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Bioconversion of Cheese Whey into Polyhydroxyalkanoates

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Sumário

Os Polihidroxialcanoatos (PHAs) podem substituir os plásticos convencionais, devido às suas propriedades semelhantes. A utilização de matérias-primas de baixo custo, tais como subprodutos indústrias, pode contribuir para a redução dos custos de produção destes biopolímeros. Neste trabalho, o soro de leite doce e ácido, um subproduto da indústria do queijo, rico em lactose, foi utilizado como fonte de carbono por *Escherichia coli* recombinante e *Haloferax mediterranei* para a produção de PHAs.

A bactéria *E. coli* foi geneticamente modificada através da inserção dos genes de PHB da bactéria *Cupriavidus necator*, com o objetivo de obter estirpes com a capacidade de converter lactose em polihidroxibutirato (P(3HB)). Das várias estirpes recombinantes obtidas e testadas em frascos de agitação, a estirpe CML3-1 foi a escolhida devido à sua alta produção de P(3HB). Demonstrou-se que o meio definido (MR), suplementado com soro de leite, era adequado para o cultivo da estirpe selecionada. No entanto, foi verificado que parte da fonte de carbono era desviada para uma elevada produção de ácidos orgânicos (OA). A limitação em oxigénio dissolvido e a alimentação em contínuo de soro de leite foram estratégias adotadas e contribuíram para o aumento da produção de P(3HB) e de OA. A quantidade de P(3HB) obtida (28,68 g/L) com a limitação em oxigénio dissolvido foi quase três vezes superior à obtida (10,72 g/L) sem essa limitação. Com o modo de alimentação em contínuo, foi obtida uma maior quantidade de P(3HB) (38,55 g/L), no entanto, a quantidade produzida de OA (115,76 g/L) foi a mais elevada deste estudo.

Com o objetivo de reduzir a capacidade de produção de OA, foi utilizado o método do protão suicida de forma a dirigir o metabolismo para a síntese de PHAs. Foram obtidos treze mutantes e testados em ensaios em frascos de agitação. A estirpe P8-X8 foi selecionada como a melhor candidata para testes em biorreator. Esta estirpe apresentou um menor rendimento em OA e um maior rendimento em P(3HB) relativamente ao substrato (0,04 $Cmol_{OA}/Cmol_{Lac}$ e 0,28 $Cmol_{P(3HB)}/Cmol_{Lac}$, respetivamente) em comparação com a estirpe recombinante de origem (0,11 $Cmol_{OA}/Cmol_{Lac}$ e 0,10 $Cmol_{P(3HB)}/Cmol_{Lac}$, respetivamente).

Para a produção de poli-hidroxibutirato-co-valerato P(3HB-co-3HV) pela bactéria *H. mediterranei*, foi otimizado um meio altamente salino (HS) para melhorar a conversão do soro de leite, quimicamente hidrolisado, em PHAs. Os resultados mostraram que a suplementação do meio HS com 10 mL de uma solução de micronutrientes (MS) melhorou a produtividade de PHA. A utilização de soro de leite hidrolisado como substrato, melhorou o rendimento em PHA ($0.61 g_{PHA}/g_{sugar}$) e, consequentemente, a produtividade do processo (4,04 g/L.dia).

Palavras-chave: polihidroxialcanoatos (PHAs), polihidroxibutirato (P(3HB)), polihidroxibutirato-co-valerato P(3HB-co-3HV), soro de leite, *Escherichia coli* recombinante, *Haloferax mediterranei*.

Summary

Polyhydroxyalkanoates (PHAs) can substitute conventional plastics due to their similar properties. The use of cheap raw materials, such as industrial by-products, can contribute for the reduction of production costs. In this work, sweet and acid cheese whey, a by-product of cheese industry rich in lactose, were used as carbon sources by recombinant *Escherichia coli* and *Haloferax mediterranei* for the production of PHAs.

E. coli was engineered through the insertion of *Cupriavidus necator* P(3HB)-synthesis genes, in order to obtain strains with the ability to convert lactose into polyhydroxybutyrate (P(3HB)). Recombinant strains were obtained and tested in shake flask experiments. Strain CML3-1 was selected due to its high P(3HB) production. Defined medium (MR), supplemented with cheese whey, showed to be suitable for this recombinant *E. coli* strain cultivation. However, a high amount of organic acids (OA) production was detected, which deviated part of the carbon source for its synthesis. Oxygen limitation and continuous feeding of cheese whey showed to increase P(3HB) and OA productions. The amount of P(3HB) obtained (28.68 g/L) with oxygen limitation was almost three times higher than that obtained (10.72 g/L) without that limitation. With the continuous feeding mode a higher amount of P(3HB) was attained (38.55 g/L), however, using this strategy, the amount of OA produced (115.76 g/L) was the highest.

The proton suicide method was used as a strategy to obtain an *E. coli* mutant strain with a reduced OA-producing capacity, aiming at driving bacterial metabolism towards PHAs synthesis. Thirteen *E. coli* mutant strains were obtained and tested in shake flask assays. Strain P8-X8 was selected as the best candidate strain for bioreactor fed-batch tests. A lower yield of OA on substrate and a higher P(3HB) production (0.04 $Cmol_{OA}/Cmol_{Lac}$ and 0.28 $Cmol_{P(3HB)}/Cmol_{Lac}$, respectively) were achieved, comparing to the original recombinant strain (0.11 $Cmol_{OA}/Cmol_{Lac}$ and 0.10 $Cmol_{P(3HB)}/Cmol_{Lac}$, respectively). This methodology showed to be effective on the reduction of OA yield by consequently improving the P(3HB) yield on lactose.

For the production of polyhydroxybutyrate-*co*-valerate P(3HB-co-3HV) by *Haloferax mediterranei*, a highly saline medium (HS) was optimized to improve the conversion of chemically hydrolyzed cheese whey into PHAs. The results showed that supplementation of HS with 10 mL of a micronutrients solution (MS) improved PHA productivity. The use of hydrolyzed cheese whey as substrate further improved the yield of polymer production ($0.61 g_{PHA}/g_{sugar}$) and consequently a higher process productivity (4.04 g/L.day) was achieved.

Key words: Polyhydroxyalkanoates (PHAs), Polyhydroxybutyrate (P(3HB)), Polyhydroxybutyrate-*co*-valerate P(3HB-co-3HV), Cheese whey, recombinant *Escherichia coli*, *Haloferax mediterranei*.

Abreviations

AcetylCoA – Acetyl-Coenzyme-A

ATP – Adenosine Triphosphate

BLAST - Basic local alignment tool

CFU – Colony forming units

CW – Cheese whey

DCW – Dry cell weight

DGGE - Denaturing gradient gel electrophoresis

DSC – Differential scanning calorimetry

FID – Flame ionization detector

GC – Gas chromatography

HPLC – High performance liquid chromatography

HS - High saline medium

LB - Luria Bertani

LB+Lac - Luria Bertani medium supplemented with lactose

LB^k+Lac – Luria Bertani medium supplemented with kanamycin and lactose

LB+Whey – Luria Bertani medium supplemented with cheese whey

lcl-PHA - long chain length Polyhydroxyalkanoates

Mw - Molecular mass

mOA – Organic Acids mass

mPHA – Polyhydroxyalkanoates mass

MR – Defined medium for recombinant *E.coli*

MR+Lac - Defined medium supplemented with lactose

MR^k+Lac – Defined medium supplemented with kanamycin and lactose

MR+Whey – Defined medium supplemented with cheese whey

MS – Micronutrients solution

NADH – Nicotinamide Adenine Dinucleotide

NPCM – non Polyhydroxyalkanoates cell mass

OA – Organic acids

PDI - Polydispersity index

PHA – Polyhydroxyalkanoates

P(3HB) – Polyhydroxy-3-butyrate

P(3HB-*co*-3HV) - Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

PP – polypropylene

PS – polystyrene

$q_{P(3HB)}$ – Specific production rate of Polyhydroxy-3-butyrate

$q_{P(3HB\text{-}co\text{-}3HV)}$ - Specific production rate of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

q_{lac} - Specific lactose consumption rate

q_{sugars} - Specific sugars consumption rate

r_{PHA}- Polyhydroxyalkanoate volumetric production rate

$r_{P(3HB)}$ – Polyhydroxy-3-butyrate volumetric production rate

$r_{P(3HB\text{-}co\text{-}3HV)}$ – Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) volumetric production rate

r_{OA}- Organic Acids volumetric production rate

r_{Lac}- Lactose volumetric consumption rate

r_{sugars}- Sugars volumetric consumption rate

SEC – Size Exclusion Chromatography

scl-PHA - Short chain length Polyhydroxyalkanoate

TCA – Tricarboxilic Acid

Tm - Melting temperature

Tg - Glass transition temperature

$Y_{OA/lac}$ – Organic Acids production yield on lactose

$Y_{OA/X}$ – Specific Organic Acids yield

$Y_{P(3HB)/lac}$ – Storage yield on lactose

$Y_{P(3HB\text{-}co\text{-}3HV)/sugars}$ – Storage yield on sugars

$Y_{X/lac}$ – Growth yield on lactose

$Y_{X/sugars}$ – Growth yield on sugars

X – Active biomass

% pO_2 – Dissolved oxygen (%)

% PHA – Polyhydroxyalkanoate content in dry cell weight

μ_{max} – maxim specific growth rate

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Chapter 1

1. Thesis outline

Polyhydroxyalkanoates (PHAs) are produced from renewable sources and can replace synthetic plastics in numerous applications. Nowadays, the PHA production is expensive and the process is far from optimized. To reduce the production costs, it is essential to focus the research in more productive strains, efficient fermentations, low-cost downstream processes and inexpensive substrates. Cheese whey (sweet or acid) is a cheap by-product from the cheese industry. It may cause environmental problems, due to its high organic matter content, thus needing to be treated before disposal or be economically valorized. Many microorganisms are described to produce PHA but few are able to convert lactose from cheese whey into PHA.

In this work a recombinant *E.coli*, was used to convert sweet whey into P(3HB), while *Haloferax mediterranei* was the strain chosen for the production of P(3HB-co-3HV) from acid whey that contains a higher salinity.

A genetic engineered *E. coli* strain harboring *Cupriavidus necator* P(3HB) synthesis genes was developed to produce P(3HB) from cheese whey. Different recombinant strains were obtained and evaluated for their ability to convert lactose into P(3HB). The objective of this work was the optimization of polymer productivity from cheese whey. With this propose several feeding and aeration strategies were tested to obtain high productivity and high P(3HB) concentration.

During the process to produce PHA from lactose, high production of organic acids (OA) was detected thus reducing the amount of carbon used for PHA production. In order to increase PHA productivity and yield, the deviation of carbon source for organic acids must be avoided. With this purpose, the Proton suicide method was applied. This is a simple and easy method developed by Winkelman and Clark (1984) to select mutants with a reduced or suppressed OA production capacity. This method relies on the toxicity of bromine (Br_2) for cells. Br_2 is formed from bromide (Br^-) and bromate (BrO_3^-) as a result of the increase of the protons concentration during the formation of OA. Hence, only mutants unable to produce OA are able to survive in such conditions (Winkelman and Clark, 1984; Cueto et al. 1990).

In a second case study, the conversion of high salinity cheese whey was evaluated. In this study, a high saline medium (HS) was optimized to improve the conversion of hydrolyzed cheese whey into PHAs by the archea *Haloferax mediterranei*. However, this strain is not able use lactose thus whey lactose was converted into glucose and galactose by chemical hydrolysis.

