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Licenciada

Production of polyhydroxyalkanoates from cheese whey -

pH effect on the acidogenic fermentation stage and nutrient needs of the culture selection stage

Dissertação para obtenção do Grau de Mestre
em Biotecnologia

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II

Universidade Nova de Lisboa

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nutrient needs of the culture selection stage

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Thesis for the Master degree in Biotechnology

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Acknowledgments

Resumo

Polihidroxialcanoatos (PHA) são poliésteres produzidos por uma grande variedade de microrganismos. São produzidos e armazenados intracelularmente como fonte de carbono e energia. Actualmente, os PHA são produzidos industrialmente de forma eficiente recorrendo a matérias-primas quimicamente definidas fermentadas por culturas puras, o que implica elevados custos de produção, tanto pela fonte de carbono como pelas condições de esterilidade exigidas. As culturas microbianas mistas surgem como uma alternativa, usando subprodutos industriais sem necessidade de condições de esterilidade. Contudo, a produção de PHA através de culturas mistas necessita de optimização. Com esta alternativa, os custos de produção de PHA serão reduzidos em relação à via de produção actual, tornando possível que os PHA possam competir com os plásticos convencionais.

O estudo descrito nesta tese teve como objectivo a optimização de um processo de produção de PHA passando pelo estudo da influência de diferentes condições de operação com o intuito de reduzir os custos de produção. O processo para produção de PHA utilizado envolve duas etapas: (1) fermentação acidogénica do soro de leite num reactor membranar com produção de ácidos orgânicos e etanol e (2) selecção da cultura acumuladora de PHA sob condições de fartura e fome num reactor descontínuo sequencial.

Numa primeira fase, a fermentação acidogénica de soro de leite foi alvo de estudo. Diferentes pH (6 e 5) foram aplicados e verificou-se que o pH influencia o perfil de ácidos orgânicos e etanol produzidos. A pH 6 o acetato era o ácido em maior concentração enquanto a pH 5, a concentração de acetato decresce e o butirato assume a posição de ácido dominante. Verificou-se também, que a pH 6 a eficiência de produção era superior, apresentando um rendimento de fermentação de 0.79 enquanto a pH 5 o rendimento era de 0.69 g-C g-C^{-1}. Posteriormente, avaliou-se a influência dos diferentes perfis de ácidos na fase de selecção da cultura acumuladora de PHA. Observou-se um decréscimo no rendimento de acumulação de PHA, de 0.64 para 0.20 C-mol PHA C-mol S^{-1} quando o butirato predominava a concentração de ácidos.

Numa segunda fase, investigou-se a necessidade de suplementar a cultura acumuladora de PHA com uma fonte externa de nutrientes, estudando duas possibilidades: (1) se a cultura seria capaz de utilizar componentes do soro de leite (proteínas) como fonte de nutrientes, sendo que o custo de produção iria diminuir excluindo a necessidade de suplementação externa de nutrientes e (2) se a cultura for incapaz de utilizar as proteínas provenientes do soro do leite, estas poderiam ser removidas tornando o processo ainda mais rentável. A cultura foi sujeita a cinco fases de operação, em que na primeira fase não havia limitação de nutrientes, na segunda a concentração foi diminuída para metade e assim sucessivamente, até que na quinta fase não houve suplementação de nutrientes. De facto, observou-se que a cultura é capaz de consumir proteínas à medida que a fonte externa de nutrientes é retirada, a

velocidade específica de consumo de proteína aumentou de 0.010 (primeira fase) para 0.033 g-Prot g-X^{-1} h^{-1} (quinta fase). Verificou-se também a capacidade de acumulação foi-se perdendo, na primeira fase a cultura apresentava um rendimento em PHA de 0.48, na segunda de 0.42, na terceira de 0.25 na quarta de 0.21 C-mol PHA C-mol S^{-1} e na quinta não foi sequer detectável. O que indica que a pressão selectiva aplicada ao longo desta experiência levou a uma selecção de uma cultura não acumuladora de PHA apesar de consumidora da proteína. Sendo que a proteína não é utilizada para produção de PHA, esta poderá ser removida aumentando assim a rentabilidade do processo.

Palavras-chave: Polihidroxialcanoatos; culturas microbianas mistas; fermentação acidogénica; soro de leite; fartura e fome

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters produced and stored intracellularly as carbon and energy source by a large amount of microorganisms. Nowadays, PHA are produced industrially in an efficient way using refined feedstocks fermented by pure cultures, which implies high production costs, from both the carbon source and the required sterility conditions. The mixed microbial culture (MMC) emerged as an alternative, using industrial byproducts without the needs of sterile conditions. However, PHA production by MMC requires optimisation. With this alternative, the cost of PHA production will be reduced as compared to the current production, making possible the competition between the PHA and the conventional plastics.

This study aimed at optimising the MMC PHA production process from cheese whey (CW). The work focused on two stages of the MMC PHA production process : (1) the acidogenic fermentation in a membrane bioreactor (AnMBR) producing organic acids and ethanol and (2) the MMC PHA-accumulating selection under feast and famine conditions in a sequencing batch reactor fed with fermented CW.

Initially, the pH influence on CW acidogenic fermentation was studied. Two pH, 6 and 5, were applied in the AnMBR, and it was noticed that the pH affects the organic acids and ethanol profile. At pH 6 the acetate was the higher acid concentration while at pH 5, the concentration of acetate decreases and butyrate assumes the position of dominant acid. It was also observed that at pH 6 production efficiency was higher, showing a fermentation yield of 0.79 while for pH 5, the yield was 0.69 g-C g-C^{-1}. Subsequently, the influence of different acid profiles in the selection phase of the PHA-accumulating culture were study. There was a decrease in the PHA accumulation yield from 0.64 to 0.20 C-mol PHA C-mol S^{-1} when butyrate prevailed in a higher concentration.

In a second study, the need to supplement the PHA-accumulating MMC with an external source of nutrients was investigated by studying two possibilities: (1) if the culture would be able to use CW proteins as nutrients source, given that the exclusion of the need to supply nutrients would decrease the production cost, or (2) if the culture would be incapable of using CW proteins. The latest would open the possibility of recovering the CW proteins, producing protein concentrates, an added-value product, making the process even more cost effective. The culture was subjected to five operation phases, where in the first phase excess nutrients were supplied, and in the following phases the supplementation was decreased until in the fifth phase there was no nutrients supply. It was observed that the MMC was capable of consuming the proteins when the external source of nutrients was removed, the specific protein uptake rate increased from 0.010 (first phase) to 0.033 g-Prot g-X^{-1} h^{-1} (fifth phase). However, the accumulation capacity was lost, the culture's PHA yield continuously decreased from 0.48 C-mol PHA C-mol S^{-1} until no PHA storage being detected in the fifth phase. In conclusion, the selection pressure applied

during this experiment led to the selection of a non PHA-accumulating MMC despite consuming the protein. Since the fCW protein was not used for PHA production, it can be removed thereby increasing the profitability of the process.

List of Abbreviations

AD- Degree of acidification

AnMBR- Anaerobic membrane biorector

C/N/P- Ratio of carbon/nitrogen/phosphurus, in C-mol /N-mol/ P-mol

CSTR- Continuous stirred tank reactor

CW- Cheese Whey

DO- Dissolved oxygen

EPS- Exopolysaccharides

EtOH- Ethanol

fCW- Fermented cheese whey

FF- Feast and famine regime

F/F- Feast and famine ratio, in $h\ h^{-1}$

GC- Gas chromatography

HAcet- Acetic acid

HB- Hydroxybutyrate

HBut- Butyric acid

HLac- Lactic acid

HOrgs- Organic acids

HPLC- High performance liquid chromatography

HProp- Propionic acid

HRT- Hydraulic retention time, in days

HV- hydroxyvalerate

HVal- Valeric acid

mcl-PHA- Medium chain length PHA

MMC- Mixed microbial culture

OLR- Organic loading rate, in $g\text{-}C\ L^{-1}d^{-1}$

P-Product

PB- Polybutene

PBAT- Poly(butylene adipate-co-terphthalate)

PE-LD- Polyethylene low density

PE-LLD- Polyethylene linear low density

PE-HD- Polyethylene high density

PET- Polyethylene terephthalate

PHA- Polyhydroxyalkanoates

PLA- Polylactic acid

PP- Polypropylene

PS- Polystyrene

PVC- Polyvinyl chloride

q_{fCW}- Specific production rate, in g-C fCW g-C X^{-1} h^{-1}

q_{PHA}- PHA storage rate, in C-mol PHA C-mol $X^{-1}h^{-1}$

q_{Prot} -Specific protein uptake rate, in g-Prot g-X^{-1} h^{-1}

q_S- Specific substrate uptake rate, in C-mol fCW C-mol $X^{-1}h^{-1}$ or g-C S g-C X^{-1} h^{-1}

q_X- Specific biomass growth rate, in C-mol C-mol^{-1} h^{-1}

r_{fCW}- fCW volumetric productivity, in g-C fCW L^{-1} d^{-1}

r_S- Volumetric substrate uptake rate

S- Substrate

SBR- Sequencing batch reactor

scl-PHA- Short chain length PHA

SRT- Sludge retention time, in days

TCA- Tricarboxylic acid cycle

TOC- Total organic carbon

VFA- Volatile fatty acids

VSS- Volatile suspended solids, in g L^{-1}

X- Active biomass

$Y_{PHA/S}$- PHA storage yield in C-mol PHA C-mol fCW $^{-1}$ or g-C g-C^{-1}

$Y_{X/S}$- biomass yields, in C-mol X C-mol fCW $^{-1}$ or g-C g-C^{-1}

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Chapter 1

Motivation and Thesis Outline

1.1 Motivation

The increasing demand of alternatives to replace plastics is growing due to the economic and ecological instability and environmental issues. Bioplastics, biodegradables and biobased are a promising alternative.

Currently, bioplastics as polyhydroxyalkanoates are already in the market, Biomer™ and Biocycle™ are companies trying to compete with the conventional polymers. The difference between the price due to the PHA production costs by pure cultures, the high price of carbon sources and the costs of maintenance process are problems which need to be solved. The solution to decrease the PHA costs goes through the replacement of refined carbon sources by raw materials and the substitution of pure culture by mixed microbial cultures.

Trying to implement this kind of process, many efforts have been done but unfortunately, nowadays there is not any company cable of producing PHA by mixed microbial culture with raw materials. It is necessary to optimise this process.

The present thesis aims to optimise a PHA production by mixed microbial culture using cheese whey as feedstock going through the study of the pH influence in the acidogenic fermentation stage and the study of the nutrient needs in the culture selection stage.

1.2 Thesis Outline

This thesis is composed of five chapters, including the current introductory chapter describing the motivation and the outline of the work developed during the master project. Chapter 2 is a general introduction where the necessity of PHA development was remarked, their properties, synthesis and applications were described.

Chapter 3 and 4 are dedicated to the main work, the optimisation of the PHA production process. In the cheese whey acidogenic fermentation the effect of pH in the fermented products profile and on polymer composition was studied (Chapter 3). In the chapter 4 the influence of nutrients in PHA-accumulating culture selection was investigated.

Chapter 5 is a general conclusion and a future perspective summary.

Chapter 2

General Introduction

2.1 Plastics

The plastics industry has changed the world, and it continues doing so. According to Plastics Europe (2012), in Europe 27 there are 59000 plastic companies, where around 145 million people work, which create an estimated annual turnover of about 300 billion euros. The global plastic production has been exponentially increasing (about 9% per annum since 1950) (Figure 2.1) over the last years, because of the growing demand (Plastics Europe 2012).

