	

4.2.4. PHA Accumulation: batch assays

Three PHA accumulation assays were carried out using a pulse wise feeding to assess the PHA accumulation performance of the three selected cultures using each of feedstocks. These assays were performed in batch mode (500 mL working volume), inoculated with SBR enriched cultures which were collected from the SBR at the end of the cycle, immediately after the withdrawing phase, in order to guaranteed that the biomass was endogenous mode. Feed pH was adjusted to 8 before reactor feeding.

Air was supplied through a ceramic diffuser and stirring was kept at 300 rpm. The assays were carried out with no pH control and at room controlled-temperature (23-25°C). The O₂ consumption was measured with a gas analyser. Whenever, O₂ was no longer consumed a new pulse was added. This procedure was repeated until cultures have reached the PHA saturation.

4.2.5. Analytical procedures

Biomass concentration was estimated as volatile suspended solids (VSS), determined according to Standard Methods (APHA 1995).

Ammonium concentration was measured in filtered samples (0.45 µm) with IDS3000 DIONEX ion chromatograph, equipped with a conductivity detector and a CSRS 300 (4 mm)

DIONEX self-regenerating suppressor, and a IonPac CS16 column and a IonPac CG16 pre column (column temperature 30 °C, methanesulfonic acid 48 mM eluent, flow rate 1 mL/min). A calibration curve was obtained with NH₄Cl standards (0.6-6 Nmmol/L).

Organic acids (acetate, propionate, butyrate, valerate and lactate), as well as lactose concentrations were determined by high performance liquid chromatography (HPLC) using a Merck-Hitachi chromatographer equipped with a RI detector and Aminex HPX-87H pre-column and column (BioRad, USA). Sulphuric acid 0.01 M was used as eluent at a flow rate of 0.6 mL/min and 50°C operating temperature. The organic acids concentrations were calculated using a standard curves of 15 – 1000 mg/L.

Polyhydroxyalkanoates were determined by gas chromatography using the method described by Albuquerque et al., (2010b). Briefly, lyophilized biomass was incubated for methanolysis during 3.5 h in chloroform and a 20% sulphuric acid in methanol solution. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) of each sample was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID Varian CP-3800). A ZBWax-Plus column was used at a flow rate of 1ml/min. The oven temperature program was as follows: 40 °C; then 20 °C/min until 100 °C; then 3 °C/min until 175 °C; and finally 20 °C/min until 220 °C. The detector temperature was set at 250 °C. Hydroxybutyrate and hydroxyvalerate concentrations were determined using commercial P(HB-HV) (88%/12%) (Sigma) standards (concentration of approximately 0.1-8 mg/mL) and corrected using a heptadecane internal standard.

4.3.6. Calculation of kinetic and stoichiometric parameters

The sludge PHA content was calculated as a percentage of VSS on mass basis, where VSS includes active biomass (Liu) and PHA. Active biomass was estimated by subtracting PHA from VSS, assumed to be represented by the typical molecular formula C₅H₇NO₂ (Henze et al., 1995), and based on ammonia uptake curve

$$\% \text{PHA} = 100 \times \frac{\text{PHA}}{\text{VSS}} \quad [9]$$

$$X = g\text{VSS} - g\text{PHA} \quad [10]$$

$$X_{(n+1)} = X_n - (\text{NH}_{4,n} - \text{NH}_{4,(n+1)}) \times 5 \quad [11]$$

where , and are the concentration of ammonium (in N-mol /L), in , and periods, respectively.

Total organic acids concentration corresponds to the sum of all organic acids concentrations (TOAs, in C-mmol/L).

$$TOAs = \sum(HAc + HBut + HPro + HVal + HLac) \quad [12]$$

The maximum specific substrate uptake (in C-mol OAs/C-mol X.h) and specific PHA storage rates (in C-mol PHA/C-mol X.h) were determined by adjusting a linear function to the experimental data of TOAs fraction per active biomass estimated (, in Cmol PHA/C-mol X), and PHA fraction per active biomass estimated (, in Cmol PHA/C-mol X), plotted over time. Specific rates correspond to the slope of each the linear fits.

PHA concentration corresponds to the sum of HB and HV monomers concentrations (in C-mmol/L)

The PHA storage yield on substrate consumed ($Y_{PHA/OAs}$ in C-mol PHA/C-mol OAs) was calculated by dividing the amount of PHA formed by the total amount of substrate consumed. This parameter is expressed by the equation [13]:

$$Y_{PHA} = \frac{tPHA_{n,feast} - tPHA_{0,feast}}{TOAs_{0,feast} - TOAs_{n,feast}} \quad [143]$$

where , , and , are the concentration of PHA (in C-mol PHA/L), and the organic acids concentration, in the begin, and the end of feast phase, respectively.

$$Y_X = \frac{tX_{n,feast} - tX_{0,feast}}{TOAs_{0,feast} - TOAs_{n,feast}} \quad [14]$$

where , , are the concentration of active biomass (in C-mol X/L), in the begin, and the end of feast phase, respectively.

The feast / famine ratio (F/F, h/h) was calculated by the ratio between the length of feast divided by the length of the famine phase in SBR cycle.

For long term SBR operation, and accumulation batches, average values were estimated for each performance parameter as a means of characterizing the reactor performance for the different periods of shift feedstock.

4.3. Results and Discussion

4.3.1. Effect of substrate shifts on the SBR's performance

The first aim of this study was to understand how a substrate shift affects the performance of the culture selection SBR. Therefore, a SBR which had been operated with synthetic mixture of OAs (relative to another job) was subjected to a feedstock shifts: (1) from synthetic OAs solution to fSCM (produced during steady state of AnMBR operated with SCM); and (2) from fSCM to fCW being coincident with the feedstock shift in the AnMBR from SCM to CW.