Thus, this work was divided into three sections: “Polyhydroxyalkanoates production by a new recombinant *Escherichia coli* strain using cheese whey as carbon source” (Chapter 3),

where it was tested two different culture media and different aeration and feeding strategies; The second section, named “Improvement on the yield of polyhydroxyalkanoates production from cheese whey by a recombinant *Escherichia coli* strain using the proton suicide methodology” (Chapter 4), was focused on the Isolation of P(3HB) producing mutants with low organic acids production, selection of mutant with improved performance and cultivation of the selected mutant in bioreactor; In the last section, “Medium optimization for polyhydroxyalkanoates production by *Haloferax mediterranei* from cheese whey” (Chaper 5), it was studied the effect of increasing the concentration of micronutrients, the cultivation medium supplemented with the hydrolyzed whey and the growing in bioreactor using the optimized medium.

Chapter 2

2. State of the art

Petrochemical-derived plastic materials are of the outmost importance in our daily life due to their versatility, low price and thermoplastic properties. Those materials can replace glass, metal and wood in many applications (Fiorese et al. 2009). Per year, 25 million tons of such nondegradable plastics are discarded in landfills and several hundred thousand tons are discarded into marine environments, being a serious threat to the environment (Jacquel et al. 2008; Reddy et al. 2003). The main concern today is to develop biodegradable plastics with characteristics similar to conventional plastics that may eventually replace them in the future.

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates, which are synthesized by numerous microorganisms, as a carbon and energy reserve material (Solaiman et al. 2006). These polymers are accumulated as intracellular granules to levels as high as 90% of the cell's dry weight (Reddy et al. 2003).

PHAs have been receiving increasing worldwide attention because their production is based on renewable compounds instead of fossil fuels (Verlinden et al. 2007). Polymers like PHAs can be used in practically the same applications as conventional plastics (Koller et al. 2007; Reddy et al., 2003). The majority of possible applications for PHAs are as partially or entirely replacements of petrochemical polymers, especially on packaging and coating applications, such as films, personal hygiene products and adhesives (Reddy et al. 2003; Verlinden et al. 2007). Composites based on bioplastics are already used in the electronics industry, agriculture and chemical synthesis of optically active compounds (Verlinden et al. 2007). PHAs have also numerous medical applications, being their main advantage the fact that they are biocompatible (Zinn et al. 2001). PHAs are used as sutures, orthopedic pins, nerve guides and bone marrow scaffolds; and may be used as scaffolds in tissue engineering and as drug carriers (Verlinden et al. 2007).

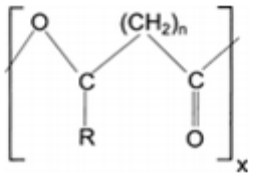
Optimization of polyhydroxyalkanoates production has received increasing attention from researchers. Some production aspects are being modified to improve productivity and to make the processes economically attractive: the utilization of new organisms and the better understanding of known ones, the use of novel and/or inexpensive substrates, more efficient fermentation process and the development of new extraction/recovery methods (Lee et al. 1996; Verlinden et al. 2007).

The carbon source used should be inexpensive because substrate costs usually correspond up to 50% of PHAs production costs (Ahn et al. 2000). The use of inexpensive substrates, such as cheese whey, could lead to significant economic advantages, when compared

with the use of primary substrates (e.g. glucose) to produce PHAs (Lee et al. 1996; Reddy et al. 2003; Tian et al. 2009; Verlinden et al. 2007).

2.1. Polyhydroxyalkanoates (PHAs)

PHAs are accumulated when an essential nutrient, like nitrogen, phosphorus, sulfur or magnesium, or oxygen, is limited and an excess amount of the carbon source is present (Braunegg et al. 1998; Lee et al. 1996). PHAs can substitute the conventional plastics due to their similar properties to various synthetic thermoplastics and elastomers (Table 2.1) and complete biodegradability after disposal. PHAs composition depends on the PHA synthases, the metabolic routes involved, the cultivation conditions and the carbon source (Braunegg et al. 1998; Fonseca et al. 2007). Their general structure is shown in Figure 2.1.



x = 100 - 30 000

n = 1	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hdroxyoctanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
n = 3	R = hydrogen	Poly(5-hydroxyvalerate)

Figure 2.1: General structure of polyhydroxyalkanoates and some representative members (adapted from Lee et al. 1996).

Due to the stereospecificity of the biosynthetic enzymes, the monomeric units are in D–(–) configuration, which is essential for biodegradability and biocompatibility of PHAs. More than 100 different monomers have been reported as PHA constituents, but only a few were produced in amounts high enough to enable the characterization of their properties and development of potential applications. The most common PHAs are poly(3-hydroxybutyrate) (P(3HB)) and the co-polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) (Lee, 1996; Zinn et al. 2001).

Renewable resources, like agricultural feedstock or wastes containing sugars and fatty acids can be used as carbon and energy sources for PHAs production. The synthesis and biodegradation of PHAs are totally compatible with carbon cycle (Figure 2.2). Thus, while for some applications the biodegradability is critical, PHAs receive general attention because their production is based on renewable compounds instead of fossil fuels (Verlinden et al. 2007).

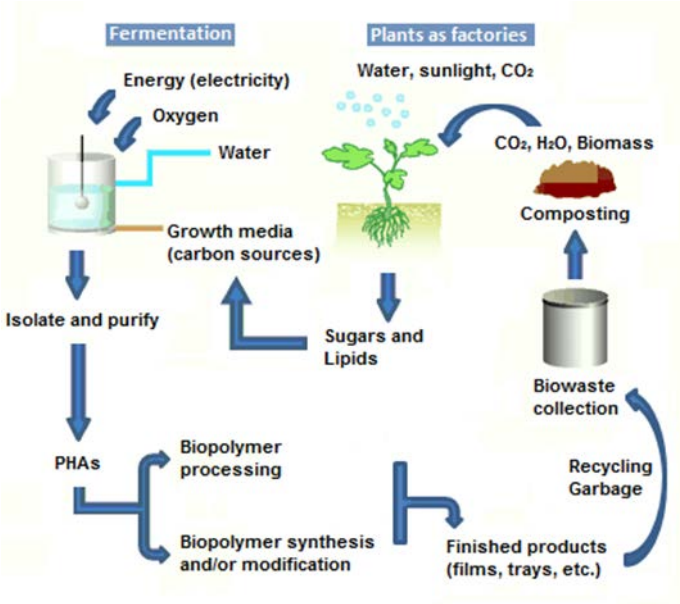


Figure 2.2: Life cycle of PHAs.

PHAs can be degraded upon exposure to soil, compost, or marine sediment. Biodegradation is dependent on a variety of factors, such as microbial activity of the environment, and the exposed surface area, temperature or pH, polymer composition and crystallinity (Reddy et al. 2003). Biodegradation of PHAs under aerobic conditions results in carbon dioxide and water, whereas in anaerobic conditions the degradation products are carbon dioxide and methane. Studies have shown that 85% of PHAs were degraded in seven weeks (Reddy et al. 2003).

PHAs can be divided in three classes: short chain length PHA (scl-PHA), with carbon numbers of monomers ranging from 3 to 5, medium chain length PHA (mcl-PHA), with 6 to 14 carbons in monomers, and long chain length PHA (lcl-PHA), with more than 14 carbons in monomers. The length of the side chain and its functional group influence the properties of the PHAs, as the melting point, the glass transition temperature and crystallinity (Zinn et al. 2001).

The molecular mass (Mw) of PHAs typically ranges between 2×10^5 and 3×10^6, varying with the PHA producer (Braunegg et al. 1998), while the polydispersity index is about 1.5 to 2.0 (Koller et al. 2007a; Fiorese et al. 2009). The properties of P(3HB), P(3HB-co-3HV) and polypropylene (PP) are compared in Table 2.1. The values of melting and glass transition temperatures, cristalinitty and tensile strength of P(3HB) and P(3HB-co-3HV) are near to those of PP. This similarity of properties between these biopolymers and synthetic propylene suggests similar applications.

PHAs are insoluble in water and have no toxic effects in living organisms (Verlinden et al. 2007). They have a high degree of polymerization and are optically active, piezoelectric and isotactic (stereochemical regularity in repeating units) (Reddy et al. 2003).

Table 2.1: Properties of P(3HB), P(3HB-co-HV) (containing 20% of 3HV monomers) and PP (adapted from Verlinden et al. 2007).

Parameter	P(3HB)	P(3HB-*co*-HV)	PP
Melting temperature (°C)	177	145	176
Glass transition temperature (°C)	2	-1	-10
Crystallinity (%)	60	56	50-70
Tensile strength (MPa)	43	20	38
Extension to break (%)	5	50	400

2.1.1. Poly(3-hydroxybutyrate)

Poly(3-hydroxybutyrate), P(3HB), is a highly crystalline thermoplastic, with a melting point around 177°C (Table 2.1). P(3HB) has similar properties to polypropylene, polyethylene or polyvinylchloride, although the biopolymer is stiffer and more brittle (Fiorese et al. 2009; Braunegg et al. 1998).

2.1.2. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

The properties of copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate, P(3HB-co-3HV), vary with their HV content. The melting temperature is minimum (≈ 80°C) when the molar fraction of the monomer 3HV is about 30% (Silva et al. 2005). P(3HB-co-3HV) bears mechanical properties, such as toughness and softness, which make them more interesting than pure P(3HB) (Braunegg et al. 1998). P(3HB-co-3HV) is more flexible than P(3HB) and can be used in films, coated paper and board, and molded products such as bottles and razors (Lee et al. 1996). The P(3HB) decomposition starts at 246.3°C, while the P(3HB-*co*-HV) decomposition starts at 260.4°C, which indicates that the presence of 3HV increases the thermal stability of the polymers (Verlinden et al. 2007).

2.2. Economic and environmental aspects of PHAs

The price of the product depends significantly on the substrate cost, on the PHA yield and on downstream processing (Reddy et al. 2003). Using a natural producer, as *Cupriavidus necator*, the cost of PHA, depending on the amount purchased, may reach US$16 per Kg, while

the cost of polypropylene is about US$0.9 per Kg. The cost of PHA decreases to around US$4 per Kg if recombinant *Escherichia coli* is used as producer, which is close to the value of other biodegradable plastic materials. To turn PHA production commercially viable, the price of the final product should be US$3-5 per Kg (Reddy et al. 2003).

The synthesis and biodegradation of PHAs is compatible to the carbon cycle (see Figure 2.2). Nevertheless, some studies showed that the PHAs production may not be any better for the environment than the production of conventional plastics. During the life cycle of PHAs, more energy would be needed comparing with the life cycle of conventional polymers. However, the production process of PHAs is still not optimized, while the production of petrochemical-derived plastic materials is fully developed (Verlinden et al. 2007).

2.3. PHAs applications

PHAs are versatile and can be combined with other chemicals to achieve specific properties. They can be easily depolymerized to a rich source of hydroxyl acids, which are optically active and pure, and can be used for the synthesis of new chemical products (Reddy et al. 2003). PHAs can be used in disposable items such as razors, utensils, diapers, feminine hygiene products and cosmetic containers. They have also been processed into tonners for printing applications. In agriculture, PHAs can be used to encapsulate seeds and fertilizers for slow release, to produce biodegradable plastics films for crop protection and biodegradable containers for greenhouse facilities (Verlinden et al. 2007).

These polyesters have the potential to become an important compound for medical applications, due to its biocompatibility. In human blood and tissue monomers of PHA (3HB) are present, which is an advantage in medical applications for PHAs, in comparison to other polymers (Koller et al. 2007). PHAs can be used in orthopedic (scaffolds for cartilage engineering, bone graft substitutes), urology, dentistry, in vascular system (heart valves, cardiovascular fabrics, pericardial patches and vascular grafts), in drug delivery and wound management (sutures, skin substitutes, nerve cuffs, surgical meshes, staples and swabs) (Zinn et al. 2001).