	1950	1976	1989	2002	2009	2010	2011
	1,7	47	99	204	250	~270	~280
	0,35	19,8	27,4	56,1	55	57	~58
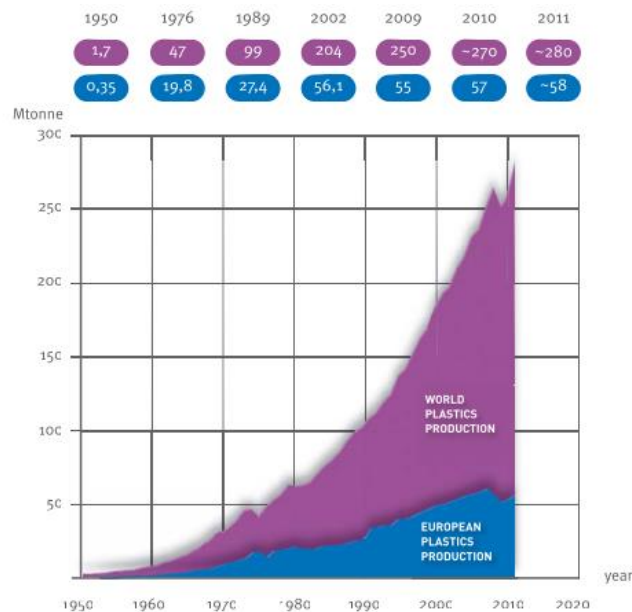
Figure 2.1 World plastics production. Adapted from Plastics Europe (2012).

Different types of plastics have been developed, and they can be divided in two groups: thermoplastics (polymers that can be melted), and thermosets (polymers that decompose after heating). There are plastics with big differences, with specific properties to cover each application that the industry needs. Polyethylene (low density PE-LD, linear low density PE-LLD and high density PE-HD), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyethylene terephthalate (PET) are the most commonly used.

Generally, the plastics have a bad image in comparison with other materials, particularly considering the environmental impact and the use of resources. All these plastics are oil crude, coal, or natural gas derived, which are limited and non renewable sources. The production involves distillation process, chemical compounds, and specific catalysts. As the final result of this production, fossil hydrocarbons are transformed into CO_2 and released into the atmosphere (Mulder et al. 1998; Digregorio et al. 2009). Consequently, the pollution originated with plastics production is a big environmental world problem. They are durable and persist in the earthen and marine environment causing an amount of damages, primarily in animals. The ocean pollution with plastics is growing in the last four decades and will remain for centuries due to their high recalcitrance (Morét-Ferguson et al. 2010).

2.2 Bioplastics

The bioplastics industry is expected to grow significantly in the next years. According to European Bioplastics, the global bioplastics production capacity is set to grow 500% until 2016. In 2011, the worldwide use of bioplastics was 85000 metric tons. Until 2016 the production of PHA is expected to grow 34 % to reach 3.7 million metric tons (BBC Research 2012). Bioplastics are biobased, biodegradable polymers, or both, made of renewable and/or biodegradable materials. They have the necessary properties to replace the conventional plastics, covering all the same applications. Their production is sustainable and environmentally friendly, they reduce significantly the harmful remaining caused by petrol plastics, the CO_2 production and the global warming. The bioplastics family (Figure 2.2) can be divided in three groups:

1) Fully or partly biobased and non biodegradable polymers, such as biobased Bio-PE, Bio-PET or Bio-PP;

2) Polymers that are biobased and biodegradable, including polylactic acid (PLA) and polyhydroxyalkanoates (PHA);

3) Polymers that are based on fossil resources and are fully biodegradable, such as Poly(butylene adipate-co-terphthalate) (PBAT) or polybutene (PB).
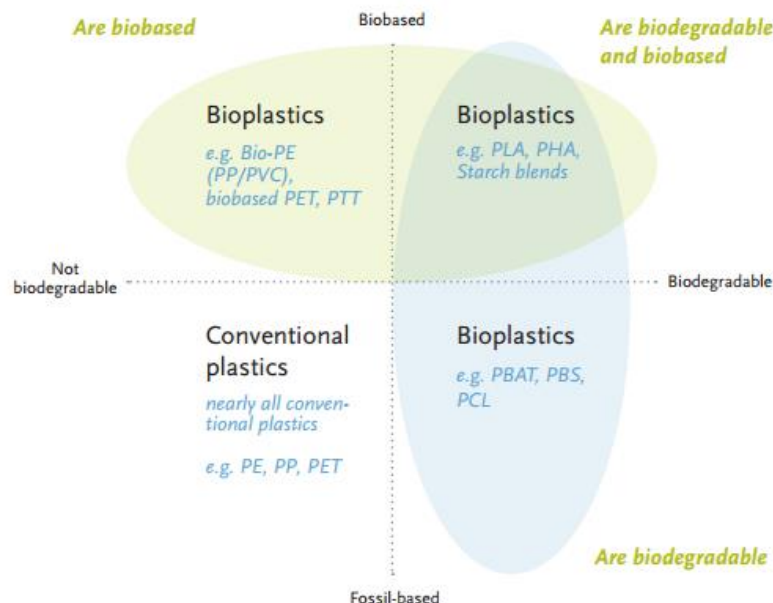
Figure 2.2 Groups of bioplastics. Adapted from Bioplastics (2012).

2.3 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a unique family of biopolymers, which are bacterially synthesised, with biodegradability, biocompatibility and thermoprocessibilty. They may be degraded in biological medium to form products innocuous to the environment: water and carbon dioxide under aerobic conditions, or water and methane under anaerobic conditions (Volova et al. 2010). The most common PHA, poly(3-hydroxybutyrate) (P3HB), was first described by Lemoingne in 1925 (Doi 1990). Since then, various bacterial strains have been identified as PHA-accumulating and various other types of PHA have been discovered (Chee et al. 2010).

2.3.1 Chemical structure and properties

PHA can be classified according to the monomer size. Usually, they are divided in two main groups which include the short chain length PHA (scl-PHA) that is constituted by monomer units with 3-5 carbon atoms, and the medium chain length PHA (mcl-PHA) that contain monomer units of 6-18 carbon atoms, as showed in Figure 2.3 (Laycock et al. 2012). Scl-PHA exhibit higher crystallinity, stiff and brittle, demonstrating thermoplastic-like properties, while mcl-PHA present lower crystallinity and more elasticity (Sudesh et al. 2000).
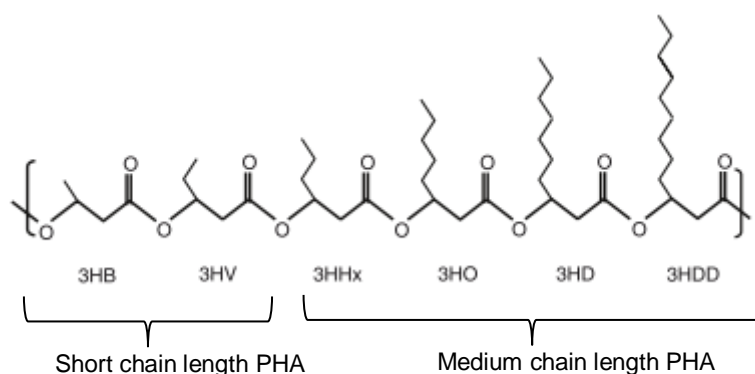
Figure 2.3 PHA monomer structures. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV). Medium-chain-length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD). Adapted from Chen (2010).

About 150 PHA monomers have been reported (Chen 2010). P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV) are the most common PHA. Due to the diversity of monomeric units and varying the type and proportion of PHA monomers, the physical and mechanical properties (such as glass and melting transition temperatures) are affected. P(3HB) have good thermoplastic properties. Glass transition temperature is around 4 ºC and melting temperature is about 180 ºC (Laycock et al. 2012; Lee 1996; Sudesh et al.

2000). This polymer is limited to some applications due to its high crystallinity (55-80%), it is fairly stiff and brittle. Vogel et al. (2007) succeeded when they improved crystallinity properties of PHB, which overcame the brittleness of PHA and created very strong monomers with new perspective applications. Copolymer P(3HB-co-3HV) (Figure 2.4), are attractive since they have mechanical properties similar to PP and PE: they have a partially crystalline structure (degree of crystallinity between 40% and 80%); the glass and melting transition temperatures are also lower when compared to P(3HB); they present a higher melt viscosity, which is a desirable property for extrusion blowing and higher toughness (Laycock et al. 2012; Sudesh et al. 2000; Yang et al. 2012).
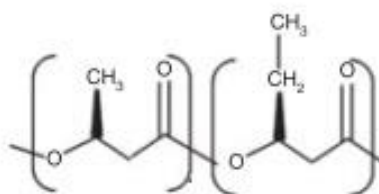
Figure 2.4 Structure of copolymer P(3HB-co-3HV). Adapted from Yang et al. (2012).

2.3.2 Biosynthesis

There are more than 250 different natural PHA producing microorganisms, but only a few bacteria have been employed for the industrial biosynthesis of PHA. This biopolymer is stored as granules in the cell cytoplasm (Figure 2.5) in insoluble inclusions as carbon and energy source (Laycock et al. 2012).
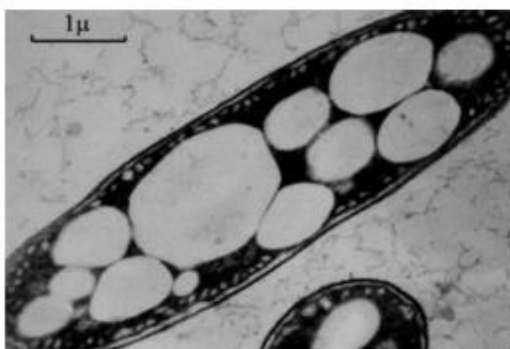
Figure 2.5 PHA granules in *Azotobacter chroococcum* cell treated with phenylacetic acid. Adapted from Laycock et al. (2012).

Biosynthesis by pure cultures

Alcaligenes latus, *B. megaterium*, *C. necator* and *P. oleovorans*, are microorganisms capable of using the carbon sources to produce PHA (Chee et al. 2010). Currently, commercial PHA is produced by pure cultures in their natural state or using genetically modified strains. The

company Biomer™ (PHB) and Biocycle™ (PHB and P(HB-co-HV)) are using natural strains. Biopol™ (P(HB-co-HV)) and Nodax™ (P(HB-co-HHx) are using recombinant strains (Lemos et al. 2006). Unfortunately, this biotechnology process using pure cultures is unfavourable comparing PHA with conventional plastics due to their costs. The industrial production process of PHA by pure cultures results in high costs, expensive equipment and high energy consumption, due to the requirement of refined feedstocks and aseptic process conditions (Johnson et al. 2009). The final price of PHA depends on the substrate price, conditions of production, the PHA yield on substrate, and on downstream process efficiency (Lee 1996). Basically, PHA production using pure cultures can be described as a two stage batch production process. Under sterile conditions, a bacteria inoculum is introduced into a medium solution (carbon source and nutrients) where the first stage occurs – growth. In the second stage, there is a limitation of an essential nutrient (such as N, P, or O_2) and this circumstances favour the PHA accumulation (Laycock et al. 2012). Pure cultures, specifically *C. neactor*, can store up to 90% of their cell dry weight (Lee et al. 2008). The properties of the final PHA depends on the carbon source used to feed the culture, the metabolic pathways that culture use for the conversion, and the substrate specificities of the involved enzymes. Many factors have to be considered for the industrial production. The ability of the cell to use the carbon source, the growth rate and the maximum polymer accumulation have to be considered and are very important parameters to choose a microorganism to produce PHA (Ojumu et al. 2004).

Biosynthesis by mixed microbial cultures

To make PHA a competitive bioplastic to replace conventional plastic, a reduction on the final cost is necessary. The strategy to contour this problem would be substituting the refined feedstocks for raw materials and appeal to mixed microbial cultures (MMC), abolishing the sterile conditions. Wallen and Rohwedder (1974), detected PHA in MMC in a wastewater treatment plant for the first time. Since then, great efforts to develop and optimise production processes using MMC fed with raw materials as carbon source have been done. The maximum MMC PHA cell content was reported by Serafim et al. (2004), 78.5% obtained in activated sludge using a pulse substrate feed strategy.

To induce the PHA synthesis and select a culture able of storing polymer, pressure conditions need to be applied. There are some strategies to create a selective pressure, such as the feast and famine conditions (FF) (also called aerobic dynamic feeding), or aerobic/anaerobic conditions (Bengtsson et al. 2010). In the FF strategy the culture is exposed to a long period of famine, during which the culture consumes all the non essentials metabolites as maintenance energy because they don't have substrate to grow, so the growth metabolites are non essential. After this period, a limited quantity of substrate is supplied to the culture (feast period) in a short period of time. When the culture has substrate in the broth the microorganisms can respond in two ways: (1) they can consume the substrate and store it intracellularly as PHA or (2) they can grow, but first of all they need to synthetise the growth metabolites. As the first option is faster than the second one, the microorganisms capable to store PHA have advantage because they

can collect more substrate than the others. The organisms that are able to accumulate the substrate during the feast period can grow during the famine period and the others just can grow during the feast period which is a very short period. Therefore, in repeated cycles of FF regime, only the accumulating microorganisms are favoured and start to be predominant. The non accumulating microorganisms can not grow with a growth rate which allows being in the reactor, so the washout happens. A common cycle of FF strategy is showed in Figure 2.6 (a).