Figure 4.2 to 4.4 presents the OAs consumption and PHA production in enrichment SBR cycles for each of different substrates fed. The selection reactor was operated for a total period of 117 days, where between days 0 to 29 a synthetic OAs solution was used as substrate, from days 29 to 71 fermented sugar cane molasses was fed and, finally, between days 71 to 117 fermented CW was used as feedstock Figure 4.5.

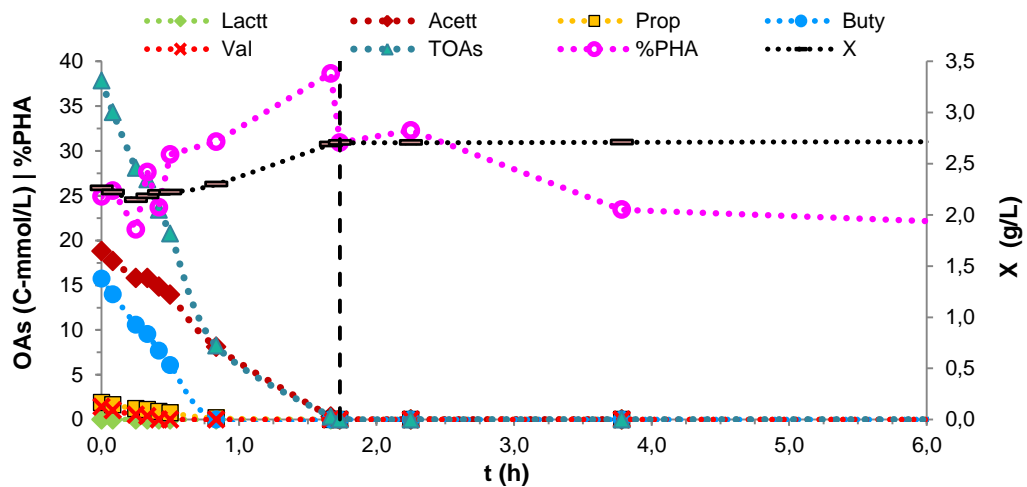


Figure 4.2 –Typical feast phase of a SBR cycle of PHA-accumulating culture selection operated under FF conditions and fed with synthetic OAs solution (dashed line marks the end of the feast phase).

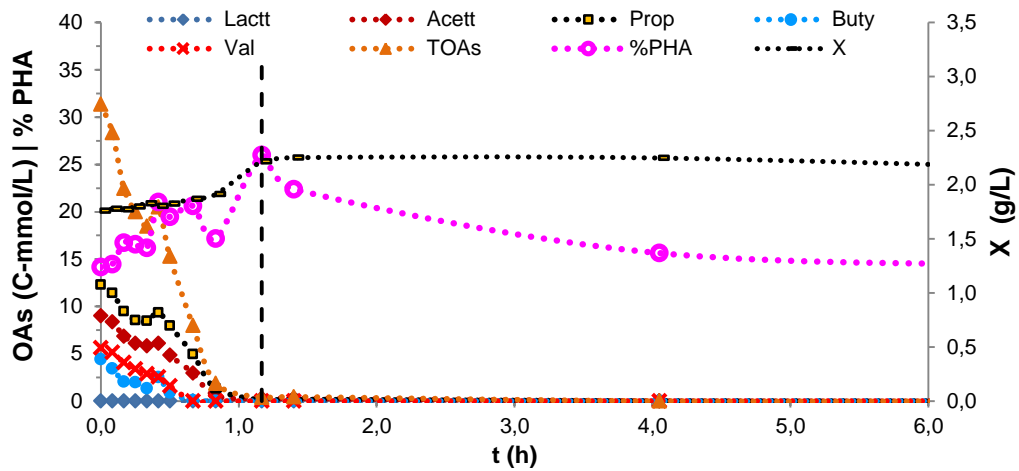


Figure 4.3 –Typical feast phase of a SBR cycle of PHA-accumulating culture selection operated under FF conditions and fed with clarified fSCM (dashed line marks the end of the feast phase).

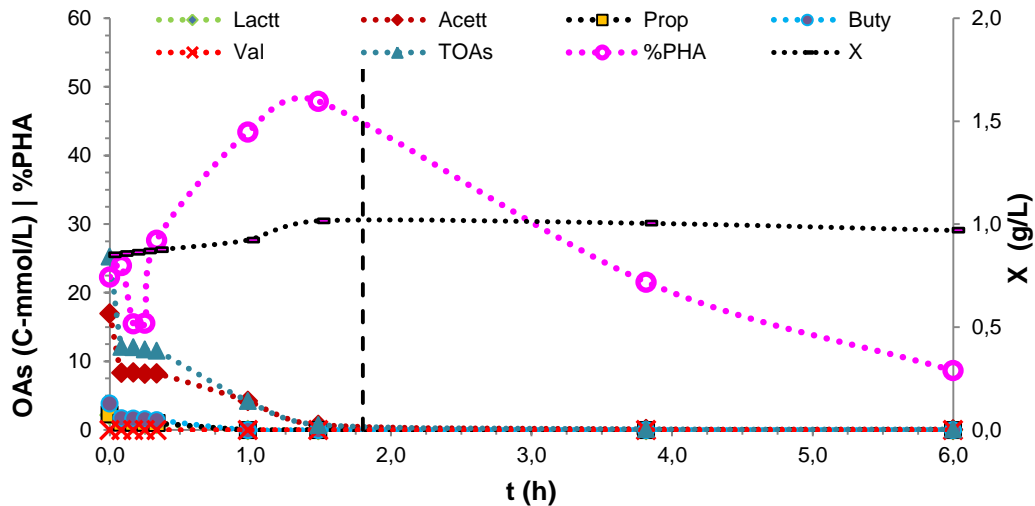


Figure 4.4 –Typical feast phase of a SBR cycle of PHA-accumulating culture selection operated under FF conditions and fed with clarified fCW (dashed line marks the end of the feast phase).