2.4. Bacterial strains for PHA production

In 1926, the French scientist Lemoigne reported the bacterium *Bacillus megaterium* to accumulate P(3HB). Since then, more than 90 genera of Archae and Eubacteria ($Gram^+$ and $Gram^-$) have been recognized as PHAs producers, including *Cupriavidus necator*, *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans* and recombinant *Escherichia coli* (Lee, 1996). These bacteria are industrially used for the production of PHAs because they can reach

high cell densities with a high PHA content in a short period of time, resulting in high PHA productivity (Lee, 1996; Zinn et al. 2001).

The most well-known PHA producer is *Cupriavidus necator*, which accumulates PHAs up to 80% of its cell dry weight, when nitrogen or phosphorous is completely depleted (Lee, 1996). Currently, this bacterium is used to produce P(3HB) in a large scale. *C. necator* presents a high PHA production, its genome is already sequenced, especially the genes responsible for the PHA biosynthesis (Lee, 1996).

Bacteria, such as *Escherichia coli*, grow fast, even at high temperature, and are easy to lyse. *E. coli* can grow on various carbon sources, such as glucose, sucrose, lactose and xylose. However, they are unable to produce or degrade PHAs. For these reasons, *E. coli* is considered the ideal host for harboring the *C. necator* PHA biosynthesis genes, in order to achieve high PHA productivity (Reddy et al. 2003). The P(3HB) content obtained in recombinant *E. coli* is about 90% on cell dry weight. This high intracellular polymer content may simplify the extraction and purification of PHAs due to easy lysis of the cells, reducing the costs of the purification of the biopolymer (Dias et al. 2006; Reddy et al. 2003).

Some members of the Archae kingdom are able to produce PHAs. Bacteria of the genus *Haloferax* are interesting because, comparing with related organisms, these bacteria grow faster and can utilize various substrates for PHAs production. *Haloferax mediterranei* requires a highly saline medium (2 – 5M of NaCl) for growth, which minimizes the risk of microbial contamination. These bacteria are extremely sensitive when exposed to hypotonic media and, in distilled water, the cells lyse immediately. In previous works, (Koller et al. 2007a) *H. mediterranei* was cultivated for several weeks without sterilization conditions and no contamination was observed. These facts may reduce drastically the production costs since no sterilization is required and downstream is easier than for other bacteria without this capacity (Koller et al. 2007a). Moreover, *H. mediterranei* produces P(3HB-co-3HV) without the need of co-substrate addition, which is another advantage over most of PHAs-producing organisms (Koller et al. 2007b).

2.5. Carbon sources

As mentioned before, the carbon source used for PHAs production should be inexpensive. Carbohydrates, oils, alcohols, acids and hydrocarbons are used by various bacteria as carbon sources (Du et al. 2012), but their costs are high. Therefore, they should be replaced by crude carbon substrates, such as cane and beet molasses, cheese whey, glycerol, plant oils, hydrolysates of starch, sucrose, triacylglycerols, cellulose and hemicellulose, industrial and food wastes (Du et al. 2012). Other carbon sources, such as ethanol, oleic acid, methane and a mixture of hydrogen and carbon dioxide can be used, but the PHA contents and productivity values achieved are usually low (Lee, 1996; Reddy et al. 2003; Tian et al. 2009; Verlinden et al. 2007). Cheese whey has been used as carbon source for PHA production (Lee et al. 1997; Wong et al. 1998; Kim et al. 2000; Ahn et al. 2001; Nikel et al. 2005).

2.5.1. Cheese whey as carbon source for PHAs production

Cheese whey is the liquid part of milk that separates from the curd at the beginning of the manufacture of cheese. Cheese whey is available in large amounts as by-product stream and is rich in fermentable nutrients, such as lactose, lipids and soluble proteins. The composition of cheese whey is presented in Table 2.2. In addition to the nutrients listed in Table 2.2, cheese whey also contains citric acid, non-protein nitrogen compounds, such as urea and uric acid, B group vitamins and other nutrients (Siso, 1996).

From the feed stock milk, skimmed whey is produced after the casein precipitation and the major part of lipids are removed. After removing 80% of water from the skimmed whey, the concentrated whey is ultra-filtrated to obtain whey permeate (the lactose fraction) and whey retentate (the protein fraction, with lactose residues). Lactoalbumin and lactoferrin are found in the retentate and are of pharmaceutical interest. Other solid parts from whey solution can be used as fertilizer or as animal food supplement. Whey permeate (which contains 81% of the original lactose in milk) is a potential carbon source for the bioproduction of PHAs (Ahn et al. 2001; Povolo et al. 2010).

Depending on the casein precipitation method, whey produced can be acid (pH<5) or sweet (pH=6-7). The acid whey has higher salt content and lower protein content than sweet whey, and its use in feeding is limited due to its acidic flavor (Siso et al. 1996).

The cheese whey used in this work was supplied by the Portuguese company, Lactogal. The composition and main characteristics of cheese whey determined by the manufacturer are presented in Table 2.2.

Table 2.2: Characterization of sweet cheese whey powder provided by Lactogal.

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

Per year, about 6 million tons of cheese are produced in European Union, which correspond approximately to 40 million tons of whey. The major part of whey is used for lactose and feed production, but 13 million tons of whey, containing about 0.6 million tons of lactose, constitute a surplus product which is discarded as effluent (Koller et al. 2008). The disposal of these high amounts of whey is expensive and represents an environmental problem. Cheese whey has high organic matter content, with a BOD_5 = 30–50 g/l and a COD = 60–80 g/l, being lactose largely responsible for the high BOD_5 and COD content. Lactose from whey can be converted in single cell protein, ethanol or methane, reducing the BOD_5, but the resulting effluent is not ready for disposal (Siso, 1996).

Cheese whey can be used as carbon source by many bacteria, such as recombinant *E. coli*, *Hydrogenophaga pseudoflava* and *Methylobacterium*. These microorganisms are able to synthetize PHAs directly from lactose because they have sufficient β-galactosidase activity (Povolo et al. 2010). Other microorganisms, such as *Pseudomonas hydrogenovora* and *Halferax mediterranei* (Koller et al. 2007b) are unable to use lactose. Thus, in theses microorganisms lactose has to be enzymatic or chemically hydrolysed to galactose and glucose. The monosaccharides can then be converted to PHAs. The third option to produce PHAs involves the anaerobic conversion of lactose to lactic acid by lactobacilli. Bacteria such as *C. necator* and *Alcaligenes latus* can convert lactic acid to PHAs (Koller et al. 2007b).

2.6. Bioproduction process

There are four biosynthetic approaches to produce PHAs (Figure 2.3). The continuous cultures are the most controlled of the cultivation methods; however, this method is not yet applied to PHA production on a large scale. The batch cultures are suitable for growth studies and screenings for potential PHA accumulating organisms. The fed-batch cultures are used on a large scale to produce PHAs, with high productivity (Zinn et al. 2001).

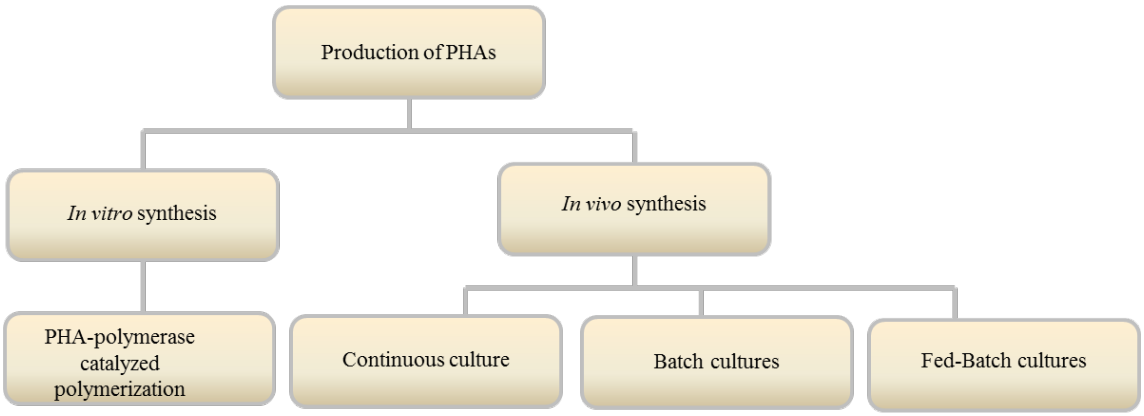


Figure 2.3: Biosynthetic approaches to produce PHAs (adapted from Zinn et al. 2001).

Fed-batch cultivation is common asset up in two-step: in the first step, cells are grown to a desired concentration, without nutrient limitation; In the second step, an essential nutrient is limited to allow PHA production (Lee, 1996). For many bacteria, the most common limitation is nitrogen, while for fewer bacteria, such as *Azotobacter spp.*, oxygen limitation is the most effective (Verlinden et al. 2007). The nutrient limitation activates a metabolic pathway, which shunts acetyl-CoA units from the Krebs cycle into the P(3HB) production (Lenz et al. 2005).

2.7. Bioproduction metabolism

In bacteria, acetyl-coenzyme-A (acetyl-CoA) is converted to P(3HB) by three enzymatic steps, as is shown in Figure 2.4. In first step, two molecules of acetyl-CoA are combined by 3-ketothiolase (encoded by *pha*A) to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (encoded by *pha*B) allows the reduction of acetoacetyl-CoA by NADH to 3-hydroxybutyril-CoA. In third step, P(3HB) synthase (encoded by *pha*C) polymerizes 3-hydroxybutyril-CoA to P(3HB) and coenzyme-A is liberated (Verlinden et al. 2007). The genes of the *P(3HB)*CAB operon encode the three enzymes that catalyze the three enzymatic reactions of P(3HB) production. The promoter upstream of *P(3HB)*C transcribes the complete operon *P(3HB)*CAB (Reddy et al. 2003).

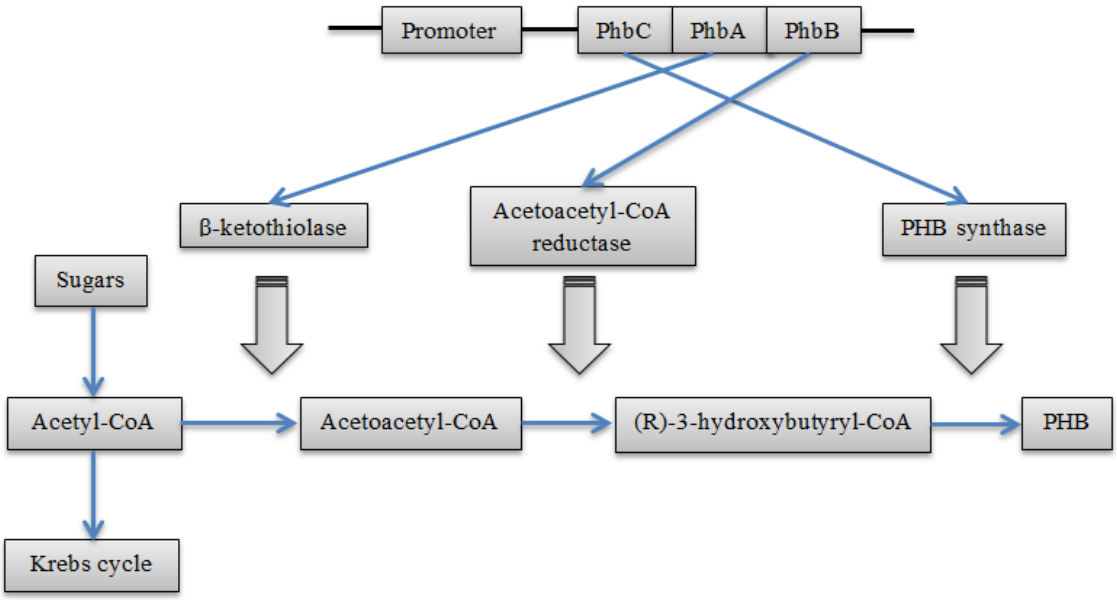


Figure 2.4: Biosynthetic pathway to P(3HB) production (adapted from Madison & Huisman, 1999).