The aerobic/anaerobic strategy has been performed in biological nitrogen and phosphorus removal systems. Under this conditions, polyphosphate and glycogen accumulating organisms are capable to accumulate PHA (Laycock et al. 2012) (Figure 2.6 (b)).
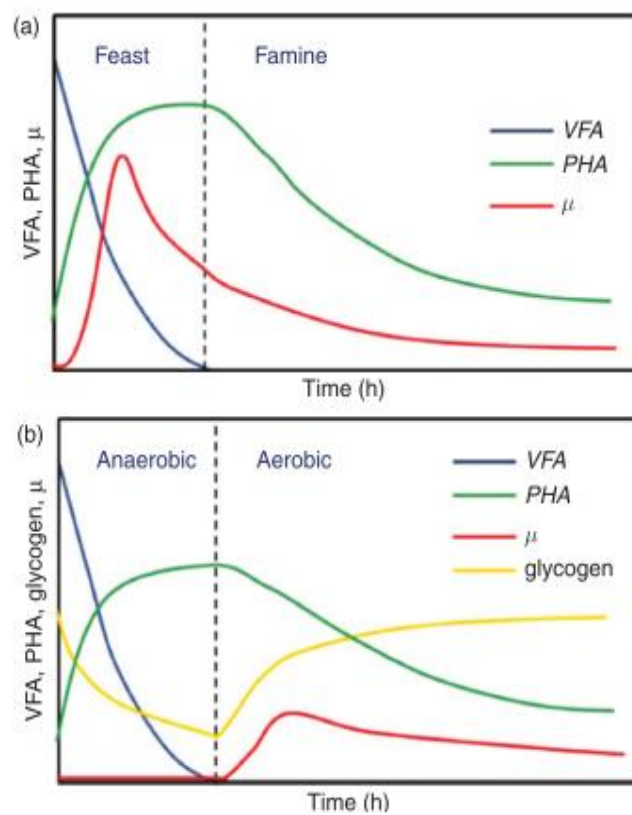
Figure 2.6 Usual behaviour of MMC under feast and famine (a) and aerobic/anaerobic (b) conditions. VFA represent the carbon source. Adapted from Reis and Albuquerque (2011).

The PHA accumulating MMC have the capacity to produce PHA from diverse carbon sources from raw materials, such as plant oils, fatty acids, alkanes, and simple carbohydrates. The waste material from agricultural and food processing industries, that are generally discharged, can be used as carbon source for PHA production, saving costs and the environment from their waste disposal (Chee et al. 2010).

Volatile fatty acids (VFA) are the favourite substrates for PHA production. Depending on raw material, an anaerobic fermentation can be applied to convert some organic compounds to VFA (Bengtsson et al. 2008). Figure 2.7 represents a three stage PHA production process: (1) the conversion of raw materials used as feedstock is done in an anaerobic stage; (2) in the selection stage the PHA-accumulating MMC is selected; and (3) in the accumulation stage the maximum PHA storage is reached.
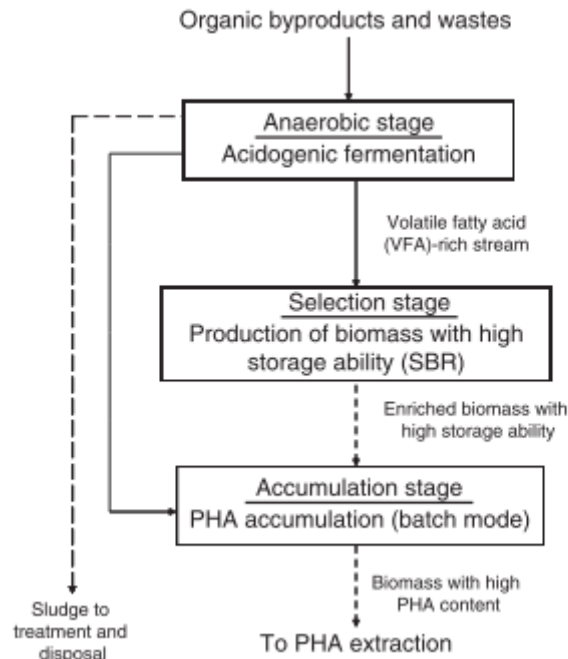
Figure 2.7 Scheme of a usual PHA production process. Adapted from Reis and Albuquerque (2011).

2.3.3 Metabolic pathways

PHA production process occurs under stress conditions, when, despite the availability of substrate (carbohydrate), cells are unable to grow. The stress conditions can be caused by the external limitation of essential nutrients such as oxygen, phosphorous or nitrogen, or by an internal limitation anabolic enzyme levels or activity (Sudesh et al. 2000). In most PHA producing pure cultures (*C.necator* and *A. latus*), the carbohydrate is degraded by catabolic pathway, resulting in the production of pyruvate, energy (adenosine triphosphate), and reducing equivalents (reduced nicotinamide adenine dinucleotide). When the system has all the conditions to grow, pyruvate is converted to acetyl-CoA, which is oxidised into CO_2 in the tricarboxylic acid (TCA) cycle with generation of anabolic precursors and more energy reducing equivalents. When the system has growth limitations, acetyl-CoA can be converted in PHB instead of being oxidised to CO_2. During this process, if protein synthesis decreased, caused by an external limitation, reducing equivalents would accumulate in the cell, inhibiting the TCA

cycle enzymes, and the acetyl-CoA would be directed just for PHB production (Reis and Albuquerque 2011). Three enzymes are involved in PHB production from acetyl-CoA: 3-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC) (Sudesh et al. 2000). PhaA is responsible for the condensation of two units of acetyl-CoA to produced acetoacetyl-CoA, which is reduced by PhaB to 3-hydroxybutyryl-CoA, which is then incorporated into a polymer chain as HB by PhaC (Figure 2.8).
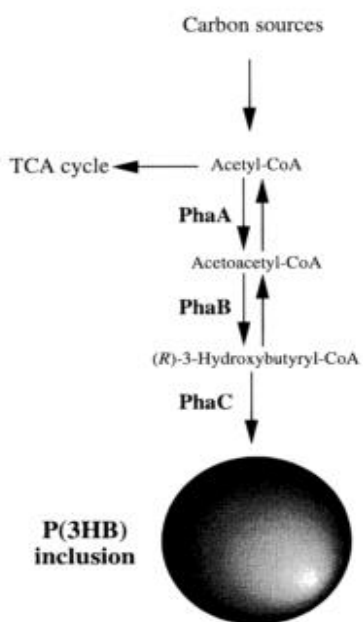
Figure 2.8 Scheme of P(3HB) accumulation, highlight on the enzymes involved. 3-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC). Adapted from Sudesh et al.(2000).

In MMC the PHA production occurs through the same pathways as in pure cultures. The production of copolymer P(3HB-co-3HV) when the system is fed with VFA can be expected, as showed in Figure 2.9. The acetate is converted in 3HB, as described above. Butyrate and valerate can be converted in 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, which form 3HB and 3HV, respectively. Propionate can be converted in 3HB or 3HV. If one unit of propionyl-CoA is combined with one unit of acetyl-CoA, a 3-hydroxy-2-methylbutyrate is formed. If two units propionyl-CoA are combined, a 3-hydroxy-2-methylvalerate is formed. For the HB syntheses, the previous production of acetyl-CoA is always necessary (Lemos et al. 2006; Reis and Albuquerque 2011).
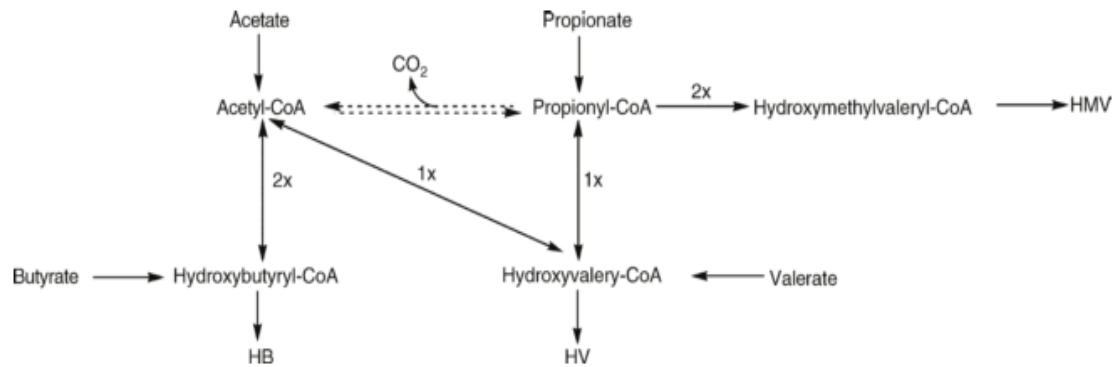
Figure 2.9 Representation of metabolic pathways of P(HB-co-HV) from VFA. The 1x and 2x means one or two units, respectively. Adapted from Lemos et al. (2006).

2.3.4 Downstream process

PHA extraction is a critical point of costs in PHA production, but very weak efforts have been done to develop cheaper, more efficient and environmentally friendly PHA extraction methods. The separation of PHA-containing cells from the broth can be done by conventional procedures such as centrifugation, filtration or flocculation (Kessler et al. 2001). A good strategy to recover PHA should minimise polymer degradation, and maximise extraction yield and final product purity. Good results have been reported, reaching efficiencies of recovery and purity up to 90% and 97%, respectively. Two different principles have to be taken in consideration in PHA recovery: the polymer solubility in appropriate solvents; and the disruption of the cell membrane (Dias et al. 2006). The most used methods to extract PHA from cells involve solvents, such as chloroform, methylene chloride, propylene carbonate and dichloroethane. These methods can result in a very pure PHA, but they have disadvantages due to the used solvents, which not only increase the final cost, but also have adverse environmental consequences due to their toxicity (Reis and Albuquerque 2011). An alternative method uses hypochlorite: first of all, PHA granules are isolated from the cell by centrifugation; and then biomass is treated with a sodium hypochlorite solution, which degrades the cellular material other than PHA (Berger et al. 1989). The main disadvantage of this method is the possible degradation of PHA, yielding PHA with a lower molecular weight (Chee et al. 2010). Another method to extract PHA is the enzymatic digestion. Enzymes like protease, lysozyme and phospholipase can be used being very specific, with no polymer degradation, and resulting in the efficient recovery of high purity polymers (Dias et al. 2006; Reis and Albuquerque 2011). This method requires a short period of heat shock treatment to the culture broth to break the cells (Dias et al. 2006).

There is not a preferable method to extract PHA from pure or mixed cultures. It is necessary to adapt a strategy for PHA extraction in each process.

2.3.5 Applications

According to Plastics Europe (2012), packaging is the major application for PHA, representing more than 39% of the whole demand in Europe. Nevertheless, PHA have more applications in many areas such as medical and pharmaceutical industries, textile industry, fine chemical industry, food industry, and biofuels (Chen 2009). Medical industry is the main area of research and improvement for PHA use. In the last years, PHA and their composites have been used to develop many medical devices, such as bone plates, cardiovascular patches, orthopedic pins (Dai et al. 2009), and in a specific area of drug delivery. In this last application, PHA have been used as microspheres and as an ingredient to nanoparticles (Chen 2010). Zhang et al. (2009), describes that monomers (3-hydroxybutyrate methyl ester and 3-hydroxyalkanoate methyl ester) obtained from PHA esterification could be used as biofuel. The authors showed that the studied PHA had a combustion heat of about 30 kJ g^{-1}, which is good when compared with ethanol, which has a combustion heat of 27 KJ g^{-1}.

Many PHA applications have been showed and investigated, which can increase the demand for this polymer and therefore intensify the improvement and the optimisation of PHA production.