The performance of the selected cultures, obtained with the three feedstocks used in this study, was monitored over time in terms of substrate uptake rate, PHA production yields and PHA production rates (B, Figure 4.5). When clarified fermented molasses replaced synthetic OAs solution, a stable performance of the selection reactor was observed (days 29 to 71). In terms of PHA storage rate and PHA production yield no significant variations were observed. However, when fermented molasses was replaced by fermented CW an acclimatization period was observed (of approximately 6 days). After steady state had been reached, PHA storage yield remained approximately in 0.59 C-mol PHA/C-mol OAs. This adaption period can be because the CW presents a more complex matrix, *i.e.*, 13.62 % (w/w CW) of protein, 78 % (w/w CW) of lactose, and 1.21 % (w/w CW) of lipids,

than sugar molasses which is mainly composed by sugars (fructose (38%) and sucrose (62%)). Consequently, from the acidogenic fermentation of these feedstocks results two different fermented substrates. The ferment molasses is mainly composed by OAs while the fermented CW still presents a high concentration of protein (about 47 %(g/g) of total protein supplied (Table 3.1). Therefore, the SBR culture had to adapt its metabolism to the presence of protein in fCW. Average volatile suspended solids remained approximately constant during the entire experiment (Table 4.3).

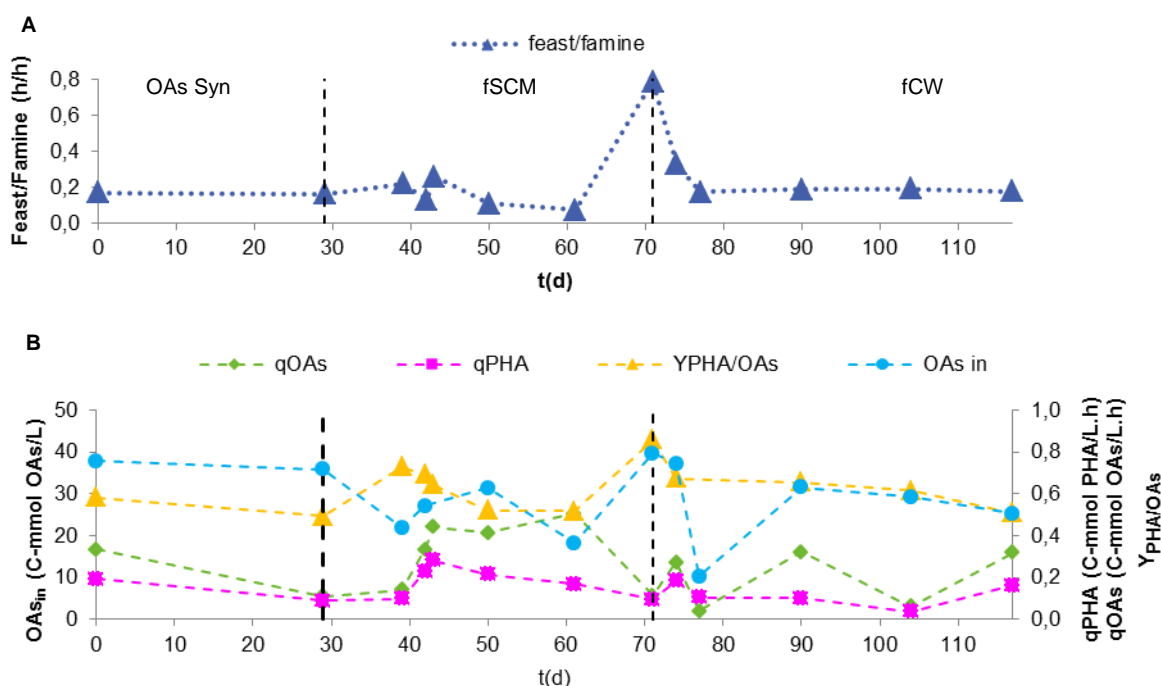


Figure 4.5 - A: F/F ratio of the SBR operating cycles; B: Effect of a substrate shift on performance over the time of PHA enrichment culture selection SBR subjected to ADF conditions. fed with a synthetic OAs solution (OAs Syn), then fermented molasses (fSCM), and finally fermented CW (fCW).

Table 4.2 – Average OAs feedstocks profiles and substrate uptake rates on the performance of PHA-accumulating culture selection SBR subjected to an FF conditions.