2.8. Conversion of cheese whey into PHA by recombinant *Escherichia coli* and *Haloferax mediterranei*

Ahn et al. (2001) described the use of recombinant *E. coli* for P(3HB) production from lactose present in cheese whey that resulted in a final P(3HB) concentration of 168 g/L and a final cell concentration of 194 g/L, corresponding to a P(3HB) content of 87% and a productivity of 4.6 $g_{P(3HB)}$/L.h (Ahn et al. 2001).

Using *Haloferax mediterranei* as a P(3HB-*co*-HV) producer from hydrolyzed whey, by Koller (Koller et al. 2007a) reported the production of 12.2 g/L of PHAs, which corresponded to 72.8% of PHA in biomass and a low productivity, 0.09 $g_{P(3HB)}$/L.h.

Comparing the aforementioned strains, *H. mediterranei* is able to produce an HB and HV copolymer, without adding precursors to the medium (Chen et al. 2006), while recombinant *E. coli* produces a homopolymer, P(3HB). Furthermore, *H. mediterranei* is easy to cultivate, with minimal sterility precautions. Thus, this strain may be economically attractive, due to copolymer applications and low production cost. On the other hand, *H. mediterranei* produces an exocellular polysaccharide which is released to the medium, decreasing the maximum yield of PHA (Rodriguez-Valera et al. 1992). The high saline concentration required for *H.mediterranei* growth is also a disadvantage since salts may corrode the stainless steel used in large-scale fermenters. This has been prevented in bench-scale cultivations by the use of bioreactors made of polyetherether ketone, glass, and silicium nitrite ceramics for the production of PHA by halophilic archaeon strain 56 (Hezayen et al. 2000). Salts are needed in

considerable amounts (2-5M of NaCl) and they cost roughly equals the synthetic carbon source (Rodriguez-Valera et al. 1992; Koller et al. 2007a).

Cultivation of *E. coli* at high cell concentrations may require the use of pure oxygen to maintain the dissolved O_2 concentration at an ideal value, which is not economically attractive (Park et al. 2002).

2.9. Downstream process

Reduced downstream costs, together with high purity recovery of PHAs will accelerate the commercialization of high quality PHA-based products (Ridiel et al. 2013). Separation and purification of PHA polymers from non-PHA cell mass (NPCM) presents a technical challenge due to the solid phase of both PHA granules and NPCM. Two strategies are usually adopted in the downstream processing: PHA solubilization and NPCM dissolution. In the former, the PHA macromolecules are dissolved in appropriate organic solvents and extracted from the cells, and in the latter, NPCM is digested and/or dissolved by chemical agents while PHA granules are left in the solid state (Yu & Chen. 2006). The generated solid and liquid phases are then separated by unit operations, such as filtration and centrifugation. Solvent extraction is widely used in the laboratories but with limited success in pilot-plant and large-scale processing (Gorenflo et al. 2001). The cost of PHA recovery with solvent extraction may reach up to 50% of the overall production cost (Chen et al. 2001). This high cost can be significantly reduced by using NPCM solubilization, such as sequential surfactant and hypochlorite digestion (Choi et al. 1997).

Recovering of PHA can be obtained by two methods: water-based separation or solvent-based extraction. In the first method, cells need to be broken up and various chemical additives added. The final PHA obtained may reach about 95% of purity and can be used as coating material. In solvent-based extraction, the PHA is extracted directly from biomass, by dissolving it in an organic solvent, such as chloroform, dichloroethane or methylene chloride. After filtration and precipitation, the polymer may reach a purity of more than 98%, which is required for medical applications (Zinn et al. 2001).

In alternative to solvent-based extraction, digestion methods can be used (such as chemical digestion with surfactants or sodium hypochloride and enzymatic digestion), mechanical cell disruption (high pressure homogenization, ultrasonication, centrifugation and chemical treatment) and supercritical fluid disruption. Another approach is based on the cell fragility that is usually verified after the accumulation of large amount of PHA, to get a simple procedure for PHA extraction. Recombinant strains of *E. coli* have been developed to release P(3HB) granules gently and efficiently (Jacquel et al. 2008).

Solvent extraction is the most common method to extract PHA from the cells. The use of solvents destroys the natural morphology of PHA granules but does not degrade the polymer.

The purity is high once the endotoxins from bacteria are eliminated and then the PHA can be used in medical applications. The use of solvents, mostly halogenated solvents, is hazardous to the environment and is not applicable in large scale. Other methods based on digestion of non-PHA cell material have been investigated, but by using hypochlorite polymer degradation was observed (Jaquel et al. 2008). Best results were obtained by combining hypochlorite with chloroform or surfactant treatment. Enzymatic digestion method was also reported, economically unattractive due to the cost of enzymes (Holmen & Lim, 1990). Mechanical cell disruption methods like using bead mills and high pressure homogenization appear to be more cost effective (Tamer et al. 1998). Recovery of P(3HB) by the use of supercritical CO_2 has been also reported (Hezazi et al. 2003), but is still expensive in comparison with other methods. Recently, new methods like spontaneous liberation of P(3HB) (Juang et al. 2005), dissolved air flotation (Hee et al. 2006), or air classification (Noda, 1998) are being investigated . Improvement of these new extraction and purification methods should lead to an optimal recovery of PHA, with a high purity and recovery level at a low production cost (Jaquel et al. 2008).

In the case of halophilic bacteria, such as *Haloferax mediterranei*, polymer can be recovered by only using water to disrupt the cells and isolate the PHAs produced, thus facilitating the downstream process (Koller et al. 2007a).

Chapter 3

This chapter was adapted from the manuscript: Pais J., Farinha I., Gameiro T., Freitas F., Serafim L., Arévalo-Rodríguez M., M., Prieto M. A., Reis A.M.R. Polyhydroxyalkanoates production by a new recombinant *Escherichia coli* strain using cheese whey as carbon source. (in preparation)

3. Polyhydroxyalkanoates production by a new recombinant *Escherichia coli* strain using cheese whey as carbon source

3.1. Summary

In this work, *E. coli* was engineered through the insertion of *Cupriavidus necator* P(3HB)-synthesis genes, fused to a lactose-inducible promoter, into the chromosome, via transposition-mediated mechanism in order to obtain strains with the ability to convert lactose into P(3HB). Recombinant strains were obtained and tested in shake flask experiments. Strain CML3-1 was selected due to its high P(3HB) production (2.14 g/L).

Defined medium (MR) supplemented with cheese whey in a bioreactor without oxygen limitation showed to be a good medium for this recombinant *E. coli* strain that achieved active biomass concentration of 39.28g/L with a P(3HB) content of 21.97%. However, a high amount of organic acids (OA) was also produced (46.74 g/L), thus deviating part of the carbon source from P(3HB) production.

To increase the P(3HB) production, oxygen concentration (pO_2) was reduced from 60% to 30% of . The lower oxygen availabily did not affect the culture growth, on the contrary, a higher biomass concentration (54.77 g/L) was achieved. On the other hand, P(3HB) production increased three-times (28.63 g/L), comparing to the assay where the oxygen was maintained above 60% of pO_2 (10.72 g/L). Despite the P(3HB) production improvement, OA were still produced (78.60 g/L).

Aiming at improving P(3HB) in detriment to OA production, different reactor feeding strategies were evaluated: The culture was fed by pulses to maintain the lactose concentration in the ranges of 0 to 10 g/L and 10 to 20 g/L. A continuous fed-batch mode was also tested to maintain the lactose concentration above 30 g/L. While the latter strategy enhanced P(3HB) production (38.55 g/L), this operation mode led to a high level of OA production (115.76 g/L). The best productivity (0.93 g/L.h) and the low amount of OA produced (16.74 g/L) were obtained when the culture was fed by pulses maintaining the lactose concentration between 10 and 20 g/L, avoiding periods with absence of lactose.

3.2. Introduction

E. coli can be easily manipulated and improved by means of recombinant DNA methodologies. It is a suitable host for the heterologous expression of background genes and high cell density cultivation strategies for numerous *E. coli* strains have already been well established (Nikel et al. 2006). Ahn and co-workers (2001) cultivated, in a fed-batch bioreactor, a recombinant *E. coli* fed by carbon source pulses, using the pH feeding strategy and a sequential oxygen limitation (40, 30 and 15% of pO_2). They obtained 119.5 g/L of DCW, a polymer content of 80% and productivity of 2.57 g/L.h. Using the same feeding strategy and maintaining the oxygen above 30% of pO_2, Wong and Lee (1998) obtained 87 g/L of DCW with 80% of P(3HB) content.

Another advantage of using *E. coli* cells is that after accumulating large amounts of P(3HB), they become fragile, facilitating the isolation and purification of the biopolymer (Nikel et al. 2006). Furthermore, the bacterium does not express PHA-degrading enzymes (Nikel et al. 2006).

In the present work, recombinant *E. coli* strains were constructed, where the genes that encode for the enzymes responsible for P(3HB) production were inserted into the chromosome. This type of construction allows maintain the stability of the genes inserted. The *Ptrc* promoter is associated to those genes, which is an advantage because the carbon source and inducer of the expression system used in this study is lactose from cheese whey. Different strains were screened in shake flask assays and the one showing the highest P(3HB) storage was tested in bioreactor. The performance of the selected strain was evaluated in rich and mineral medium supplemented with both commercial lactose and cheese whey. The medium that allowed for the best results was chosen for the subsequent studies, where the imposition of oxygen limitation and different fed-batch feeding strategies were studied aiming to achieve the highest P(3HB) productivity.

During cultivation Organic Acids (OA) production was detected. It was already reported in literature the production of acetic acid during aerobic growth of *E. coli* on glucose. (March et al. 2002; Akesson et al. 1999). The impact of OA excretion on P(3HB) production was evaluated.

3.3. Materials and methods

3.3.1. Microorganism

The recombinant *Escherichia coli* strains used in this work were obtained from the lab of A. Prieto (CSIC-CIB) and modified by BIOMEDAL (Spain). A genetic construct, plasmid pMAB26, was obtained in order to integrate the P(3HB)-producing genes of *Cupriavidus*

necator into the chromosome of recombinant *E. coli*, via transposition-mediated mechanism. This new construct contained the P(3HB) operon (with *phbC, phbA* and *phbB* genes) inserted in the mini-Tn*5* element of plasmid pCNB5 and located under the lactose-inducible Ptrc promoter of this element allowed a stable integration of these genes and the expression of the corresponding enzymes in the *E. coli* strains. Plasmid pMAB26 was transferred by conjugation to four rifampicin-resistant derivates of *E. coli* strains: BL21(DE3), C, MG1655 and ET8000.