Chapter 3

PHA production by mixed microbial cultures from cheese whey: effect of pH in the fermented products profile and on polymer composition

Abstract

Dairy surplus disposal represents a big environmental issue. Cheese whey acidogenic fermentation represents an advantage to treat their high organic content. The acidogenic fermentation can serve as a first step in a PHA production from mixed microbial culture. To produce PHA from cheese whey, a two stage process was used: (1) the cheese whey acidogenic fermentation and (2) the selection of PHA-accumulating mixed microbial culture. The aim of this work was to study the effect of pH on the cheese whey acidogenic fermentation and consequently the effect of the fermented cheese whey profile produced in the first stage on the PHA-accumulating culture selection stage. The acidogenic reactor was operated at pH 6 and 5. Subsequently, the PHA culture selection response to the produced fermented cheese whey was studied.

3.1 Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polymers able to replace the petrochemical polymers (Bengtsson et al. 2010). Nowadays, this polymer is industrially produced by pure microbial cultures fed with expensive subtracts, which increases the production costs compared to the conventional polymers. Therefore, PHA can not compete with the petrochemical plastics. The main PHA production cost is the substrate price, which is a critical factor that determines the performance of fermentation and consequently the final process cost. Renewable raw materials have been explored to reduce the costs in the PHA production. The waste materials from agricultural and food industries (sugar cane molasses, wastewater containing spent coffee grounds, cheese whey, effluent paper mill, etc.) can provide double benefits: (1) reduces the PHA production cost and (2) saves and reduces the environmental problem concerning their high organic content (Arroja et al. 2012; Chee et al. 2010). The PHA production from waste and surplus feedstocks requires a previous anaerobic fermentation stage, these waste materials can be converted into organic acids (lactic, acetic, propionic, butyric, valeric acid) and other fermented products, such as alcohols. Since organic acids are the favourite substrates for PHA production by mixed microbial cultures, acidogenic fermentation might be an important step for PHA production (Bengtsson et al. 2008). Reis and Albuquerque (2011) described a three stage process to produce PHA (1) the acidogenic fermentation, (2) the PHA-accumulating culture selection stage and (3) PHA production stage. The set up system used for PHA production from sugar cane molasses has already been performed by Albuquerque et al. (2007, 2010a, 2010b, 2011) The authors used an acidogenic continuous stirred tank reactor (CSTR) to ferment molasses to produce organic acids and these were used as feedstock for PHA-accumulating culture selection. Cheese whey, a dairy industry by-product (about 9 kg of whey per 1 kg cheese), is a substrate rich in lactose, which represents about 85 - 95% of the processed milk volume and retains 55 % of milk nutrients. Traditionally, cheese whey has been used to feed animals, but this end is not sustainable and the lactose intolerance of farm animals also limits the use of cheese whey (Kisielewska 2009). Using the cheese whey to produce organic acids both the high biological oxygen demand (about 50000 mg L^{-1} - 80000 mg L^{-1}) and disposal cost can be abated (Arroja et al. 2012). Bengtsson et al. (2008), who studied the hydraulic retention time (HRT) and the pH influence on organic acids production from cheese whey, have shown that the main fermentation products were acetate, propionate and butyrate at pH 6 – 3.5 and HRT of 8 - 35 h .

This work aimed to study the pH influence in the CW acidogenic fermentation. Two pH (6 and 5) were applied in the AnMBR and the fCW profiles were analysed. Subsequently, the influence of different acid profiles in the selection stage of the PHA-accumulating culture were study.

3.2 Materials and Methods

3.2.1 Cheese whey preparation

The anaerobic culture was fed with cheese whey powder supplied by Lactogal (Porto, Portugal). The composition and characteristics of cheese whey are described in Table 3.1. Cheese whey solution was prepared by diluting this feedstock in tap water at a final concentration of 15 g sugar L^{-1}. The medium was kept in the fridge at 4 ºC in a bottle continuously stirred.

Table 3.1 Cheese whey powder characterization by Lactogal.

Cheese whey powder	
Lactose content (% w/w)	78.4
Protein content (%w/w)	13.62
Fat content (%w/w)	1.21
Acidity (cm^3 per 100 g, NaOH 1 M)	11.4
Moisture content (% w/w)	1.8
Specific weight (g L^{-1})	570
Insolubility index (cm^3)	<0.1

3.2.2 Experimental set up

The experiments were carried out in a two lab-scale reactors system (Figure 3.1). The acidogenic fermentation was performed in an anaerobic membrane bioreactor (AnMBR) to convert lactose in fermented cheese whey products. The second step, PHA-accumulating culture selection, consisted in a sequencing batch reactor (SBR), subjected to selective pressure conditions.
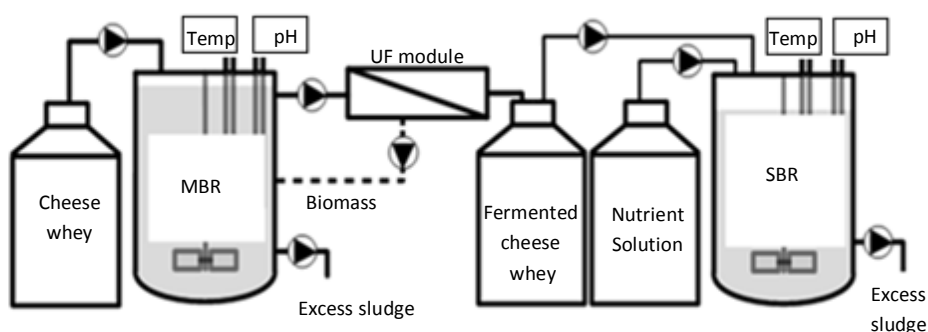
Figure 3.1 Two-stage experimental set up for PHA production using cheese whey as feedstock.

Step 1 - Acidogenic fermentation

The cheese whey acidogenic fermentation converts lactose in organic acids (lactate, acetate, propionate, butyrate and valerate) and ethanol under anaerobic conditions. The anaerobic fermentation of cheese whey was carried out in an AnMBR, consisting of a glass continuous stirred tank reactor (CSTR) with a working volume of 1250 mL coupled to an ultrafiltration hollow fiber membrane module (5×10^5 MW cut-off, GE). From time to time, when perturbations in the permeate rate were noticed, meaning that biomass and protein were attached to the membrane surface, the membrane was washed with 0.5 M NaOH and then with tap water until the pH reached the value 7. The AnMBR was inoculated with biomass from another AnMBR operating with cheese whey (CW) (unpublished data). The AnMBR was continuously operated at 30 ºC and mixing was provided at 300 rpm. The temperature was maintained using a water jacket and a thermostat bath. The sludge retention time (SRT) was kept at 3 days controlled by over flow and the hydraulic retention time (HRT) at 0.5 day controlled by the permeate flow rate (Table 3.2). The organic loading rate was kept at 15 g-sugar $L^{-1}d^{-1}$. The reactor was operated at two different pH values, 6 and 5 (Table 3.2), which was controlled by dosing a 2 M NaOH solution. The clarified fermented feedstock, named permeate, was collected under sterile conditions and kept at 4 ºC until its further utilization in the next step.

Table 3.2 Conditions and parameters measured in the cheese whey anaerobic fermentation in each phase.

Phase	pH	Operation days	OLR (g-C L^{-1}d^{-1})	HRT (days)	SRT (days)
I	6	21	11.2 (2.9)	0.49 (0.02)	3.03 (1.29)
II	5	51	19.9 (5.4)	0.50 (0.03)	2.46 (1.54)
III	6	28	17.9 (3.7)	0.51 (0.01)	3.15 (2.02)

(Standard Deviation)

Step 2 – PHA-accumulating culture selection

During five days, an activated sludge from a wastewater treatment plant "Beirolas" (Lisboa, Portugal) was acclimatized with 25 C-mmol L^{-1} of synthetic acids (acetate, propionate, butyrate and valerate) in two cycles (12 hours) per day. The culture was supplied with nutrients, namely ammonia (NH_4Cl) and phosphate (KH_2PO_4), in a C/N/P molar ratio of 100/10/1 and 10 mg L^{-1} allylthiourea to avoid nitrification.

After the acclimatization process, the PHA-accumulating organisms previously selected were transferred to a SBR with a working volume of 1 L and started being fed with the fermented cheese whey (fCW) produced in the AnMBR. The SBR was operated under feast and famine (FF) regime. Each SBR cycle consisted in 12 hours, divided in four phases: feeding (5 min),

aerobiosis reaction (1400 min), settling (20 min) and draw (15 min). The cycles were automatically controlled by a computer program developed in the group. The HRT was maintained at 1 day, while the SRT was kept at 4 days. The reactor was operated at an aeration rate of 1 L min^{-1} supplied through a ceramic diffuser, at room temperature and stirring was kept at 450 rpm. pH was not controlled. The mixed microbial culture (MMC) was fed, in each cycle, with 25 C-mmol L^{-1} of fCW and supplied with the same nutrient solution described above. The concentration of dissolved oxygen (DO) and temperature in the reactor was measured with a DO meter (HANNA Instruments, Portugal) and the pH was monitored with a pH meter (Mettler Toledo, USA). The SBR was operated in the first phase during 9 days, in the second phase during 58 days and the last phase was operated during 25 days.

3.2.3 Analytical Procedures

Lactose, lactate, acetate, propionate, butyrate, valerate and ethanol concentrations of filtered samples (0.45 μm) were measured by high performance liquid chromatography (HPLC) using a Merck-Hitachi chromatographer equipped with an RI detector and Metacarb 87-H Varian pre-column and column. Sulphuric acid 0.001 M was used as eluent with an elution flow rate of 0.5 mL min^{-1} and an operating temperature of 30 ºC. The fCW concentrations were calculated through a standard calibration curve (25-1000 mg L^{-1} of each acid and EtOH).

PHA determination was performed according to Serafim et al. (2004). Briefly, biomass taken to analyse PHA was lyophilized then was incubated with 1 mL of acidic methanol (20 % sulphuric acid v/v). To this solution was added 1 mL of chloroform with 1 g L^{-1} of heptadecane (as internal standard), and the mixture was digested in a dry-heat thermoblock at 100 ºC for 3.5 h. After cooling, 1 mL of water was added. The organic phase (methylated monomers dissolved in chloroform) was extracted and injected (2 μL) into a gas chromatograph equipped with a flame ionization detector (Bruker 430-GC) and a BR-SWax column (60 m, 0.53 mm internal diameter, 1 μm film thickness, Bruker, USA) coupled with a guard-column (0.32 mm internal diameter). Samples were analysed using helium as carrier gas at 1 mL min^{-1}, under a temperature regime starting at 40 ºC, increasing to 100 ºC at a rate of 20 ºC min^{-1}, to 175 ºC at a rate of 3 ºC min^{-1} and reaching a final temperature of 220 ºC at a rate of 20 ºC min^{-1} to ensure the cleaning of the column after each injection. Injector and detector temperatures were 280 ºC and 230 ºC, respectively. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through two calibration curves, one for HB and other for HV, using standards (0.1-10 g L^{-1}) of a commercial P(HB-co-HV) (88% / 12%) (Sigma), and corrected with the internal standard.

The biomass concentration was measured as volatile suspended solids (VSS) by filtration according to Standard Methods (ALPHA 1998).

Phosphate was determined using a colorimetric method implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, Netherlands).

The total polysaccharides were determined according to Dubois et al. (1956). Phenol 5% (w/w) and sulphuric acid were added to the samples and the absorbance measured at 490 nm. Lactose standards (0-200 mg L^{-1}) were used to determine total sugars concentration.

Proteins were determined spectrophotometrically at 750 nm using the Folin phenol reagent as described by Lowry et al. (1951). Bovine serum albumin standards (0-200 mg L^{-1}) were used for the determination of the protein content.

Total organic carbon from clarified samples was analysed in TOC-Vsh (Shimadzu, Republic of China).

3.2.4 Calculations
Acidogenic fermentation

Biomass concentration was calculated through the volatile suspended solids and converted to carbon (C) units. The fCW concentrations were measured by HPLC and converted to C units. The fCW yield on substrate ($Y_{fCW/S}$) was calculated by the equation 1, where T(fCW) are the total fermentation products produced (g-C L^{-1}) and TOC_{in}, TOC_{out} and TOC_{fCW} are the influent TOC, the permeate TOC and the fCW produced, respectively.

$$Y_{fCW/S} = \frac{T(fCW)}{TOC_{in} - (TOC_{out} - TOC_{fCW})} \; (gC \; gC^{-1}) \qquad \text{Eq. 1}$$

The yield of biomass on carbon ($Y_{X/S}$) was calculated as described in equation 1 but T(fCW) was replaced by the amount of biomass produced.

fCW volumetric productivity (r_{fCW}) and volumetric substrate uptake rate ($-r_S$) were calculate dividing the fCW produced and substrate consumed, respectively, converted to C units, by the HRT.