Feedstock	OAs_{in} (C-mmol OAs/L)	Lact	Acet	Prop	Buty	Val	-qLac	-qAce	-qPro	-qBut	-qVal
			(C-mol HA/l)				(C-mol /C-mol X h)				
OAs Syn	37,85	nd	18,8	1,9	15,73	1,43	nd	0,08	0,02	0,00	0,04
fSCM	34,14	nd	12,71	10,60	6,21	4,63	nd	0,12	0,19	0,07	0,04
	(15,3)		(4,6)	(9,3)	(2,4)	(4,2)		(0,0)	(0,2)	(0,1)	(0,2)
fCW	28,68	2,25	20,40	1,93	3,75	0,34	0,01	0,15	0,03	0,16	0,01
	(3,15)	(2,19)	(3,02)	(0,89)	(3,68)	(0,30)	(0,01)	(0,10)	(0,03)	(0,19)	(0,01)

*Values under brackets are the standard deviation

Figure 4.2 to 4.4 show that the organic acids consumption for both feedstocks fed to SBR were fully exhausted approximately in 1.7 to 2h after steady state had been reached, resulting an F/F ratio of 0.17 to 0.2 h/h (Figure 4.5). Nevertheless, when fermented CW replaced fermented molasses, an acclimatization period of 6 days was observed, and F/F range from 0.79 to 0.18 h/h between 71 and 77 days, respectively. This behaviour can be explained by the high F/F ratios resulting from higher OLR/influent substrate concentration during that period: when fermented molasses was replaced by fermented CW, organic acids concentrations increased from 18.27 to 39.7 C-mmol OAs/L (Figure 4.5, B) which favored long feast phases. However, the mechanism through which the F/F ratio affects the selective pressure for PHA-storing microorganisms is related to a decrease of the internal growth limitation that is necessary to induce PHA storage (Dionisi et al., 2006; Albuquerque, 2009; Reis et al., 2011). In this manner, if the famine phase is not long enough (high F/F ratios), such an internal limitation is not ensured and microorganisms will be better fit to grow when supplied with external carbon source and will therefore, accumulate less PHA. Furthermore, the competition among microorganisms will not be based on the PHA storage rate and yield since both PHA-storing and non-storing microorganisms will be able to grow unrestrained during the feast phase and the selective pressure favoring the best storing microorganisms will be partially lost (Reis et al., 2011).

The performance of the selected cultures obtained with the different substrates, was unstable in terms of average specific substrate uptake rate ($-q_{OAs}$), and when fermented molasses was fed to bioreactor reached the higher value obtained in this study, 0.453 Cmol OAs/C-mol X.h (Table 4.3). This result is similar to those obtained by Albuquerque et al., (2010b). The authors studied the effect of influent substrate concentration of fermented molasses (30, 45, and 60 C-mmol VFA/L) in a PHA-accumulating selection culture and reported an organic acids uptake rate of 0.54 C-mol OAs/C-mol X.h. On the other hand, specific storage rates, as well as PHA production yield were stable over reactor operation, varying between 0.216 and 0.101 C-mol PHA/C-mol X.h and 0.56 to 0.59 C-mol PHA/C-mol OAs, respectively.

Acetate was the most abundant acid in the synthetic OAs solution and fermented CW (Table 4.2). On the other hand, propionate was the most concentrated acid in fermented molasses (Chapter III). A copolymer of P(HB-co-HV) was produced in all SBR cycles. However, when fermented CW or synthetic OAs solution were fed to the SBR, and after the system had reached steady state, HB was the main constituent of PHA polymer produced. This result was expectable since acetate and butyrate (HB precursors) represented 85 to 87% of the total OAs fed in both feedstocks (Table 4.2). Propionate and valerate which are the HV monomer precursors represented 57% of the total OAs in fermented molasses. Thus, HV was the main constituent of the polymer composition produced when this feedstock was fed (Table 4.2).

Table 4.3 - Average performance of PHA-accumulating culture selection SBR subjected to a ADF conditions and a feedstock shift with a synthetic OAs solution (OAs Syn), fermented molasses (fSCM), and fermented CW (fCW).

Feedstock	F/F ratio	VSS	OAs	X_i	$Y_{PHA/OAs}$	-qOAs	qHB	qHV	qPHA	HB	HV	PHAm _{ax}
	(h/h)	(g VSS/L)	(C-mmol OAs/L)	(g/L)	(C-mol PHA/C-mol OAs)	(C-mol OAs/C-mol X h)	(C-mol HA/C-mol X h)	(C-mol PHA/C-mol X h)			(%)	
OAs Syn	0.17	3.52	37.85	2.41	0.58	0.333	0.169	0.026	0.194	0.862	0.138	0.386
fSCM	0.18	2.24	34.31	1.70	0.56	0.453	0.098	0.125	0.223	0.424	0.576	0.225
	(0.1)	(0.6)	(17.7)	(0.2)	(0.1)	(0.05)	(0.04)	(0.02)	(0.06)	(0.04)	(0.04)	(0.04)
fCW	0.19	2.34	28.68	2.34	0.59	0.234	0.068	0.033	0.101	0.810	0.190	0.399
	(0.01)	(0.11)	(3.2)	(0.3)	(0.08)	(0.15)	(0.04)	(0.02)	(0.06)	(0.06)	(0.06)	(0.08)

*Values under brackets are the *standard deviation*

4.3.2. Effect of feedstock shift on PHA accumulation.

The potential of the mixed microbial cultures selected with the OAs synthetic solution, the fermented SCM and fermented CW, to store PHA, was accessed through batch assays using growth limiting conditions (nitrogen limitations). The fSCM molasses, and fCW, produced in acidogenic fermentation (Chapter III), as well as a synthetic OAs solution were used as substrate. The aim of this study was to evaluate the PHA-accumulating performance of the cultures selected on the SBR with the different feedstocks.

4.1.1.1. PHA accumulation assays

Figure 4.6 to Figure 4.8 present the accumulation batch assays using the synthetic OAs solution, fermented SCM and fermented CW, respectively. The results of the three batch tests are summarized in Table 4.4.

In the PHA accumulation assay using the synthetic OAs solution as substrate, acetate and butyrate (HAc+HBut), the most concentrated acids, were consumed at higher rates, 0.424 to 0.176 C-mol HA/C-mol X.h (data not shown), until the 6th pulse. Propionate and valerate, at lower concentration in OAs synthetic solution, were totally consumed at the beginning of each pulse (0.86 to 0.30 C-mol HA/C-mol X.h (data not shown)).