Plasmid pMAB26, harbouring the P(3HB) biosynthesis genes *phbC, phbA* and *phbB* from *C. necator* was constructed by binding the 4632 bp *Hin*dIII-*Bam*HI, blunt-ended fragment of plasmid pAV1 (M. A. Prieto, unpublished), containing the *phaCAB* operon, to mini-Tn5 delivery vector pCNB5, (de Lorenz*o et* al. 1993), linearized with *Not*I and blunt-ended. *E. coli* strains DH5 λ*pir* (Biomedal, Spain) and S17 1 λ*pir* (de Lorenz*o et* al. 1993) were used for propagation and conjugative transfer of pMAB26, respectively. Insertion of *phaCAB* operon of *C. necator* in the chromosome of *E. coli* strain MG1655 (Hayashi et al. 2006) was carried out using S17 1 λ*pir* as the donor strain for pMAB26 and MG1655-RIF-1 (a spontaneous, rifampicin-resistant derivative of MG1655) as the recipient strain. Transconjugant strains were selected as kanamycin-resistant, rifampicin-resistant, and ampicillin-sensitive colonies.

Derivates of the *E. coli* strains obtained were tested for their ability in storing PHAs from lactose by observation of P(3HB) granules by optical microscopy. The five best strains, in terms of lactose consumption (data not shown), were selected for this work: CML 1-1A from BL21 (DE3), CML 2-3A from C, CML 3-1 and CML 3-2A from MG1655 and CML 4-1A from ET8000. These strains carried in their chromosome a single copy of the expression cassette *lacI*-Ptrc::*phbCAB* that allowed for a maximal expression of the *phb* genes when lactose was present in the culture medium.

3.3.2. Media

LB (Luria-Bertani) medium (bactotriptone 10g, yeast extract 5g, NaCl 10g per L) (Sambrook and Russell, 2001) was prepared and autoclaved at 121°C for 20 minutes. pH was adjusted at 6.8 with NaOH 5N. LB^k was prepared by supplementing LB medium with kanamycin solution (1mL/L). This solution was prepared by solubilizing 1.25 g of kanamycin sulfate (Sigma-Aldrich) in 50 mL of distilled water and filtered under sterile conditions (Sartorius Stedim Minisart, 0.2 μm). Solid LB^k was prepared by adding 15g/L of agar prior to autoclaving. LB^k+Lac medium was supplemented with 1% (w/v) lactose autoclaved separately.

A defined medium (MR medium) was also used in some assays. MR medium had the following composition (per liter): KH_2PO_4, 13.5g ; $(NH_4)_2HPO_4$, 4.0g; citric acid monohydrate, 1.9 g; 10 mL mineral solution (Lee et al. 1993). The mineral solution had the following composition (per liter of HCl 1M): $FeSO_4.7H_2O$, 10.0g; $CaCl_2.2H_2O$, 2.0g; $ZnSO_4.7H_2O$, 2.2g;

$MnSO_4.H_2O$, 0.5g; $CuSO_4.5H_2O$, 1.0g; $(NH_4)_6MO_7O_{24}.4H_2O$, 0.1g; $Na_2B_4O_7.10H_2O$, 0.02g). The pH was adjusted to 6.9 by adding NaOH pellets prior to autoclaving at 121ºC, for 20 min. Lactose 20% (w/v) and $MgSO_4.7H_2O$ 20% (w/v) solutions were prepared and autoclaved separately. When commercial lactose was used as the carbon source, 1 mL of a filter sterilized proline and thiamine solution was added (400 mg of Proline and 6.740 g of Thiamine-HCl dissolved in 20 mL distilled water). 1 mL/L of kanamycin (25g/L) solution were also added to MR medium after sterilization.

For the fed-batch reactor assay, MR medium was supplemented with cheese whey to obtain the desired concentration of lactose. The cheese whey used in this work was supplied by Lactogal (Portugal).

Before its use, cheese whey was deproteinized according to the procedure of Ahn et al. (2000), with some modifications. Briefly, a cheese whey solution was prepared by dissolving 300 - 400 g whey powder in 1 L of deionized water. Then the solution was autoclaved at 121ºC for 15 minutes, followed by centrifugation at 8000 g for 1 hour in sterile bottles for removal of the precipitated protein aggregates. The solution was filtered (SartoLab-P20 plus, 0.2 μm) to remove the remaining small protein aggregates and assure its sterility.

3.3.3. Inocula preparation

The inocula were prepared after two adaptation steps. First, a single colony of the recombinant strain grown on LB^k+Lac agar plates was inoculated in 100 mL shake flasks containing 10 mL of medium and incubated at 37ºC and 200 rpm, during 16 hours in the assays with LB^k+Lac medium and 32 hours with MR^k+Lac. Then, 20 mL of each culture were centrifuged and the pellet was re-suspended in fresh LB^k+Lac or MR^k+Lac media (100 mL of the medium in 500 mL flask) and incubated for 10 hours under the same conditions.

3.3.4. Recombinant strains screening

The screening for the recombinant strain presenting the highest PHAs storage capacity was performed with recombinant strains CML 1-1A, CML 2-3A, CML 3-1, CML 3-2A, CML 4-1A. The experiments were performed by incubating 2.5 mL of inoculum in exponential phase (with 10 h of incubation time) in 500 mL flasks with 100 ml of medium LB^k+Lac, in an orbital shaker (200 rpm) at 37ºC.

The assays were run for 75 h and samples were taken at the beginning and at the end of the experiments. Samples were analyzed for P(3HB), biomass and lactose quantification.

3.5. Bioreactor operation

The assays were performed in a 2-L reactor (BioStat® B-Plus, Sartorius, Germany) with 1 L working volume inoculated with 200 mL of the selected culture. The temperature was controlled at 37ºC and pH at 6.9±0.1, by the automatic addition of NH_4OH 28% (v/v) solution. A constant aeration rate of 3 vvm was maintained during the experiments. The dissolved oxygen concentration (% pO_2) was controlled by automatically increasing the stirring rate from 200 to 1000 rpm and, when necessary, supplementation of the air stream with pure oxygen. Foam formation was suppressed by the automatic addition of Antifoam A (Fluka). In experiments A to E the reactor was operated in a discontinuous fed-batch mode with the addition of several pulses of lactose or cheese whey after the exhaustion of the previous one. In experiment F, a continuous fed-batch mode was implemented in order to maintain the lactose concentration above 20g/L. In experiment G several pulses were added in order to maintain lactose concentration between 10 and 20 g/L. In experiments A, B, C and D the %pO_2 was maintained at 60%. An oxygen limitation was implemented (%pO2 at 30%) in experiments E, F and G. (Table 3.1)

Table 3. 1: Medium, carbon source, feeding strategy and pO_2 control used in each bioreactor experiment.

Experiment	Medium	Carbon source	Feeding strategy	pO_2 control
A	LB	Lactose	By pulses (0-10 g/L)	60%
B	LB	Cheese whey	By pulses (0-10 g/L)	60%
C	MR	Lactose	By pulses (0-10 g/L)	60%
D	MR	Cheese whey	By pulses (0-10 g/L)	60%
E	MR	Cheese whey	By pulses (0-10 g/L)	30%
F	MR	Cheese whey	Continues mode (above 30 g/L)	30%
G	MR	Cheese whey	By pulses (10-20 g/L)	30%

The purpose of experiments A, B, C and D was to choose the best medium for growth and PHA storage by recombinant *E. coli* strain. LB medium supplemented with lactose or cheese whey was used in experiments A and B, respectively. MR medium supplemented with lactose was used for experiment C, while in experiment D MR medium was supplemented with cheese whey. In Experiment E, oxygen limitation (30% of pO2) was applied to increase the P(3HB) production. In Experiment F, the pulses feeding strategy was replaced by continuous mode feeding (0.2-11.7 g/L.h of lactose) in order to stimulate P(3HB) production. The biopolymer production was associated to a lactose promoter that was induced by lactose, for this

reason the last feeding strategy was tested. In experiment G, the feeding strategy by pulses was implemented again to maintain the lactose concentration between 10 and 20 g/L, to avoid periods with lactose absence (Table 3.1).

The experiments were run for 50-60 h and 10 mL samples were periodically taken for P(3HB), biomass, lactose and organic acids quantification.

3.3.6. Analytical methods

The dry cell weight (DCW), defined as the dry weight of cells per litre of culture broth, was gravimetrically determined by filtering a sample, after cell wash with NaCl 0.9% (w/v), through 0.2 μm membranes (GVS cellulose acetate) and drying at 100ºC until constant weight.

PHA was quantified by gas chromatography (GC) using the method proposed by Braunegg et al. (1978) and Comeau et al. (1998), with minor modifications introduced by Satoh et al. (1992) and Lemos et al. (2006). Briefly, the lyophilized biomass (~2 mg) was resuspended in methanol with 20% of sulfuric acid solution (1 mL) and chloroform (1 mL) containing 0.88 mg/mL of heptadecane (as internal standard). Then, the samples were hydrolysed at 100ºC for 3.5 h. After cooling, 500 μL of deionized water were added and the samples were shaken for 1 minute in a vortex. 800 μL of the chloroform phase were extracted and transferred to a 2 mL vial with molecular sieves (0.3 nm, Merck).

The samples (2 μL) were injected in a Varian CP-3800 gas chromatograph (Varian, CA, USA), equipped with a FID detector and a ZB-WAX plus column (60 m, 0.53 mm internal diameter, 1 mm film thickness, Phenomenex, USA) coupled with a guard-column (0.32 mm internal diameter). Helium was used as a carrier gas, at constant pressure (14.5 psi). The temperature of injection was 280 ºC, the temperature of the detector was 230 ºC and the temperature ramp started at 40 ºC, increased at a rate of 20 ºC/min until 100 ºC, further increased at a rate of 3 ºC/min until 155 ºC and finally increased again at 20 ºC/min until 220 ºC, to ensure a cleaning step of the column after each injection and 220 ºC during 1 minute.

A calibration curve, correlating the ratio between the peak areas of monomers (HB and HV) and heptadecane and the ratio between their concentrations, was obtained by preparing standards of HB/HV copolymer (88%/12%; Merck) and subjecting them to the same treatment as the samples.

Lactose and OA were quantified in the cell-free supernatant by high-performance liquid chromatography, using an Aminex HPX-87H (Biorad) column, coupled to a Refractive Index detector. The mobile phase was H_2SO_4 0.01 N, with an elution rate of 0.6 mL/min and an operating temperature of 50 °C. Lactose, pyruvate, formate, succinate, acetate, butyrate, lactate, propionate (Sigma Aldrich) standard solutions (1.0 - 0.125 g/L) were used.

3.3.7. Calculations

PHA content (%PHA) was determined by Equation 1:

$$\%PHA = \frac{[PHA]}{DCW} \times 100 \quad (1)$$

where DCW includes active biomass(X) and PHA.

The active biomass (X, g/L) concentration was determined as follows:

$$X = DCW - [PHA] \quad (2)$$

The maximum specific growth rate (μ_{max}) was determined from the linear regression slope of the exponential phase of ln X versus time, where X (g/L) is the active biomass.

The PHA volumetric productivity (r_{PHA}, g/L.h) was determined by Equation 4:

$$r_{PHA} = \frac{m_{PHA\,max}}{V \cdot t} \quad (3)$$

where m_{PHAmax} (g) is the maximum amount of PHA produced, V (L) is the working volume and t (h), the time needed to obtain m_{PHAmax}.

The OA volumetric productivity (r_{OA}, g/L.h) was determined by Equation 5:

$$r_{OA} = \frac{m_{OA\,max}}{V \cdot t} \quad (4)$$

where m_{OAmax} (g) is the maximum amount of OA produced.