Specific substrate uptake rate ($-q_S$) and specific production rate (q_{fCW}) were calculated dividing the volumetric rates by the biomass concentration.

Degree of acidification was calculated by dividing the fCW produced, converted to C units, by the influent TOC.

Protein removal was calculated as the difference on the inlet protein concentration and the permeate concentration divided by the inlet concentration. Biological protein removal efficiency was calculated as the difference on the inlet protein concentration and the reactor concentration divided by the inlet concentration.

PHA-accumulating culture selection

The calculation of PHA content was calculated by the equation 2 and considering some assumptions (i- iii):

$$\%PHA = \frac{PHA}{VSS} \times 100 \ (gPHA \ gVSS^{-1}) \qquad \text{Eq. 2}$$

(i) $m(VSS) = m(X) + m(PHA)$

(ii) $X = C_5H_7NO_2$, $M_X = 113,1 \ g \ mol^{-1}$

(iii)   PHA is a P(HB-co-HV) polymer

The maximum specific substrate uptake rate ($-q_S$ in C-mol fCW C-mol $X^{-1}h^{-1}$) and the PHA storage rate (q_{PHA} in C-mol PHA C-mol $X^{-1}h^{-1}$) were determined by adjusting a linear function to the experimental data of specific fCW and PHA concentrations plotted over time. fCW concentration corresponds to the sum of all the organic acids concentrations (fCW in C-mmol L^{-1}), is equal to Σ HLac, HAct, HProp, HBut, HVal and EtOH. PHA concentration (in C-mol L^{-1}) corresponds to the sum of HB and HV monomers concentrations. The yields of active biomass ($Y_{X/S}$ C-mol X C-mol fCW $^{-1}$) and PHA ($Y_{PHA/S}$ in C-mol PHA C-mol fCW^{-1}) on substrate consumed were calculated by dividing the specific biomass growth rate and the PHA storage rate by the specific substrate uptake rate, respectively.

3.3 Results and Discussion

3.3.1 Effect of pH in the acidogenic fermentation

Cheese whey fermentation was performed in an anaerobic membrane bioreactor divided in three different phases (Table 3.2), as described above. Briefly, the AnMBR was firstly operated at pH 6 (phase I), then changed to pH 5 (phase II) and finally changed back to pH 6 (phase III). The AnMBR was operated for 21 days at pH 6 until reaching similar fCW profiles compared to the ones had in a previous AnMBR, operating under the same conditions (unpublished data). Then the pH was changed to 5 and the AnMBR was operated until a pseudo-steady state was reached. After 51 days of operation at pH 5, pH was turned back to 6 for 28 days. The overall AnMBR performance along the three phases of operation is shown in Table 3.3.

Table 3.3 Parameters measured during the pseudo-steady state in the three operation phases of acidogenic fermentation.

Phase	I	II	III
VSS (C-mmol L^{-1})	480.70 (186.67	289.80 (74.95)	590.07 (206.73)
Total fCW (g-C L^{-1})	5.14 (0.59)	5.19 (0.88)	5.33 (0.43)
-q_S (g-C S g-C $X^{-1} h^{-1}$)	0.08 (0.04)	0.19 (0.06)	0.10 (0.05)
q_{fCW} (g-C fCW g-C $X^{-1} h^{-1}$)	0.08 (0.03)	0.13 (0.03)	0.06 (0.02)
r_{fCW} (g-C fCW $L^{-1} d^{-1}$)	10.52 (1.07)	9.94 (1.21)	10.95 (1.82)
Biological protein removed (g g^{-1})	57.66 (40.30)	79.53 (14.38)	74.28 (12.36)
Total protein removed (g g^{-1})	68.03 (18.54)	86.56 (4.81)	81.30 (10.79)
Acidification degree (g-C FP g-TOC In^{-1})	0.83 (0.27)	0.68 (0.10)	0.71 (0.17)
$Y_{X/S}$ (g-C g-C^{-1})	0.20 (0.09)	0.15 (0.08)	0.20 (0.07)
$Y_{fCW/S}$ (g-C g-C^{-1})	0.79 (0.12)	0.69 (0.07)	0.72 (0.05)

The fCW profile changed in each phase. In the first phase (pH 6), acetate was the main fCW (with 45 % -molar basis) and butyrate was near to the same concentration (Figure 3.2). Propionate, valerate and ethanol had the smaller concentrations, lactate was residual and all the sugars were fermented. Through the second phase, at pH 5, butyrate was the major product with 60 % (molar basis) of the total fCW. Acetate represents the second major concentration (25 % -molar basis) of fCW and the others fCW were in lower concentration, similar to the earlier phase. Only lactate was higher due to the lower pH and about 14 % (molar basis) of sugars were not fermented. Finally when the reactor returned to pH 6 the butyrate was still the major product (24 % -molar basis), followed by the acetate (21 %) (Figure 3.2). In this phase the higher lactate percentage (26 %) is explained by the pH change, for the disturbance caused in the system. The propionate and valerate were in higher percentage too, due to the disturbance and to the little time of operation. This result can be explained by the insufficient time to reach the stability, because in the last phase it will be expected the same profile as the one in the first phase. Probably, the culture did not have time to readapt their performance to these conditions and if the operation time in the third phase was longer, the culture would possibly have reached the same fCW profile that in the phase I. Bengtsson et al. (2008) observed that the organic acids profile was significantly affected by HRT and pH. When pH increased from 5.25 to 6, acetate and butyrate production decreased and propionate production increased which is different from the results obtained in the present study, but the HRT used was not the same and the CW used by Bengtsson et al. (2008) was deproteinized. Some lactate peaks were noticed in the third phase, which were not represented in Figure 3.2 and were not considered in the parameters calculations. These outliers were related to system disturbances, such as temperature, and pH variations. In this specific case, the lactate peaks resulted from pH disturbances, namely from pH 5 to 6. As Albuquerque et al. (2012), have shown, when different conditions were applied to a MMC, one of its microbial populations would adapt their performance to the conditions applied and different population niches were developed, being that the adaptation was not immediate.

(a)

(b)

(c)
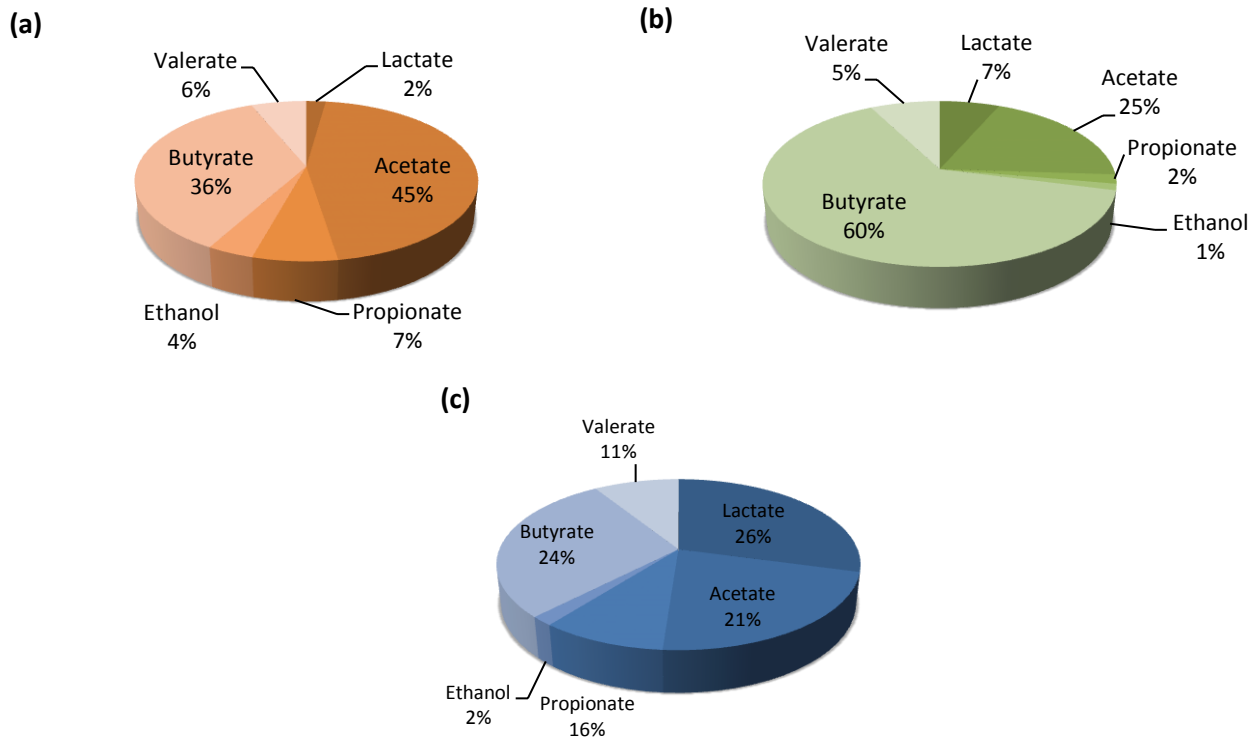
Figure 3.2 fCW profiles in pseudo-steady state at (a) pH 6 (phase I), (b) pH 5 (phase II) and (c) pH 6 (phase III).

It is quite notorious, that the pH change affects the fCW profile but not the fCW concentration, that was around 5 g-C L^{-1} (Figure 3.3 and Table 3.3) during the three phases.
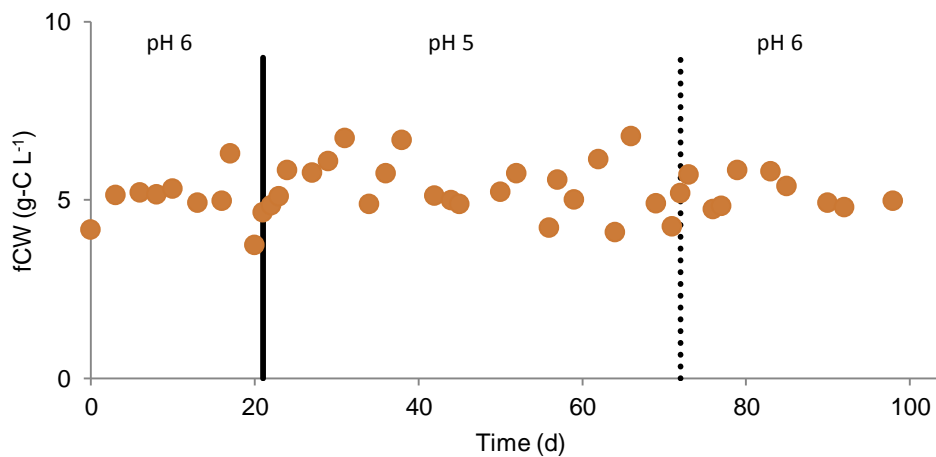
Figure 3.3 Total fermented products concentration during AnMBR operation. The straight line represents the day when pH was changed to 5 and the dotted line represents the day when pH returned to 6.

On the other hand, in the second phase the VSS (Table 3.3) decreased, while the total fCW concentration was still stable. The VSS concentration measured over the experiment was underestimated (Table 3.3) due to the fact that proteins attached to the membrane. Therefore, biomass was retained on the membrane surface causing membrane biofouling. Thus in the

27

second phase there was less biomass to convert the same concentration of sugars, suggesting that the culture used other source to maintain the fCW concentration, probably protein. Hassan and Nelson (2012) refer that hydrolytic bacteria under anaerobic conditions can degrade protein, carbohydrates and fat into simpler compounds, such as organic acids and alcohols which corroborates this theory. Subsequently, the specific substrate uptake rate ($-q_S$) and specific fCW production rate (q_{fCW}) (Table 3.3) were influenced by the VSS decrease and consequently the rates were higher in the second phase.