In the 7th pulse a short lag phase was observed in the OAs consumption which was intensified in the posteriorly pulses. Simultaneously, a decrease of the organic acids consumption rate (0.089 - 0.006 C-mol OAs/C-mol X.h) (Figure 4.6) was observed from 7th pulse onward indicating the approach of the culture's PHA synthesis saturation.

Table 4.4 – Average stoichiometric and parameters obtained in PHA accumulation assays using the cultures selected with synthetic OAs solution, fermented molasses, and fermented CW as substrate.

Feedstock	VSS	OAs	OAs profile	$Y_{PHA/OAs}$	PHA max	PHA Composition	-qOAs	qPHA	X
	(g VSS/L)	(C-mmol OAs/L)	HLac:HAc:HProp:HBut:HVal (% C-molHA/C-mol OAs)	(C-mol PHA/C-mol OAs)	(%)	%HB:%HV (C-mol/C-mol PHA)	(C-mol OAs/C-mol X h)	(C-mol OAs/C-mol X h)	(g/L)
OAs Syn	2.54 (0.83)	205	0:68:3:26:2	0.74 (0.13)	60	83:17	0.17 (0.06)	0.22 0.13	1.60 (0.54)
fSCM	3.26 (0.91)	79	0:25:30:15:31	0.49 (0.17)	56.2	51:49	0.14 (0.06)	0.11 (0.25)	2.51 (0.80)
fCW	2.59 (0.70)	108	3:64:8:22:2	0.73 (0.38)	65.1	83:17	0.23 (0.16)	0.42 (0.18)	1.84 (0.59)

*Values under brackets are the *standard deviation*

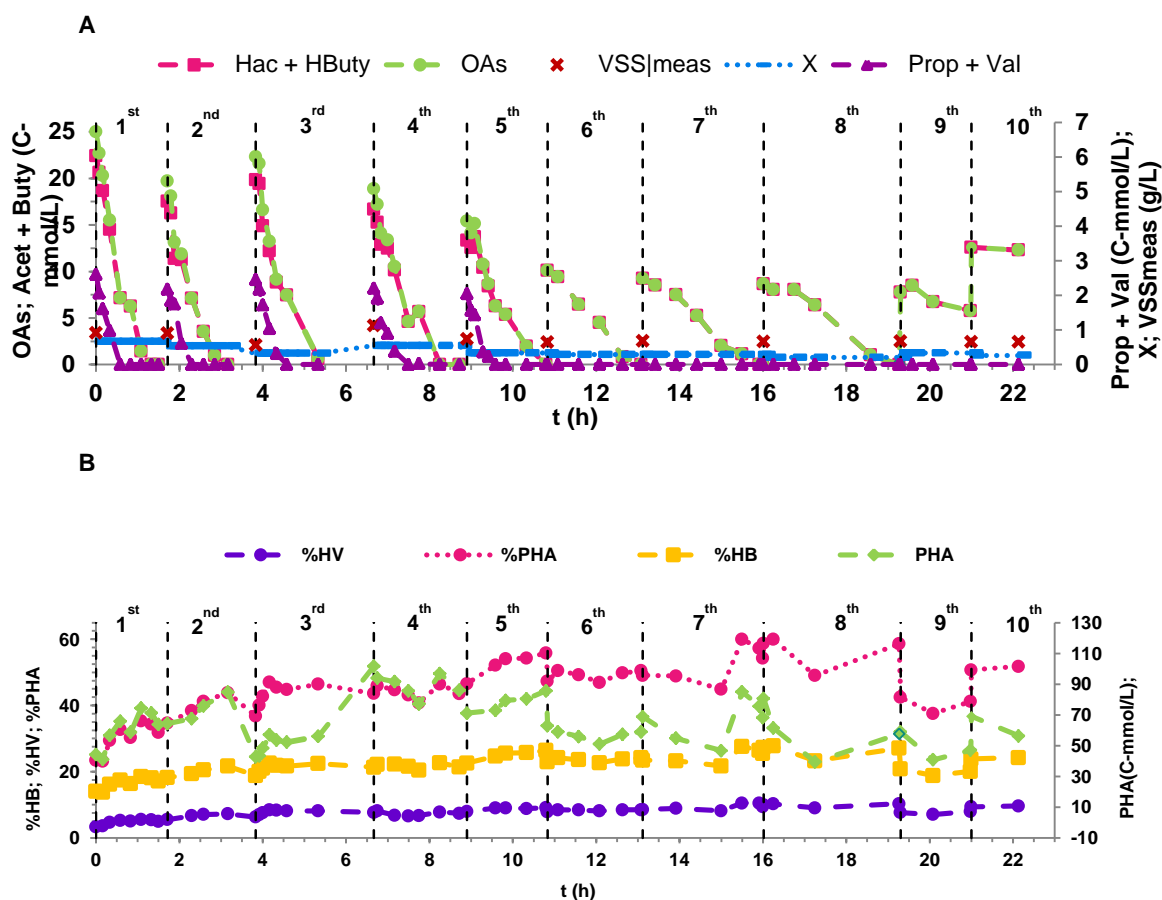


Figure 4.6 – PHA batch accumulation with the selected culture on the first period of a shift feedstock (days 0 to 29, Figure 4.5) with a synthetic OAs solution under nutrient limiting conditions. A: OAs consumption; B: PHA production.