The storage ($Y_{PHA/sugars}$, $Cmol_{PHA}/Cmol_{sugars}$ or g_{PHA}/g_{sugars}), growth ($Y_{X/sugars}$, $Cmol_X/Cmol_{sugars}$ or g_X/g_{sugars}) and OA ($Y_{OA/lac}$, $Cmol_{OA}/Cmol_{lac}$ yields on lactose were calculated using Equations 6, 7 and 8, respectively:

$$Y_{PHA/sugars} = \frac{\Delta Cmol_{PHA}}{\Delta Cmol_{sugars}} \text{ or } Y_{PHA/sugars} = \frac{\Delta[PHA]}{\Delta[sugars]} \quad (5)$$

$$Y_{X/sugars} = \frac{\Delta Cmol_{X}}{\Delta Cmol_{sugars}} \text{ or } Y_{X/sugars} = \frac{\Delta[X]}{\Delta[sugars]} \quad (6)$$

$$Y_{OA/lactose} = \frac{\Delta[OA]}{\Delta[Lactose]} \quad (7)$$

The specific OA yield ($Y_{OA/X}$, g_{OA}/g_X) was determined using the following equation:

$$Y_{OA/X} = \frac{\Delta[OA]}{\Delta[X]} \quad (8)$$

The volumetric sugars consumption rate (r_{sugars}, g/L.h) was determined from the slope of sugars concentration along the period of time considered. The specific consumption rate of sugars (q_{sugars}, g/gX.h) was calculated by dividing the sugars volumetric consumption rate of each pulse per the corresponding active biomass.

3.4. Results and discussion

3.4.1. Selection of strain

Plasmid pMAB26 was transferred by conjugation to four rifampicin-resistant derivates of *E. coli* wild type strains: BL21(DE3), C, MG1655 and ET8000. The derivates of the *E. coli* strains obtained were tested in shake flask experiments and five transconjugant were selected for this work due to their PHAs storing ability from lactose: CML 1-1A, derived from BL21 (DE3), CML 2-3A from C, CML 3-1 and CML 3-2A from MG1655, and CML 4-1A from ET8000.

The five recombinant *E. coli* strains were grown in rich medium LB^k+Lac (at 37ºC and 200 rpm), for the selection of the best P(3HB)-producing strain. The performance of the five strains is presented in Table 3.2.

Table 3.2: Results obtained with the five recombinant *E. coli* strains cultivated in LB^k+Lac medium.

Strain	DCW (g/L)	%P(3HB) (%)	[P(3HB)] (g/L)	X (g/L)	$Y_{P(3HB)/lac}$ ($Cmol_{P(3HB)}/Cmol_{Lac}$)	$Y_{x/lac}$ ($Cmol_X/Cmol_{Lac}$)	$r_{P(3HB)}$ (g/L.h)	$q_{P(3HB)}$ (h^{-1})
CML 1-1A	4.73	26.14	1.24	3.49	0.16	0.40	0.016	0.005
CML 2-3A	4.17	40.10	1.67	2.50	0.22	0.28	0.022	0.009
CML 3-1	4.07	52.65	2.14	1.92	0.28	0.22	0.029	0.015
CML 3-2A	3.66	45.15	1.65	2.01	0.22	0.23	0.022	0.011
CML 4-1A	4.27	7.65	0.33	3.94	0.04	0.45	0.004	0.001

The results obtained showed that all the strains were able to produce P(3HB) from lactose, meaning that the pMAB26 plasmid transferring was successful. CML 3-1 strain was considered the best P(3HB) producer since it presented the highest P(3HB) storage content (52.65%) and polymer production (2.14 g/L), as well as volumetric and specific productivities (0.029 $g_{P(3HB)}$/L.h and 0.015 $g_{P(3HB)}/g_X$.h, respectively) and storage yield (0.28 $Cmol_{P(3HB)}/Cmol_{lactose}$) (Table 3.2). CML 3-1 was the only strain with a storage yield higher than the growth yield (0.22 $Cmol_X/Cmol_{lactose}$), meaning that more lactose was consumed for P(3HB) synthesis than for growth. The other four strains drifted more carbon substrate for active biomass formation than for P(3HB) production. The highest growth yield and, consequently, the lowest storage yield were observed for strain CML 4-1A, 0.45 $Cmol_X/Cmol_{lactose}$ and 0.04 $Cmol_{P(3HB)}/Cmol_{lactose}$, respectively. Based on these results, strain CML 3-1was selected for the subsequent assays.

3.4.2. Bioreactor experiment

3.4.2.1. Effect of medium composition in CML3-1 performance

In order to choose the best medium for P(3HB) production by *E. coli* CML3-1, the strain was cultivated in bioreactor using either rich medium (LB) (experiments A and B) or defined medium (MR) (experiments C and D). Commercial lactose (experiments A and C) and cheese whey (experiments B and D) were tested as carbon sources in each medium. Its performance in terms of growth and P(3HB) production was evaluated in all the experiments. (Figure3.1)

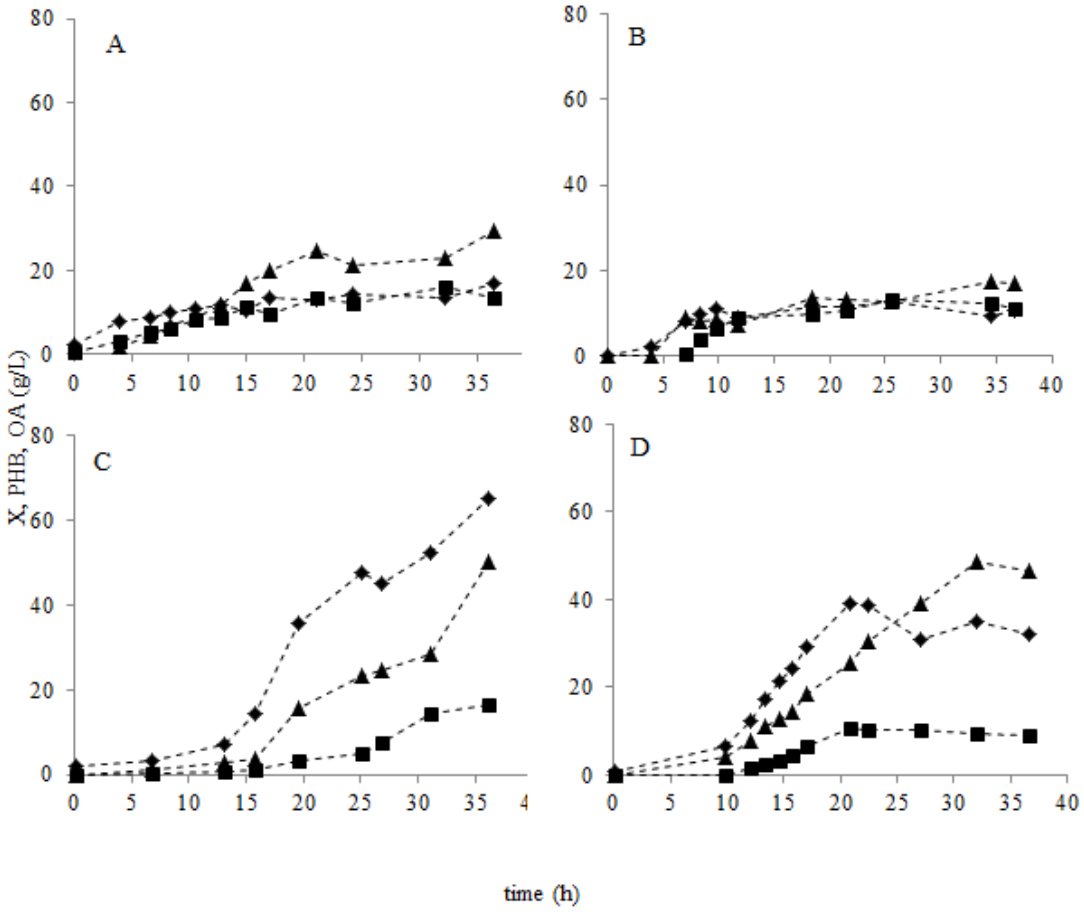


Figure 3.1: Profiles of active biomass (◆), P(3HB) (■) and OA (▲) concentration for bioreactor experiments with different media composition (A – LB Lac; B - LB Whey; C - MR Lac; D – MR Whey).

The reactor was operated in a discontinuous fed-batch mode with the addition of substrate in pulses in order to obtain 10g/L of lactose in the bioreactor along the experiment. This low concentration of lactose was used to avoid secondary products, such as organic acids (OA). March and co-workers report acetate production as an apparently nonessential metabolite that accumulates during aerobic growth of *E. coli* on glucose (March et al. 2002).

Acetate is enzymatically synthesized from acetyl coenzyme A (acetyl-CoA) in two steps-phosphotransacetylase (*pta* gene) converts acetyl-CoA to the intermediate acetyl

phosphate, which is then converted to acetate with the generation of ATP by acetate kinase (*ack)*. Although the detailed mechanism remains unknown, this by-product is generally thought to accumulate in *E. coli* fermentations as a result of the tricarboxylic acid (TCA) cycle not keeping pace with glycolysis (Akesson et al. 1999)**.** In other words, acetate accumulates as a result of insufficient oxaloacetate being present in the first step of the TCA cycle, the conversion of oxaloacetate and acetyl-CoA to citrate via citrate synthase.

Using LB medium supplemented with lactose (Figure 3.1-A), no lag phase was observed. However, the specific growth rate was very low (0.04 h^{-1}) and a low active biomass concentration was achieved at the end of the experiment (16.75 g/L) (Table 3.3). On the other hand, a high P(3HB) content in the cells was achieved (44.27%), resulting in a P(3HB) concentration of 15.96 g/L (Table 3.3).

Table 3.3: Kinetic and stoichiometric parameters obtained with the recombinant strain *E. coli* CML3-1 in bioreactor cultivation.

Experiment	A	B	C	D	E	F	G
Medium	LB+Lac	LB+whey	MR+Lac	MR+whey	MR+whey	MR+whey	MR+whey
μ_{max} (h^{-1})	0.04	0.03	0.24	0.25	0.25	0.25	0.26
X_{max} (g/L)	16.75	12.58	65.22	39.28	54.77(49h)	84.49(38h)	85.18(26h)
$P(3HB)_{max}$ (g/L)	15.96	12.97	16.47	10.72	28.68 (39h)	38.55 (42h)	25.56 (27h)
OA_{max} (g/L)	29.42	17.00	50.19	46.74	78.6 (49 h)	115.76 (47h)	16.74 (27h)
$P(3HB)_{max}$ (%)	44.27	50.14	20.17	21.97	36.13 (47h)	29.29 (47 h)	23.11 (27h)
$Y_{X/lac}$ (g/g)	0.17	0.12	0.29	0.16	0.35	0.69	0.62
$Y_{P(3HB)/lac}$ (g/g)	0.14	0.12	0.07	0.05	0.17	0.29	0.19
$Y_{OA/lac}$ (g/g)	0.30	0.18	0.22	0.23	0.51	1.05	0.12
$r_{P(3HB)max}$ (g/L h)	0.49	0.51	0.46	0.51	0.53	0.67	0.93

Similarly to experiment A, in the assay with LB supplemented with whey (experiment B), no lag phase was observed (Figure 3.1-B), but a low maximum specific growth rate (0.03h^{-1}) was observed (Table 3.3). The maximum active biomass achieved was 12.58 g/L with a P(3HB) content of 50.14%, corresponding to a P(3HB) production of 12.97 g/L (Table 3.3).

In both assays, OA production was observed, being lower in experiment B (17.00 g/L) than in experiment A (29.42g/L) (Table 3.3). These results suggest that cheese whey might have

been less favourable for OA production than lactose, when LB medium was used. In experiment A the yield of OA was 0.30 g_{OA}/g_{Lac}, while in experiment B, the same yield was only 0.18 g_{OA}/g_{Lac}, meaning that, more carbon was directed for OA when lactose was the carbon source (Table 3.2).