The acidification degree (AD) expresses the acidification efficiency of the substrate by the anaerobic culture (Arroja et al. 2012). As the substrate was the same during the three phases, similar values for the three phases would be expected. However, it was observed (Table 3.3) a reduction of the AD at pH 5 (from 0.83 to 0.68 g-C fCW g-TOC In^{-1}). Fang and Yu (2001), reported similar values of AD at pH 6 (78 %), but they have shown an increase of the AD (78 % to 81 %) when the pH decreased from 6 to 5. These different results compared with the present study could be explained by the substrate used by Fang and Yu (2001), which was lactose from wastewater, while cheese whey was the selected feedstock used in the present study.

The total protein and biological protein removed (Table 3.3) suggests that at pH 5 the cheese whey acidogenic fermentation was more efficient, but when the biomass and the fCW yields were analysed it is obviously that the best efficiency was at pH 6.

3.3.2 Effect of different FP profile in MMC selection

The aim of this experiment was to evaluate the repercussions in the PHA composition of the different fCW profiles produced in the AnMBR. The SBR was inoculated with a PHA-accumulating culture selected in another SBR operated during 7 months under the same conditions (unpublished data). The MMC was fed with the fCW from the AnMBR.

The average fCW profiles used to feed the PHA-accumulating culture in each phase are presented in Table 3.4. The feed, in the first phase, was mainly composed by acetate, in the second phase by butyrate and in the third phase by lactate, acetate and butyrate. However, the performance of the PHA-accumulating culture was very similar during all the three phases. A typical SBR cycle of the first phase is represented in Figure 3.4, in the other two phases the behaviour was very similar. The average of feast and famine ratio (F/F) in the first, second and third phase, was 0.13, 0.15 and 0.11, respectively. The F/F was very similar and was a good ratio to converge the selective pressure with the PHA-accumulating culture (Dionisi et al. 2006).

Table 3.4 Average of fCW profiles used to feed the PHA-accumulating culture.

Phase	HLac %	HAcet %	HProp %	HBut %	HVal %	EtOH %
I	0.9	49.7	5.0	35.8	4.1	4.6
II	8.2	26.1	4.7	53.7	3.3	4.0
III	25.7	22.4	16.1	23.3	10.1	2.4

(% - molar basis)
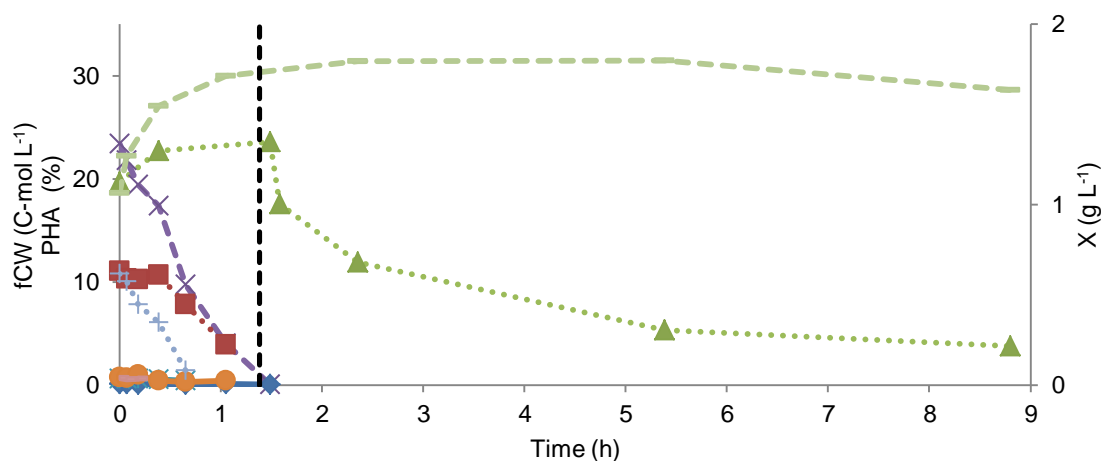
Figure 3.4 Usual FF cycle during the first phase in the SBR. The organic acids (HOrgs) and ethanol (EtOH) (x) consumption and the PHA evolution (▲) were represented. Biomass (---), HLac (♦), HAcet (■), HProp (*), HBut (+), HVal(-) and EtOH (●) were represented too. The dotted line marks the end of feast time.

The specific biomass growth rate (q_X) and the specific substrate uptake rate (q_S) decreased (Table 3.5) when butyrate represented the main fCW product (second and third phase). The MMC was adapted to high acetate concentrations and suddenly this main acid was replaced by

another one. This perturbation affected the MMC performance. As reported by Albuquerque et al. (2012), in the presence of multiples substrates, a microbial population specialized in specific substrates is favoured by adapting their performances to different niches. In the second phase q_{PHA} decreased abruptly probably because it was affected by the same cause. The PHA yield on substrate demonstrated that the MMC culture had a better performance when butyrate was in lower concentrations. Overall, butyrate seems to affect the culture on the PHA storage.

Table 3.5 Average performance of the PHA-accumulating culture in the SBR during the three operation phases.

Phase	q_X (C-mol C-mol^{-1}h^{-1})	$-q_S$ (C-mol C-mol $^{-1}$.h^{-1})	q_{PHA} (C-mol C-mol^{-1} h^{-1})	$Y_{X/S}$ (C-mol X C-mol S^{-1})	$Y_{PHA/S}$ (C-mol PHA C-mol S^{-1})
I	0.27 (0.01)	0.59 (0.13)	0.26 (0.06)	0.37 (0.01)	0.64 (0.05)
II	0.09 (0.02)	0.33 (0.17)	0.07 (0.05)	0.33 (0.15)	0.20 (0.10)
III	0.08 (0.02)	0.32 (0.04)	0.25 (0.03)	0.23 (0.05)	0.71 (0.01)

(Standard Deviation)

The polymer composition is affected by fCW profile, as described by Lemos et al. (2006). However, since the major products in all phases (acetate and butyrate) are precursors of P(HB), the final composition of the copolymer P(HB-HV) was similar. In the first phase the copolymer was 70 % of HB and 30 % of HV, in the second phase 87 % of HB and 13 % of HV and in the third phase the copolymer was 65 % of HB and 35 % HV.

3.4 Conclusions

The valorisation of cheese whey through PHA production by mixed microbial culture was successfully established. Different pH affected the cheese whey acidogenic fermentation by producing different fCW profiles. At pH 6, acetate was the main HOrg and at pH 5, butyrate was the dominant HOrg. The cheese whey acidogenic fermentation was more efficient at pH 6, showing a fermentation yield of 0.79 while at pH 5, the yield was 0.69 g-C g-C^{-1}.

The PHA-accumulating culture was clearly affected by the fCW profile. When the fCW was mainly composed by butyrate, the specific PHA production rate and the specific biomass growth rate decreased and consequently the PHA yield also decreased. There was a decrease in the PHA accumulation yield from 0.64 to 0.20 C-mol PHA C-mol S^{-1} when butyrate prevailed at a higher concentration.

Generally, the cheese whey acidogenic fermentation performed at pH 5 affected negatively the two stage PHA production.

Some suggestions for future work arise from the results obtained in this work:

1 - To study the effect of HRT at pH 5 on fCW profile.

2 -To study the effect of other pH values, such as 5.25 and 5.5, on the acidogenic fermentation step.

3 – To couple a back wash system to the membrane in order to solve the membrane biofouling.

Chapter 4

Influence of nutrients in PHA-accumulating culture selection

Abstract

Nowadays, the reduction of PHA production costs is mandatory. To achieve this, it is necessary to optimise the production process. The general aim of this study is the optimisation of the culture selection of the two stages process for production of PHA from cheese whey. The particular goal of the presented work is to study the nutrients supplementation needs of this stage, aiming at two possibilities: (1) if the culture is able to use components from cheese whey (such as proteins) as nutrients, the PHA production cost will decrease, since there will be no longer the need for external nutrient sources supplementation; (2) if the culture is unable to use proteins, these may be removed and recovered from cheese whey, allowing the production of an added-value product, the whey proteins concentrate. A PHA-accumulating culture was selected in a SBR operated under feast and famine regime, fed with fermented cheese whey, and supplied with a nutrient solution (ammonium and phosphorus), which concentration was gradually reduced. The system was monitored during the entire experiment, in terms of biomass concentration, substrate and protein consumption, and PHA production.

4.1 Introduction

Currently, polyhydroxyalkanoates (PHA) are a promising alternative to conventional plastics. PHA are commonly produced by pure cultures under sterile conditions fed with refined feedstocks. Alternatively, PHA can be produced by mixed microbial cultures (MMC) fed with raw materials. With this kind of production, conventional plastics can be replaced by PHA, because the production costs will be lower compared with the current plastics (Chakravarty et al. 2010). This way of PHA production is environmental encouraged, given that through it environmentally polluting raw materials can be converted in an eco-friendly biodegradable polymer. To make this possibility happen, many studies have been done. Albuquerque et al. (2007, 2010a, 2010b, 2011), described a three stage PHA production process, where in the first stage the raw material used (molasses) was fermented and converted into organic acids (HOrgs). In the second stage, the authors used a sequencing batch reactor (SBR) to select the PHA-accumulating culture. This aerobic reactor, which operated under feast and famine (FF) regime, was fed with the feedstock produced in the first stage and supplied with ammonia and phosphate. The FF regime is used to create an unbalanced growth and increase the PHA-accumulating capacity of MMC (Ince et al. 2012). The nutrient supply is needed to assure that the culture has nutrients to allow sufficient growth of the PHA-accumulating culture (Johnson, et al. 2010). The nutrient supply contributes to an additional increase in the final cost of PHA production and the benefits will have to justify the costs involved.

Jonhson et al. (2010) studied the influence of different ratios of carbon/nitrogen in the PHA-accumulating culture selection. These authors have shown that nitrogen limitation was a successful strategy for reaching high PHA contents during the PHA production step, but not in the PHA-accumulating culture selection. Nitrogen-limited cultures offer the advantage of a higher PHB content. Cheese whey, a dairy industry by-product, is an environmental problem concerning their high organic content. Usually, cheese whey has been used in agriculture but this end is not sustainable (Kisielewska 2009). Using the cheese whey to produce organic acids both the high biological oxygen demand and disposal cost can be abated (Arroja et al. 2012; Kisielewska 2009). Cheese whey contains nitrogen and phosphorus mostly in form of protein. During the cheese whey fermentation the anaerobic culture consumes part of this protein but other part is not consume and goes through the process until the selection stage.

This study aims to evaluate the need to supplement the PHA-accumulating MMC with an external source of nutrients. Investigate if the culture would be able to use CW proteins as nutrients source, decreasing the production cost and if the culture would be incapable of using CW proteins, they can be removed adding an added-value product increasing the profitability of the process.

4.2 Materials and Methods

4.2.1 Experimental set up

The two stage experimental set up was similar to the one described in the previous chapter (Chapter 3), with two bioreactors and a hollow fiber ultrafiltration membrane module. The first stage, cheese whey acidogenic fermentation, was carried out in an AnMBR. The second stage, selection of a PHA-accumulating culture, was done in a SBR, where culture selection was done under FF regime applying selective pressure conditions.

Step 1 - Acidogenic fermentation

Since the focus of this study was on the PHA-accumulating culture selection stage, it was necessary to assure that throughout the entire operation the obtained results would not be influenced by the anaerobic fermentation stage process. Consequently, to avoid changes in the SBR feed profile, 20 L of fermented cheese whey (fCW) were collected from the stable operation of a pilot AnMBR, and frozen in individual packs. The pilot AnMBR was composed of a 10 L bioreactor (BioStat®, B-Plus,Sartorius) coupled to a hollow fibre filtration module (5×10^5 MW cut-off, GE), and operated under an OLR of 15 g-sugars L^{-1} d^{-1}, under constant temperature, 30ºC, and pH 6, conditions. The HRT and SRT were kept at, respectively, 1 and 3 days. The fCW was collected during three days of AnMBR operation, and its composition was, in %C-mol basis: 46 % of acetate, 45 % of butyrate, 5 % of propionate, and 4 % of valerate.

Step 2 – PHA-accumulating culture selection

A sequencing batch reactor (SBR) was inoculated with a PHA-accumulating culture previously selected as described in Chapter 3, section 3.2.2.