The result of the PHA batch accumulation assay, using fSCM as substrate, is presented in Figure 4.7. In this accumulation assay, organic acids were consumed at similar substrate uptake rates, 0.07 C-mol OAs/C-mol X.h to acetate and butyrate, and 0.06 C-mol OAs/C-mol X.h to propionate and valerate (data not shown), respectively. In the first pulse a higher concentration in OAs was fed to the batch reactor (85 C-mmol OAs/L) which resulted in a 6 hours of pulse until all the organic acids were totally consumed. In addition, organic acids did not begin to be consumed immediately after the reactor being fed. Instead, a lag phase was observed in the for organic acids uptake curves. This result can be explained by some inhibition by the higher initial substrate concentration. Albuquerque et al., (2011) has shown that cultures selected on fermented molasses suffer inhibition by high substrate concentrations, namely, above 90 C-mmol VFA/L. Therefore, this behavior can explain the lower PHA production yield, 0.49 C mol PHA/C-mol OAs (Table 4.4), and storage rate, 0.11 C-mmol PHA/C-mmol X.h (Table 4.4), obtained in this assay, when compared to those obtained in the SBR cycles (0.56 C-mol PHA/C-mol OAs, and 0.223 C-mmol PHA/C-mmol X.h, respectively (Table 4.3). The following pulses were more diluted, and a simultaneously consumption of organic acids was

observed without inhibition by substrate concentration (a lag phase was not observed). The culture reached PHA synthesis saturation on the 3rd pulse.

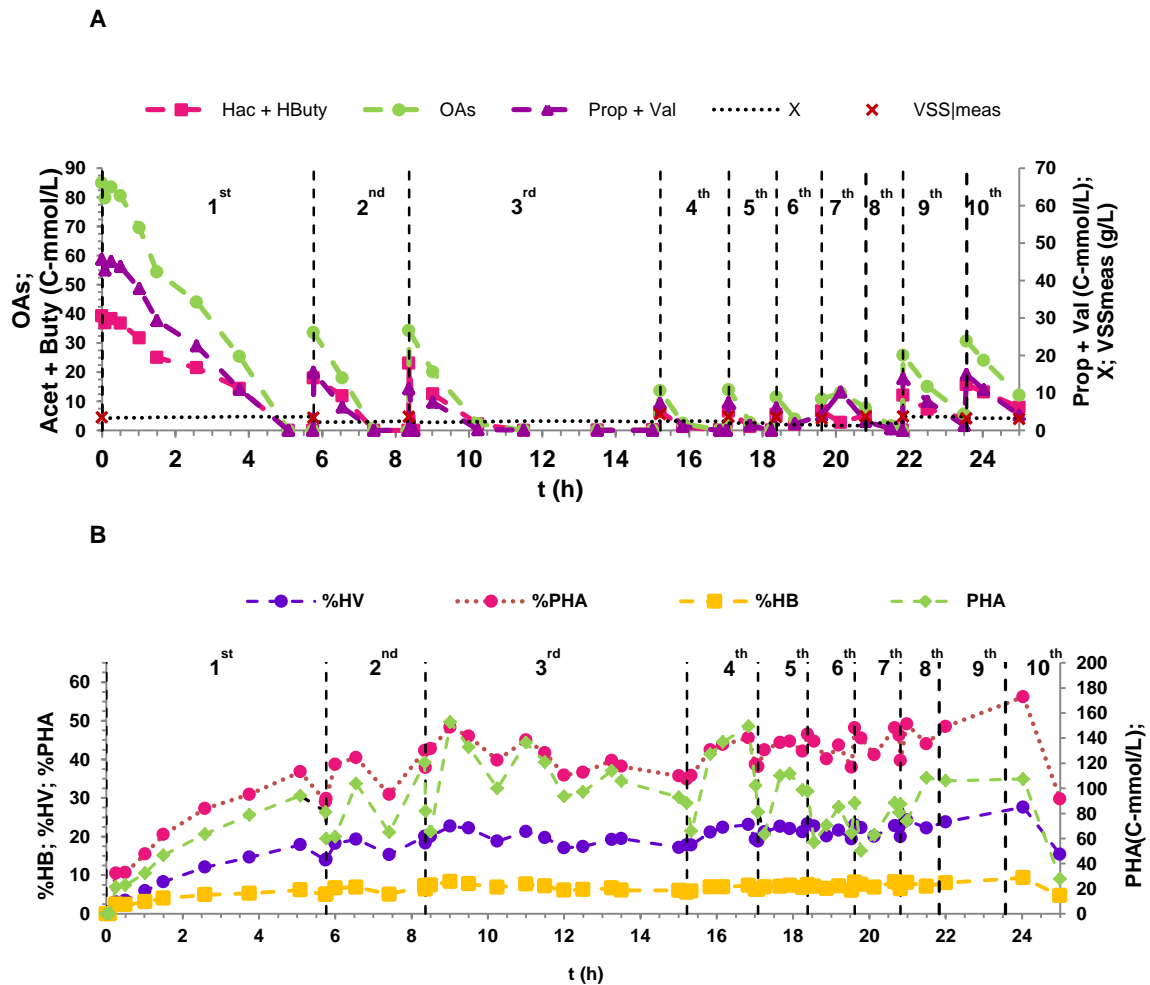


Figure 4.7 – PHA batch accumulation with the selected culture on the second period of a shift feedstock (days 29 to 71, Figure 4.5) with fermented molasses under nutrient limiting conditions. A: OAs consumption; B: PHA production.