Figure 3.1-C shows the results obtained in experiment C using MR medium supplemented with lactose (MR+Lac). The strain presented a lag phase of 13 hours, growing thereafter with a maximum specific growth rate of 0.24 h^{-1}, achieving a final active biomass concentration of 65.22 g/L (Table 3.3). Concentration of P(3HB) was 16.47 g/L and the polymer content in the biomass was 20.17%. P(3HB) production started at 9.80 hours, and the maximum P(3HB) production (10.72 g/L) was achieved at 20.90 hours (Figure 3.1-C).

The production of OA in experiment C (50.19 g/L) was considerably higher than that verified in experiment A (29.42 g/L) (Table 3.3). The yield of P(3HB) in experiment A was double than that verified in experiment C ($0.14g_{P(3HB)}/g_{Lac}$ and $0.07g_{P(3HB)}/g_{Lac}$ respectively). This could suggest that cheese whey boosted OA production, influencing negatively P(3HB) production. Despite the differences between experiments A and C in terms of growth and storage, similar volumetric productivity values were attained (0.49 and 0.46g/L.h, respectively).

Using MR medium supplemented with whey (experiment D), a shorter lag phase (10 hours) than the one observed in experiment C was observed. With this medium a higher maximum specific growth rate (0.25 h^{-1}) was observed, when comparing with experiments A and B (Table 3.2). However, the active biomass production was lower (39.28 g/L) than in the MR Lac (experiment C) (65.22 g/L) (Table 3.1). Experiment D revealed a different growth profile than the one observed in experiment C. When the culture was fed with cheese whey (experiment D) growth stopped before the end of incubation (20.9 h), on the other hand, when lactose was the carbon source (experiment C), the culture grew until the end of cultivation. In experiment D, P(3HB) production also stopped at 20.9 hours of incubation. From this time on, only OA production was detected. End of growth could be associated with the OA production, once, more than 30 g/L of OA could be toxic for the culture growth. When cheese whey was the substrate (experiments B and D), P(3HB) production stopped around 20 hours of incubation. On the contrary of experiment B and D, the feeding stock solution of lactose used in experiment A and C was supplemented with magnesium. One hypothesis for the fact that growth and storage have stopped earlier (around 20 hours) could be associated with the lack of magnesium concentration needed. Magnesium is unquestionably essential to living cells. It is required for the activity of many enzymes and is needed for the preservation of the structure of ribosomes (Lusk et al. 1967). In addition, studies of magnesium starvation, and the effects of ethyl-enediaminetetraacetate and of osmotic shock suggest that this ion is important for the maintenance of the permeability barrier of the bacterial cell. The stabilization of spheroplasts by

magnesium ions also suggests their involvement in the integrity of the cell membrane. (Lusk et al., 1967)

As reported above, more OA were produced when the culture was fed with cheese whey. The culture produces OA to balance its redox power (Akesson et al. 1999). Formation of acetate, when *E. coli* is grown under fully aerobic conditions, typically occurs at high growth rates and/or high glucose uptake rates. The acetate production is thought as an overflow phenomenon where flux of AcetylCoA is directed to acetate, via acetylphosphate, instead of entering the TCA cycle. In batch and continuous cultivations, it was observed that the specific oxygen uptake rate reached an apparent maximum at the onset of acetate formation (Andersen & Meyenburg, 1980; Reiling et al. 1985). It was suggested that the respiratory system, where NADH is reoxidized, has a limited capacity. As flux to the TCA cycle results in NADH production and as the flux to acetate does not, redirection of AcetylCoA flux to acetate would be necessary to avoid accumulation of NADH, when the respiration saturates. Another explanation that has been suggested is that the TCA cycle has a limited capacity and that this limitation is reached before that of the respiration (Majewski and Domach, 1990). When the TCA cycle saturates, increasing glucose uptake will again result in flux from AcetylCoA to acetate. In this case, NADH production and respiration can increase further until the maximum respiration capacity or the maximum glucose uptake is reached (Akesson et al. 1999). Our results seem to indicate that, with cheese whey, a higher potential redox unbalance is caused which may explain the stop of P(3HB) storage around 20 hours of experiment. Thus, to balance its redox potential the culture seemed to prefer OA secretion instead P(3HB) production, which is in accordance with the above proposed mechanism.

Despite the lower growth and P(3HB) production with cheese whey than with lactose, the productivity in experiment D (0.51 g/L.h) was higher than in C (0.46 g/L.h).

Also in assay D the carbon source was deviated for OA, with 46.74 g/L of OA produced resulting in a high yield of OA production of 0.23 g_{OA}/g_{Lac} and low yield of P(3HB) production of 0.05$g_{P(3HB)}/g_{Lac}$. These values are close to those obtained for experiment C with lactose as carbon source. The same trend was observed in in experiment C, where the production of organic acids reached 50.19 g/L, resulting in a yield of OA much higher (0.22 $g_{OA}/g_{Lac)}$ than that of P(3HB) (0.07 $g_{P(3HB)}/g_{Lac)}$.

The results obtained for P(3HB) production were far from the results achieved with other recombinant strains. Ahn et al. (2001) obtained in bioreactor 119.5 g/L of biomass and 96.2 g/L of P(3HB) using a recombinant *E. coli* strain fed with cheese whey. However, Ahn et al. (2001) had implemented an oxygen limitation strategy (20% of pO_2) that they reported to favour P(3HB) production. On the other hand, Kim and co-workers (2000) cultivated a recombinant *E. coli* in fed-batch mode, without oxygen limitation and also obtained high

biomass and P(3HB) values (55 g/L and 32 g/L, respectively). These results indicate that, aeration and feeding strategies are important conditions in P(3HB) production processes using recombinant *E. coli* and deserves further investigation.

Despite experiment D presented a high level of OA production, MR+Whey medium was chosen due the higher productivity achieved.

3.4.2.2. Effect of reducing oxygen concentration

In order to investigate the effect of oxygen on PHA production by recombinant *E. coli*, oxygen concentration was reduced from 60 % to 30% using cheese whey (experiment E). A cultivation profile similar to that of experiment D (Figure 3.1-D) was obtained for experiment E (Figure 3.2-E). After a lag phase of 8 hours, the culture grew with maximum specific growth rate of 0.25 h^{-1}, achieving an active biomass concentration of 54.77 g/L at the end of the experiment (Table 3.3). These results show that the culture was not affected by the lower oxygen availability. On the contrary, the culture achieved a higher biomass concentration.

On the other hand, oxygen limitation seemed to benefit P(3HB) production. The P(3HB) produced (28.68 g/L) at 30% oxygen was almost three-times higher than at 60%, (10.72 g/L of P(3HB)) . Furthermore, the lower pO_2 significantly benefited P(3HB) content: this increased from 21.97% (experiment D) to 36.13% (experiment E). Also the yield of P(3HB) increased from 0.05 $g_{P(3HB)}/g_{Lac}$ (experiment D) to 0.17 $g_{P(3HB)}/g_{Lac}$ (experiment E). The productivity (0.53 g/L.h) was higher than in experiment D (0.51 g/L.h).

The maximum P(3HB) content observed was still much lower than the value of 80% reported by Ahn and co-workers (2001). However, the productivity achieved in this study was higher than that obtained by Kim et al. (2000) (0.48 g/L.h), using a recombinant *E. coli* and cheese whey in a fed-batch bioreactor with oxygen limitation (30% of pO2).

It is important to notice that despite lactose was acting as an inducer of P(3HB) production, in some short periods of time, it was depleted. These short moments likely contributed for a lower P(3HB) production since the genetic construction had a lac-inducible promoter associated with the genes responsible for P(3HB) production. In absence of lactose, the generations that were being formed could fail to have the ability to produce P(3HB) (Lorenzo et al. 1993)

Despite the improvement obtained in the P(3HB) production with the pO_2 reduction, the OA production was also increased. In the final of fermentation the strain produced 78.60 g/L (Table 3.3). The obtained results show that, one again, the recombinant strain preferred to produce OA instead of producing P(3HB). Indeed, the yield of OA per lactose was 0.51 g_{OA}/g_{Lac}, while the yields of P(3HB) per lactose and active biomass per lactose were 0.17

$g_{P(3HB)}/g_{Lac}$ and 0.25 g_X/g_{Lac} respectively. The deviated carbon source to OA production resulted in low volumetric P(3HB) productivity (0.58 g/L.h) comparing with the productivity of 2.57 g/L.h obtained by Ahn and co-workers (2001), using a stronger oxygen limitation (30-10% of pO_2).

The use of a lower pO_2 showed to be a good strategy to increase P(3HB) production by *E. coli* strain CML3-1 but also contributed for a higher OA production. Strategies for the reactor operation should be defined in order to increase P(3HB) production while keeping the OA production at a lower level.

3.4.2.3. Implementation of reactor feeding strategies

The strain constructed by Biomedal S.L. carried at its chromosome a single copy of the expression cassette *lacI*-Ptrc::*phbCAB* that allows a maximal expression of the *phb* genes when lactose is present at the culture medium. In this system the repression exerted by the LacI regulator over the Ptrc promoter is relaxed in the presence of lactose in the culture medium and, therefore, associated to the P(3HB) synthesis genes. Thus, presence of higher amounts of lactose was expected to stimulate P(3HB) production by ensuring the expression of the *phb* genes. Lee and co-workers (1997) tested different lactose concentrations (10-70g/L) and the higher value of P(3HB) concentration (5.2 g/L) was achieved when the lactose concentration was 30g/L. In this line of reasoning, a continuous fed-batch mode was implemented when the exponential phase started (experiment F). The feeding flow rate was adjusted during the cultivation to maintain the lactose concentration above 20 g/L. A lactose concentration higher than in the previous experiments was chosen in order to obtain a high induction of Ptrc promoter that drives the expression of P(3HB) producing genes and, consequently, a higher P(3HB) production.

Similarly to what was observed in experiment E, the culture showed a lag phase of 8 hours. (Figure 3.3) From this moment on, the active biomass increased until 38.2 hours of fermentation, being the maximum specific growth rate ($0.25h^{-1}$) the same obtained in experiments D and E ($0.25h^{-1}$). However, the culture grew for a longer time than in experiments D and E, which was likely due the higher amount of cheese whey fed and higher availability of magnesium. This cation plays an important role in *E.coli* growth, as explained above.

The maximum of P(3HB) content and concentration obtained was 29.29% 38.55 g/L, respectively. The latter was higher than in experiment E (28.68 g/L). An improvement of biomass and P(3HB) production was achieved with a continuous feeding strategy implemented. Furthermore, this strategy also resulted in a higher biomass concentration; 84.49 g/L of active biomass in experiment F versus 54.77 g/L, in E (Table 3.3). Accordingly, productivity also increased from 0.53 g/L.h to 0.67 g/L.h (experiment E and F, respectively).

Despite the growth and storage improvements achieved with the continuous feeding strategy, that guaranteed the lactose concentration above 20 g/L, high OA production was still produced. At the end of the experiment 115.76 g/L of OA were formed, corresponding to more than the double of P(3HB) amount (38.55 g/L).