The PHA-accumulating culture selection was done in a SBR operated under FF regime. The SBR was operated at 12 hours cycles consisting in four phases: feeding (5 min), aerobiosis reaction (1400 min), settling (20 min), and supernatant withdrawal (15 min). The HRT was maintained at 1 day, while the SRT was kept at 4 days by purging 250 mL of mixed liquor per day. The reactor was operated with an aeration rate of 1 L min^{-1}, supplied by a ceramic diffuser and stirred (250 rpm) during the feeding and aerobiosis reaction phases, and kept at room temperature. The pH was maintained at 8.80, through the automatic addition of a 0.5 M HCl solution. The concentration of dissolved oxygen (DO), temperature, and pH were continuously monitored with a DO meter (HANNA Instruments, Portugal), and a pH meter (Mettler Toledo, USA). The MMC was supplied, in each cycle, with 25 C-mmol L^{-1} of fCW and with a nutrient solution, ammonia (NH_4Cl) and phosphate (KH_2PO_4), in a varying C/N/P molar ratios, and 10 mg L^{-1} allylthiourea to avoid nitrification. During the SBR operation, different C/N/P ratios were applied, as presented in Table 4.1, being all the other operation conditions constant.

Table 4.1 Operation phases of the PHA-accumulating culture selection stage.

Phase	Operation time (days)	Ratio C/N/P (C-mol /N-mol/ P-mol)
I	9	100/10/1
II	21	100/5/0.5
III	13	100/2.5/0.25
IV	7	100/1.25/0.125
V	8	100/0/0

4.2.2 Analytical Procedures

All the analytical procedures used on this study are described in Chapter 3, section 3.2.3. Briefly, lactose, lactate, acetate, propionate, butyrate, valerate and ethanol concentrations were measured by high performance liquid chromatography (HPLC), and PHA were determined by gas chromatography (GC). The biomass concentration was measured as volatile suspended solids (VSS) by filtration according to standard methods (ALPHA 1998). Proteins were determined spectrophotometrically at 750 nm following the Lowry's Method, using an alkaline solution and the Folin reagent (Lowry et al. 1951). The total polysaccharides were determined according to Dubois' Method, phenol 5% and sulphuric acid were added to the samples and the absorbance measured at 490 nm (Dubois et al. 1956). Phosphate was determined using a colorimetric method implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, Netherlands).

4.2.3 Calculations

The specific protein uptake rate (-qProt) was calculated dividing the volumetric rates of protein consumption by the biomass concentration. All the other calculations done are described in Chapter 3, section 3.2.4 - PHA-accumulating culture selection.

4.3 Results and Discussion

The culture selection in the SBR was operated under the same conditions (HRT of 1 d, SRT of 4 d, OLR of 50 C-mmol HOrgs L^{-1}, and uncontrolled pH and T) for 7 months (from October 2012 until April 2013), successfully selecting a PHA-accumulating culture. However, the selected culture was never stable on the long-term (data not shown). The reason for this instability was the changeable fCW being produced from a lab-scale AnMBR. In order to be able to study the effect of different nutrients supplementation strategies to the selection stage, a stable selection reactor was mandatory, which meant supplying it with fCW which composition did not vary along the time. To assure this, the fCW from a pilot scale AnMBR was used.

The experiment started with excess nutrients supplementation (phase I: C/N/P 100/10/1) and after the steady stage the nutrients were decreased for half concentration (phase II: C/N/P 100/5/0.5). In this second phase the MMC's performance worsted and the reactor's appearance changed with some precipitation in the broth. In fact, the nutrient solution had buffer capacity in the system, and once this solution concentration was decreased to half, the system lost this buffer, and the proteins from fermented cheese whey precipitated. This was a problem, because it was intended to test if MMC could use the fCW protein as nitrogen source when external nitrogen was not supplied. The solution for such a problem was to couple a pH controller to keep the pH in the system constant at such a pH which assured no protein precipitation. To assure no protein precipitated the pH at which proteins precipitated was investigated. Figure 4.1 demonstrates that upper than pH 9 there was protein precipitation. According to this experiment, the SBR pH was set at 8.8 to assure no protein precipitated.
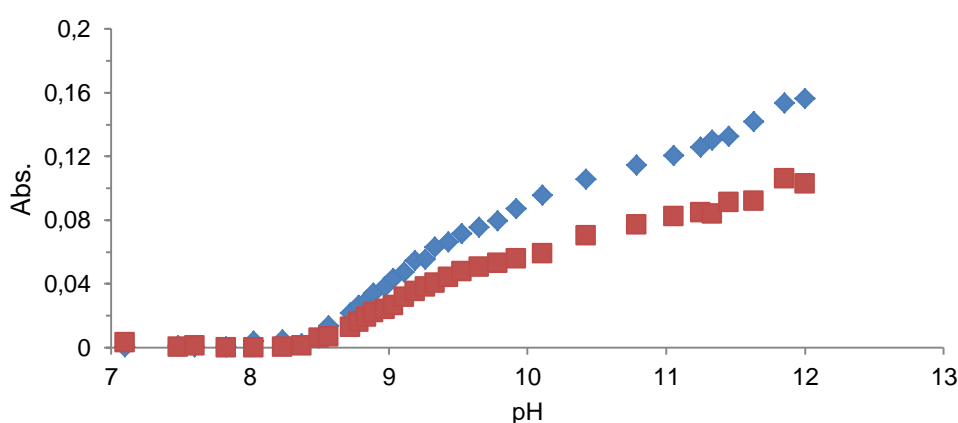

Figure 4.1 Behaviour of fCW protein at different pH. The protein absorbency was measured at 600 nm (♦) and 700 nm (■). pH was adjusted through the addition of 1M NaOH, and 1M HCl.

Once protein precipitation was solved, the plan of this work started to be done. A FF cycle is generally characterised by two periods: in the feast period the culture has external substrate

and uses it to produce and store PHA; in the famine period, after external substrate exhaustion, the PHA stored inside the cell is consumed. A usual FF cycle from the first phase is represented in Figure 4.2. During the first phase, the feast period was always less than 2 hours. So the feast to famine ratio (F/F) was 0,20 which has been found to be a good ratio for the efficient selection of PHA accumulating MMC, avoiding cells growth (Dionisi et al. 2006). Figures 4.2 until 4.6 represent FF cycles during the different operational phases, from I to V, and translate the adaptation of the MMC to each nutrients supply condition. It is notorious, that during the second and third phases the MMC had similar performances, while from the third to the fifth phases the fCW consumption time was very long and the PHA production was very low or null.

Figure 4.2 Usual FF cycle in the first phase (C/N/P of 100/10/1). The fCW (×) consumption and the PHA (▲) were represented. The vertical line notes the end of feast time.

Figure 4.3 Usual FF cycle in the second phase (C/N/P of 100/5/0.5). The fCW (×) consumption and the PHA (▲) were represented. The vertical line notes the end of feast time.

Figure 4.4 Usual FF cycle in the third phase (C/N/P of 100/2.5/0.25). The fCW (×) consumption and the PHA (▲) were represented. The vertical line notes the end of feast time.

Figure 4.5 Usual FF cycle in the fourth phase (C/N/P of 100/1.25/0.125). The fCW (×) consumption and the PHA (▲) were represented.
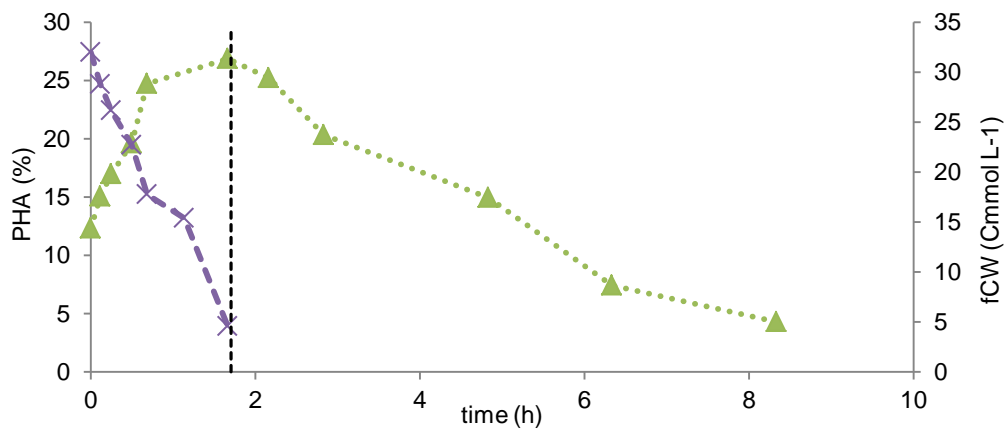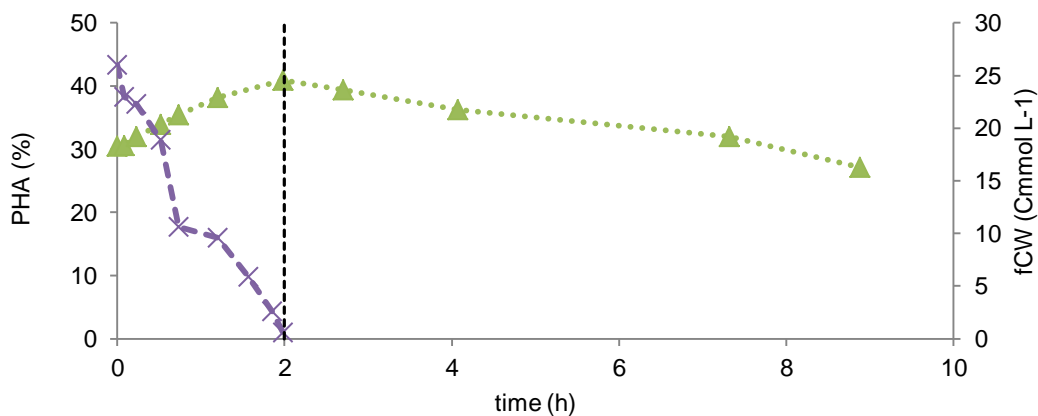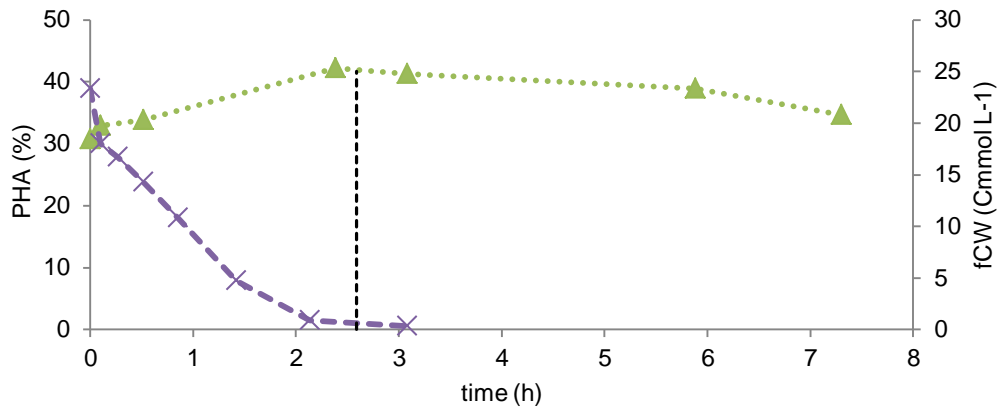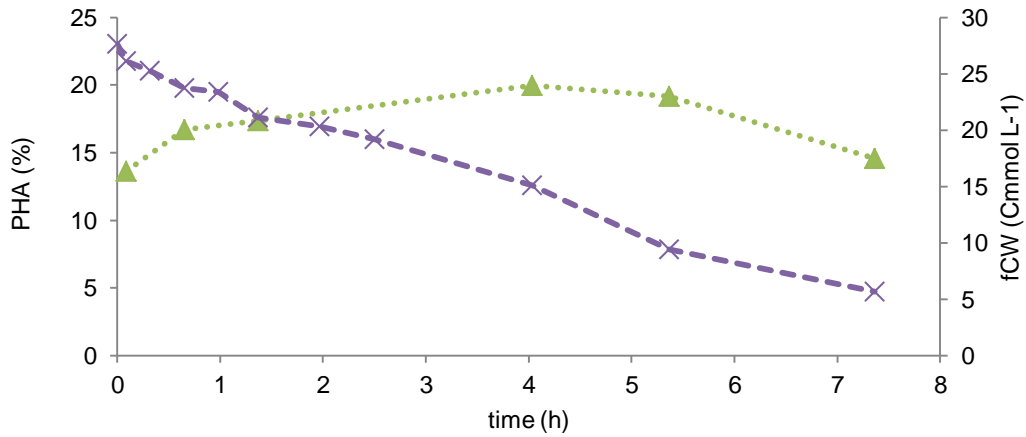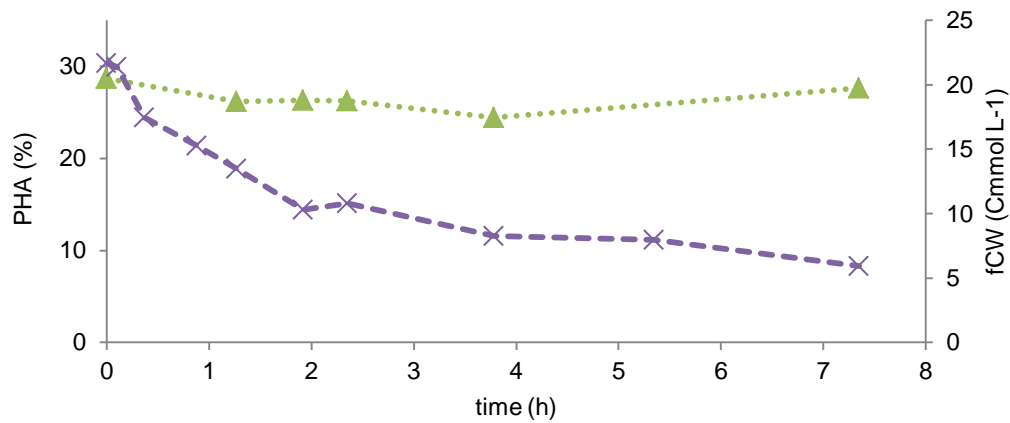
Figure 4.6 Usual FF cycle in the fifth phase (C/N/P of 100/0/0). The fCW (×) consumption and the PHA (▲) were represented.