PHA accumulation assay with selected culture on fermented CW on SBR was also evaluated (Figure 4.8). In terms of OAs consumption the selected culture showed a similar behavior to that which was obtained with the synthetic OAs solution. Indeed, the OAs concentrations were approximately the same for both experimental assays (0/68/3/26/2 and 3/64/8/22/2 (%HLact:%HAc: %HProp:%HBut:%HVal, Table 4.4, for a OAs synthetic solution and fermented CW, respectively). Acetate and butyrate were consumed at higher rate of 0.12 and 0.070 C-mol/Cmol X h, respectively. The culture reached PHA synthesis saturation in the 8th pulse.

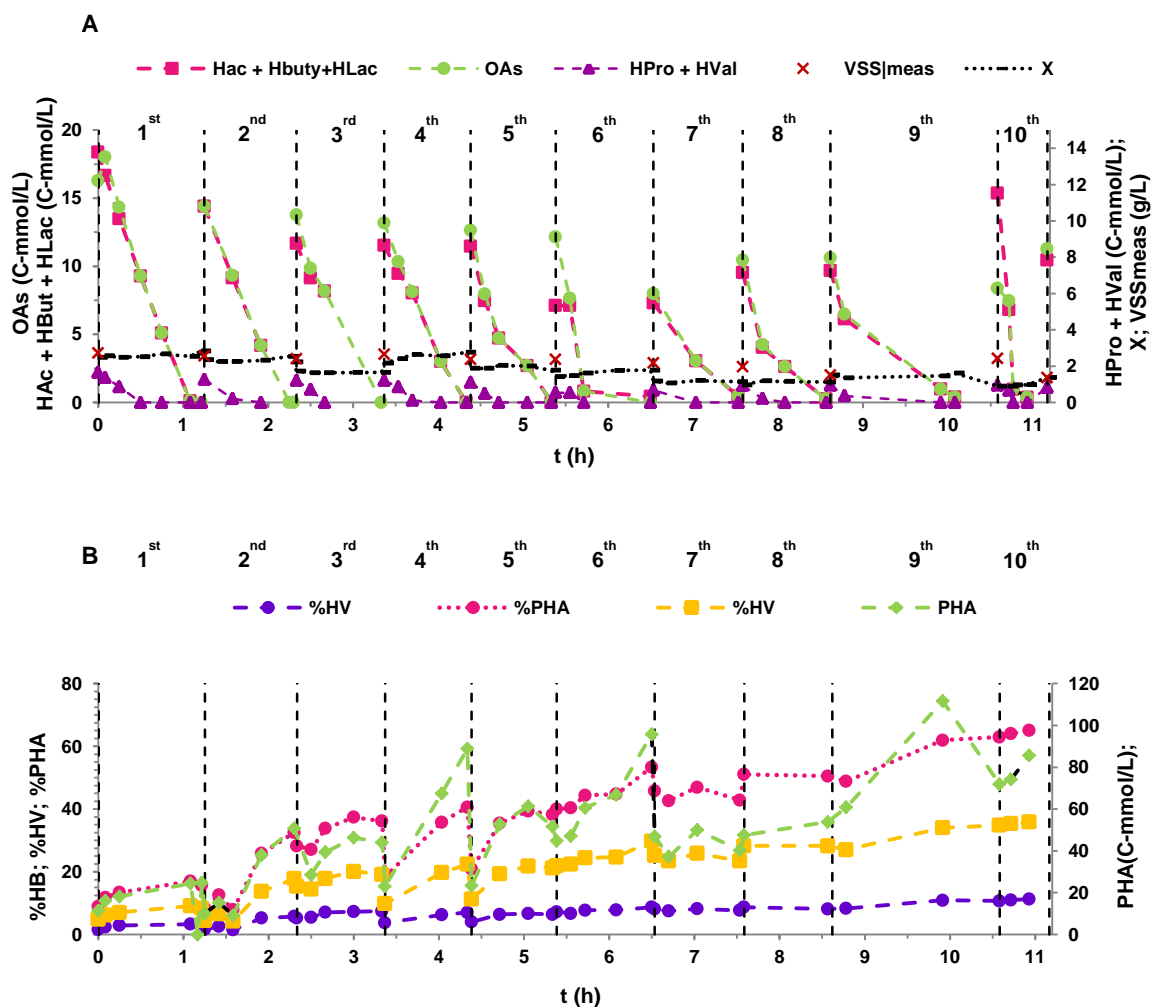


Figure 4.8 – PHA batch accumulation with the selected culture between days 71 and 117 of a shift feedstock (Figure 4.5) with a fermented CW as substrate under nutrient limiting conditions. A: OAs consumption; B: PHA production.

The maximum PHA content was obtained in the PHA accumulation assay with fermented CW, reaching to 65.1 %PHA_{max}, followed by accumulation with OAs synthetic solution (60% PHA_{max}) (Table 4.4). The lower value, 56.2 % maximum PHA content (Table 4.4), was attained with selected culture with fermented molasses. The low batch PHA accumulation performance of the culture selected on fSCM could be due to the higher OAs concentration of the first pulse fed, which may have caused inhibition and loss of PHA storage physiological capacity. Other explanation could be the major diversity of the selected culture with fermented molasses in the SBR since if non-accumulating organisms were selected they could have affected the PHA storage capacity of the PHA-accumulating culture (Reis et al., 2011)). In fact, this result much lower when compared to which was reported by Albuquerque et al., (2010b). The authors have shown that the culture selected with fermented molasses in a SBR operated at an influent substrate concentration of 45 C-mmol VFA/L attained a PHA cell content of 74.6 %(g-PHA/g-VSS) under nitrogen limiting conditions. Up to date, this is the highest PHA content obtained with MMC and complex waste based feedstocks. The PHA content attained in the

different PHA batch accumulations assays was consistent with which has been generally reported in literature for PHA production by mixed microbial cultures (65 % (g-PHA/g-VSS), Serafim et al., (2004); 48 % (g-PHA/g-VSS), Bengtsson et al., (2008); 57 % (g-PHA/g-VSS), Mengmeng et al., 2009). However, these values are still lower compared to those 89 % (g-PHA/gVSS) reported by Johnson et al., (2009) for a mixed cultures fed with single synthetic carbon source (acetate).

4.3.3. Effect of OAs concentration profiles in polymer profile and PHA production yield

The three batch accumulation assays were evaluated in terms of PHA storage yield on substrate ($Y_{\text{PHA/OAs}}$). The lower value was obtained for the production batch with fermented molasses, 0.49 C-mol PHA/C-mol OAs to which corresponded a PHA volumetric productivity of 0.31 g PHA/l h (data not shown). However, the accumulation batch assays with OAs synthetic solution and clarified fermented CW attained 0.74 and 0.73 C-mol PHA/C-mol OAs (Table 4.4) and a volumetric productivity of 0.564 and 0.561 g PHA/l h, respectively. The maximum PHA storage rate was attained for culture selected with fermented CW (0.42 C-mol OAs/C-mol X h).

Numerous authors have studied the effect of organic acids profiles in the polymer composition (%HB:%HV molar ratio) and concluded that they are directly proportional to the molar fraction of organic acids precursors present in substrates. Acetate, butyrate and lactate are HB precursors while propionate and valerate and are precursors for HV (Albuquerque et al., 2007; Serafim et al. 2008; Bengtsson et al., 2010; Johnson^{00~+ et al., 2010}). This is generally consistent with metabolic pathways for PHA production that have been reported for pure cultures (Albuquerque, 2009).

In all experimental assays, co-polymers of hydroxybutyrate and hydroxyvalerate P(HB-co-HV) were produced with molar composition of 83:17 (%HB:%HV molar fraction) for the production assays in which fermented CW, and the synthetic OAs solution were used as feedstock (Table 4.4). This result was anticipated by the fact that the acetate and butyrate (81 and 86 % (C-mol HA/C-mol OAs) are the HB precursors and were completely channelled to its production. In the batch assay with PHA-accumulating organisms selected with fermented molasses (Figure 4.7), the polymer composition attained was 51:49 (%HB:%HV molar fraction) (Table 4.4). Indeed, this result was not what would be expected since 39 % (C-mol/C-mol OAs) of organic acids on the feedstock (data not shown) represented the percentage of HB precursors (acetate and butyrate), and 61 % (C-mol/C-mol OAs) the percentage of organic acids that should have been converted in HV monomers. These assays also demonstrate that the use of different fermented carbon sources in the PHA production by mixed microbial cultures results different PHA compositions.

4.4. Conclusions

In this study the valorization of complex waste/surplus feedstock through polyhydroxyalkanoates production by mixed microbial cultures was successfully established. The effect of substrate “seasonality” shifts in the performance of a PHA-accumulating culture selection SBR, and in PHA production was investigated. Fermented cheese whey, fermented sugar cane molasses, and the synthetic organic acids solution were used as substrates for the substrate shifts study.

It was concluded that the imposition of a feedstock shift to the PHA-accumulating SBR under FF conditions, depending of the feedstock substrate fed, may need a period of acclimatization, depending on feedstocks fed. Nevertheless the aerobic enriched culture demonstrated the capacity to adapt to a substrate modification, and three stable PHA-accumulating cultures were obtained, when subjected to a substrate shift under feast and famine conditions. However, the necessity of improvements on the performance of the selection reactor has been shown.

The culture selected on fermented molasses showed lower PHA accumulation performance, *i.e.*, lower polymer storage yield (0.49 C-mol PHA/C-mol OAs), production rate (0.14 C-mol PHA/C-mol X h), substrate uptake rate (0.11 C-mol OAs/C-mol X h) and maximum PHA content (56.2%), than the cultures selected with fCW, and synthetic OAs solution.

The maximum PHA content was attained by the PHA-accumulating culture selected with fCW, reaching 65.1% of the cell dry weight.

Even though, the shift of complex feedstock in PHA-accumulating culture selection is still at a very early stage of development, this work illustrates the advantaged of favoring the selection of a culture with the capacity to adapt its metabolism to different feedstocks. This will offer the possibility of using numerous substrates and improving strategies to optimize culture selection and polymer production.

Chapter V – General conclusions and future perspectives

In this work, the capacity of MMC adapt their metabolism to a feedstock seasonality shift in a 3-step MMC PHA production process was proven. Three stable PHA-accumulating cultures were obtained. It was also demonstrated that the imposition of a feedstock shifts to the 3-step MMC PHA production process, depending of the feedstock substrate fed, may need a period of acclimatization, depending on feedstocks fed.

The shift of complex feedstock in PHA-accumulating culture selection is still at a very early stage of development. However, this work illustrates the advantaged of favoring the selection of a PHA-accumulating culture with the capacity of use different feedstocks according their availability all over the year.

Though the good results obtained with the biomass acclimatization in the selection and PHA production stages, this process might be optimized, in order to maximize the PHA production.

Moreover, it is necessity to understand if the PHA storage culture will respond equally to a different substrate from what was selected.

In the past few years, it was clearly demonstrated that the use of different fermented carbon sources in the PHA production resulted in different polymer compositions. In this manner, the possibility of utilizing feedstock mixtures in the acidogenic fermentation, PHA-accumulating culture selection, and PHA production may open the door for tailor-made material properties by fine-tuning the polyester composition and consequently different physic-chemical polymer properties which may be produced will allow the utilization of PHAs in several industrial sectors.

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