The decrease in the amount of external nutrients supplied to the SBR had a clear impact on the selected culture's performance. The feast time increased, probably due to the fact that biomass decreased (Figure 4.7). Along the first and second phase the VSS were just measured in monitoring days, once a week. From the third phase until the end, VSS were measured every day (Figure 4.7). During the first and second phase the VSS concentration were about 2 g L^{-1}, which is a usual concentration in this kind of systems, already been reported by Albuquerque et al. (2010a). In the third phase the VSS concentration decreased 10 % (1,8 g L^{-1}) relatively to the average in the first and second phase. In the fourth phase the VSS concentration (1,3 g L^{-1}) decreased 35 % relatively to average between the first and second phase. When the reactor was without nutrients supplementation (phase V) the VSS concentration decreased 70 % until 0,6 g L^{-1}.

Figure 4.7 Representation of the volatile suspended solids since from the first until the fifth phase. The five divisions measured up with the first, second, third, fourth and fifth phase, respectively.

During the last phase the VSS were in lower concentration but there was no washout, as it would be expected when all nutrients supplementation was removed. On the other hand, it 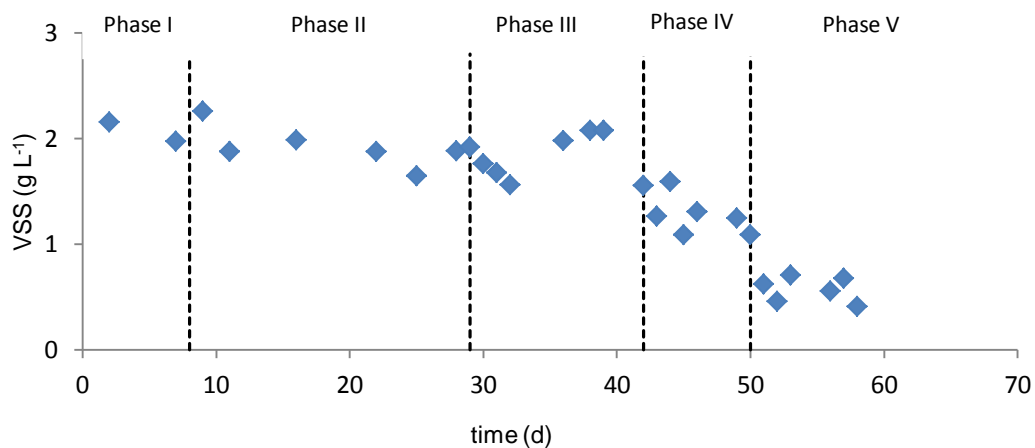was observed that proteins were consumed during the FF cycles (Figure 4.8). This means that the MMC was able to use the fCW proteins to grow. In fact, along each phase the specific protein uptake rate ($-q_{Prot}$) increased (Table 4.2) which means that the selected culture consumed some protein beyond the nutrients (except in the fifth phase, where there was no nutrients supply) during the FF cycles.

Table 4.2 Average specific protein uptake rates in each phase of the SBR operation.

Phase	$-q_{Prot}$ (g-Prot g-X^{-1} h^{-1})	Δ Protein (In-End) (g L^{-1})
I	0.010 (0.008)	0.034
II	0.017 (0.008)	0.073
III	0.018 (0.007)	0.082
IV	0.022 (0.004)	0.089
V	0.033 (0.002)	0.172

(Standard Deviation)
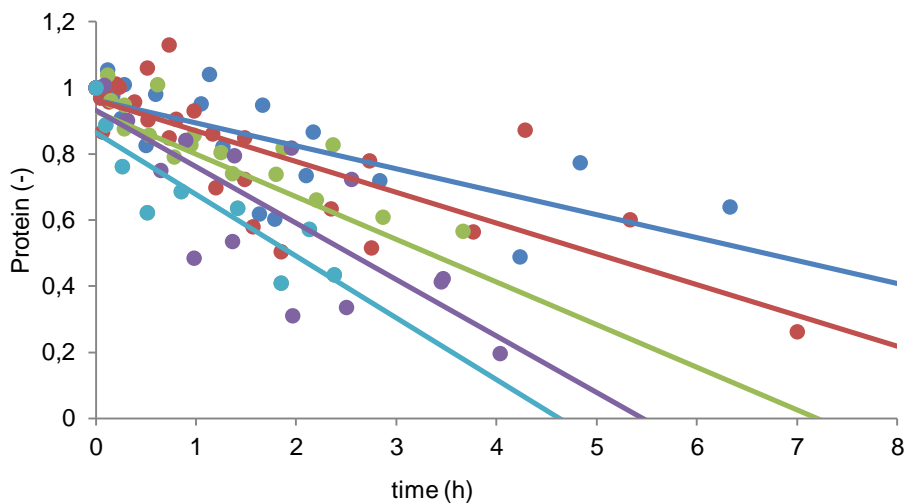
Figure 4.8 Normalised protein concentration (in terms of initial concentration) along the FF cycles in each phase. First phase (●), second phase (●), third phase (●), fourth phase (●) and fifth phase (●).

The specific biomass growth rate (q_X) (Table 4.3) proves that the MMC previously selected to store PHA is capable of using proteins as nitrogen source, but not sufficiently to grow as when it did not have nutrient limitation. The q_X decrease from 0.25 to 0.07 C-mol X C-mol X^{-1} h^{-1} from the first to the second phases was probably due to the perturbation caused by the decrease of

nutrient concentration. The increase in the next phases, is probably attributed due to the fact that the culture started to be adapted to use the nitrogen from fCW protein and increase its growth. On the other hand, the PHA production rate (q_{PHA}) was decreasing during all the experiment (Table 4.3). It is quite notorious that the MMC gradually lost the capacity to accumulate PHA, as the nutrients concentration decreased. The specific substrate uptake rate shown that fCW continued to be consumed, but the carbon is not used to produce PHA. The biomass and PHA yield just proved what is described above, the culture changed its performance and lost the capacity to be a PHA-accumulating culture from the second phase onward.

Table 4.3 Average performance of the PHA-accumulating culture selection in the SBR during the five phases.

Phase	q_X (Cmol-X Cmol-X^{-1} h^{-1})	$-q_S$ (Cmol-fCW Cmol-X^{-1} h^{-1})	q_{PHA} (Cmol-PHA Cmol-X^{-1} h^{-1})	$Y_{X/S}$ (Cmol-X Cmol-S^{-1})	$Y_{PHA/S}$ (Cmol-PHA Cmol-S^{-1})
I	0.25 (0.19)	0.91 (0.59)	0.42 (0.23)	0.26 (0.04)	0.48 (0.06)
II	0.07 (0.03)	0.31 (0.03)	0.13 (0.05)	0.23 (0.09)	0.42 (0.17)
III	0.18 (0.07)	0.35 (0.05)	0.09 (0.08)	0.51 (0.15)	0.25 (0.19)
IV	0.11 (0.04)	0.43 (0.11)	0.09 (0.08)	0.25 (0.02)	0.21 (0.19)
V	0.11 (0.01)	0.53 (0.1)	ND	0.16 (0.09)	ND

(Standard Deviation); ND-Not detectable

The MMC was able to store PHA with reasonable PHA yields. Indeed, in the first phase the culture had a performance comparable with that obtained by Albuquerque et al. (2010b) who had biomass and PHA yields higher than the obtained in the present study, but a different feedstock and HRT were used. In the next phases, still maintaining every conditions to create the right selective pressure, the nutrient concentration reduction caused a change in selective pressure and other culture, not a PHA-accumulating, was favoured. During the third phase the reactor's appearance changed and with the evolution of the experiment the biomass attached to the wall's reactor increased and the broth became viscous. All these facts seem to suggest that the selective pressures applied (FF regime and nutrients limitation) lead to the selection of an EPS producing culture, once the system was under stress conditions. This supposition is supported by the fact that EPS production from aerobic cultures is generally favoured under excess availability of a carbon source while under nutrient, namely nitrogen, limitation (Freitas et al. 2011) . Such described conditions were exactly the ones applied in phase V, considering that the nitrogen present in proteins which would be available for the cells to use was limited, which is a reasonable hypothesis.

4.4 Conclusions

In this study the influence of nutrients supplementation in the PHA-accumulating culture selection was investigated. It was observed that the PHA storage yield decreased from 0.48 (first phase) to 0.21 (fourth phase) C-mol X C-mol S^{-1} along the gradual process of nutrient removal, and in the fifth phase no PHA storage was detected. The PHA storage capacity was lost when the nutrients ratio decreased to half concentration (from 100/10/1 to 100/5/0.5 C-mol /N-mol/ P-mol). On the other hand, the selected MMC showed to be able to use fCW protein as nitrogen source when the external source of nitrogen was missing. Proving this, the specific protein uptake rate increased from 0.01 (first phase) to 0.033 (fifth phase) g-Prot g-X^{-1} h^{-1} along the study.

Concluding, the MMC needs nutrient supplementation, being incapable to use fCW protein to maintain their performance as PHA-accumulating culture.

The suggestions for future work includes:

1- Investigate the possibility to remove the fCW protein in the first stage of operation, producing an added-value product besides PHA.

2- Analyse the samples to study the community (taken during the study) to verify the EPS production hypothesis.

3- Study the influence in the PHA-accumulating culture of each nutrient (nitrogen and phosphorus) separately.

Chapter 5

General conclusions and Future perspectives

General conclusions and Future perspectives

In this study, a two stage process conditions were investigated- the pH effect in the cheese whey acidogenic fermentation stage and nutrients needs of the culture selection stage.

Two pH values (6 and 5) were applied in the cheese whey acidogenic fermentation. The influence of pH changes was majorly detected in the fCW profiles, affecting negatively the efficiency of the fermentation. Subsequently, the fCW profiles affected the PHA-accumulating culture stage but not the copolymer composition. Generally, the cheese whey acidogenic fermentation performed at pH 5 negatively affects the two stage PHA production.

The PHA-accumulating culture decreased their PHA production when the external nutrient source was taken. Notoriously, the MMC needs a nutrients supplementation during the PHA storage, being incapable to use fCW protein to maintain their performance as PHA-accumulating culture.

Though the relevant results obtained, the two stages PHA production might be improved in order to minimise the production costs, maximise the PHA content and study the possibility of an added-value production besides PHA.

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