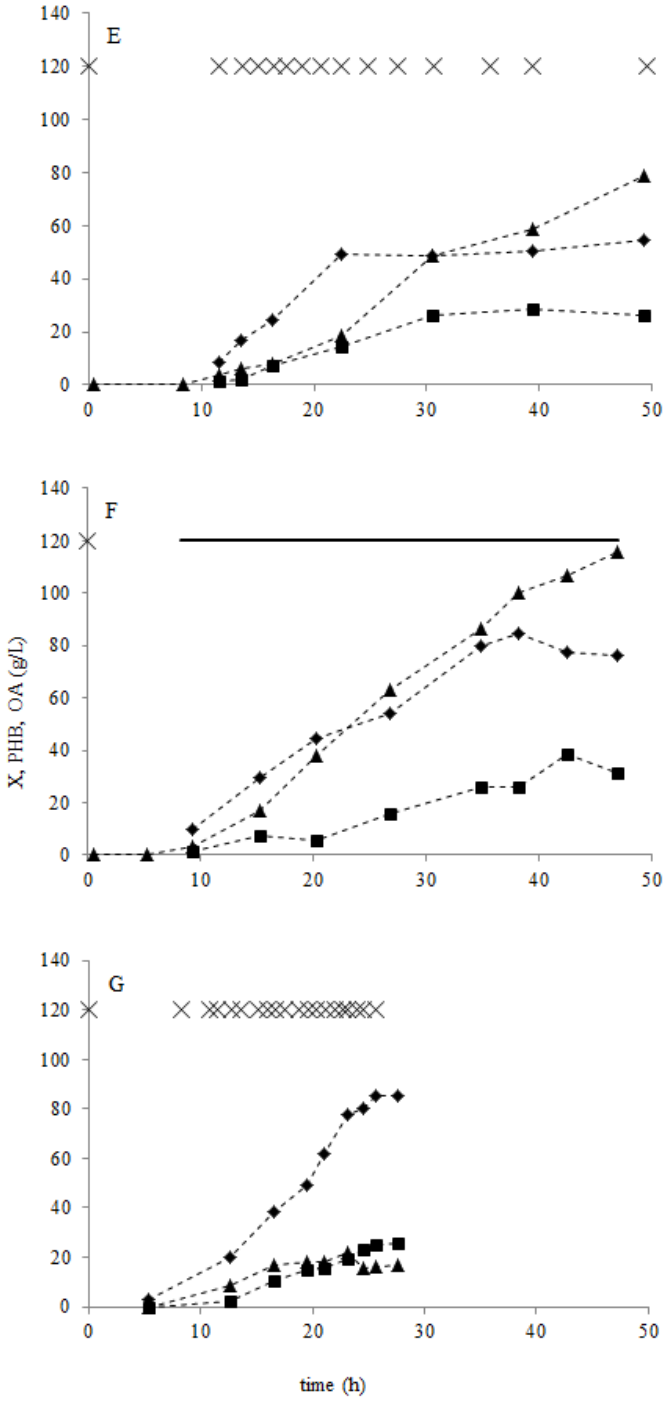


Figure 3.2: Profiles of active biomass (◆), P(3HB) (■) and OA (▲) concentration obtained in bioreactor experiments using MR medium supplemented with cheese whey under oxygen limitation (30% of pO_2), where the feeding was performed by pulses (×), to maintain the lactose concentration between 0-20 g/L (E), by continuous fed-batch mode (-) after exponential phase started, to maintain the lactose concentration above 20 g/L (F) and by pulses, to maintain the lactose concentration between 10 and 20 g/L (G).

Aiming at reducing the production of OA, a different strategy was implemented in experiment G: pulse feeding was applied, maintaining lactose concentration between 10 and 20 g/L to avoid, in one hand, periods with absence of lactose and in other hand, to avoid high lactose concentration that leds to a high OA production. Without lactose absence periods the Ptrc promoter, that allows a maximal expression of the *phb* genes, the P(3HB) production is always activated. Maintaining the lactose concentration at low values (10-20g/L) could decrease the OA production already detected, once the glycolysis is keeping in pace with TCA cycle.

Using this feeding strategy, high cell density was achieved (85.18 g/L) as it occurred for experiment F. The same value of specific growth rate achieved in experiments D to F was obtained (0.26 h^{-1}). Both P(3HB) content (23% vs 29%) and concentration (25.56 g/L *vs.* 38.55 g/L) were lower than in the continuous fed-batch assay. However, volumetric productivity was improved from 0.67 g/L.h in 0.93 g/L.h in experiment F and G, respectively, being the best value achieved in this work. These values were much lower than those obtained with recombinant *E.coli* and Whey by Choi (1997) and Lee (1998), where the productivity was 2.18 g/L.h and 1.10 g/L.h, respectively,

A significant decrease in OA production was verified in this experiment: only 16.74 g/L was produced until 27 hours of fermentation and the yield of OA per lactose ($0.12 g_{OA}/g_{Lac}$) was lower than the yield of P(3HB) per lactose ($0.19 g_{P(3HB)}/g_{Lac}$), meaning that, more lactose were directed for P(3HB) production. Thus, it may be concluded that pulse feeding, maintaining the lactose concentration between 10 and 20 g/L, was best strategy for growth and storage, reducing significantly the deviation of carbon source for OA formation.

3.5. Conclusions

Five recombinant *E. coli* strains were tested and strain CML3-1 was selected due to its good performance in terms of biomass and P(3HB) production (1.92 and 2.14 g/L of biomass and P(3HB), respectively, in shake flask assays).

Different media were tested (LB and MR) supplemented with lactose or cheese whey. The defined MR medium led to higher cell density than LB. Organic acid production was observed in both media. Aiming at improving PHA production while reducing the carbon deviated to OA synthesis, different reactor operation strategies were evaluated. The reduction of pO_2 from 60%, to 30%, almost doubled the P(3HB) concentration (from 10.72 g/L to 28.68 g/L, respectively). However OA production was also increased. Two reactor feeding strategies were further tested: continuous mode that maintained the lactose concentration above 20 g/L and pulses feeding, maintaining the lactose concentration between 10 and 20 g/L. Despite the feeding in continuous mode led to a higher P(3HB) production (38.55 g/L), it also contributed

for a very high level of OA production (115.76 g/L). Pulse feeding, resulted in a lower OA production, it also contributed to lower P(3HB) concentration (25.56 g/L).

Continuous feeding mode seems to be best way to achieve high P(3HB) production. However, this strategy was not able to reduce de OA production. The possibility of blocking or inhibiting the metabolic routs of OA production might be the way to increase the P(3HB) production. By this way, higher and continuous flux of lactose could be applied to activate the lac-inducible promoter, that it is associated with P(3HB) production genes.

Chapter 4

The contents of this chapter were adapted from the publication Pais J., Farinha I., Freitas F., Serafim L. S., Martínez V., Martínez J. C., Arévalo-Rodríguez M., M. Prieto M. A., Reis M. A. M.; (2014); Improvement on the yield of polyhydroxyalkanotes production from cheese whey by a recombinant *Escherichia coli* strain using the proton suicide methodology; Enzyme and Microbial Technology 55, 151-158 and is subject to the copyright imposed by the Enzyme and Microbial Technology Journal.

4. Improvement on the yield of polyhydroxyalkanoates production from cheese whey by a recombinant *Escherichia coli* strain using the proton suicide methodology

4.1. Summary

In this work, it was shown that polyhydroxyalkanotes (PHAs) production by this strain using cheese whey was low due to a significant organic acids (OA) synthesis (Chapter 3). The proton suicide method was used as a strategy to obtain an *E. coli* mutant strain with a reduced OA-producing capacity, aiming at driving bacterial metabolism towards PHAs synthesis.

Thirteen *E. coli* mutant strains were obtained and tested in shake flask assays, using either rich or defined media supplemented with lactose. P8-X8 was selected as the best candidate strain for bioreactor fed-batch tests using cheese whey as the sole carbon source. Although cell growth was considerably slower for this mutant strain, a lower yield of OA on substrate (0.04 $Cmol_{OA}/Cmol_{lac}$) and a higher P(3HB) production (18.88 $g_{P(3HB)}/L$) were achieved, comparing to the original recombinant strain (0.11 $Cmol_{OA}/Cmol_{lac}$ and 7.8 $g_{P(3HB)}/L$, respectively). This methodology showed to be effective on the reduction of OA yield by consequently improving the P(3HB) yield on lactose (0.28 $Cmol_{P(3HB)}/Cmol_{lac}$ vs 0.10 $Cmol_{P(3HB)}/Cmol_{lac}$ of the original strain).

4.2. Introduction

Only few bacteria are able to utilize lactose as carbon source and even fewer are able to store it as PHAs. *Escherichia coli* can grow on various carbon sources, including lactose, but is unable to produce PHAs. Nevertheless, *E. coli* genetic and metabolism routes have been extensively studied, which makes it an ideal host for harboring PHAs producing genes, namely, the *Cupriavidus necator* PHAs biosynthetic genes (Reddy et al. 2003). Recombinant *E. coli* strains were already described as being able to produce large amounts of P(3HB), representing up to 80% of the cell's dry weigh, and to achieve high cell densities (up to 119.5 g DCW/L) using cheese whey as substrate (Ahn et al. 2000).

The use of recombinant *E. coli* strains is also advantageous for downstream process, since cells can be easily disrupted, contrary to most wild type PHAs producers that are often hard to lyse (Nikel et al. 2006). Moreover, in contrast with those microorganisms, *E. coli* does not contain pathways for PHAs degradation. The high intracellular polymer content, together with easier PHAs extraction and purification procedures, can significantly reduce the overall production costs of the biopolymer (Reddy et al. 2003; Dias et al. 2006).

Aerobic cultivation of recombinant *E. coli* leads to the production of organic acids (OA), thus limiting the amount of carbon used for cell growth and PHAs production (Park et al. 2002). This inefficient behavior can be attributed to an imbalance between substrate consumption and its utilization for both biomass and energy generation (March et al. 2002).

The most commonly used strategy to decrease OA production by microorganisms involves genetic manipulation, by knocking-out the genes that code for the expression of enzymes responsible for OA production. However, it is a complex methodology that could damage other important metabolic pathway routes (Papoutsakis et al. 1999). The isolation of *Clostridium acetobutylicum* mutants, in which the genes involved in acetic acid production were deleted, has been reported (Papoutsakis et al. 1999). Although those mutants exhibited less acetate secretion, there was a simultaneous increase in pyruvate production (Papoutsakis et al. 1999). In *E.coli* the consecutively deletion of the genes responsible for mixed acids fermentation resulted in the redirection of the carbon flux to the lactate formation pathway (Jian et al. 2010). A further deletion of *ldh*A gene suppressed lactate formation, but largely increased ethanol secretion. Furthermore, complete deletion of all fermentative pathways may result in an oversupply of NADH, which could damage cell growth (Jian et al. 2010).

Aiming to improve P(3HB) production by recombinant *E. coli*, in this work, an alternative strategy was followed: the proton suicide method. This is a simple and easy method developed by Winkelman and Clark (1984) to select for mutants with a reduced or suppressed OA production capacity. This method relies on the toxicity of bromine (Br_2) for cells. Br_2 is formed from bromide (Br^-) and bromate (BrO_3^-) as a result of the increase of the protons concentration during the formation of OA. Hence, only mutants unable to produce OA are able to survive in such conditions (Winkelman & Clark, 1984; Cueto & Méndez, 1990). This methodology has been successfully used by Cueto and Méndez (1990) to obtain low acid-producing mutants of *Clostridium acetobutylcum* in the acid recycling to solventogenesis. 1,3-propanodiol production by *Clostridium butyricum* was also improved by using this method (Abbad-Andaloussi et al. 1995).

The proton suicide method was applied to the recombinant *E. coli* strain CML3-1 harboring *C. necator* P(3HB)-synthesis genes, in order to obtain mutants with a low OA production capacity. The different mutants obtained were tested in fed-batch bioreactor

cultivation using cheese whey as the sole substrate. To the best of our knowledge this method was never tested to select PHA producing strains.

4.3. Materials and methods

4.3.1. Microorganisms

The microorganism was described in section 3.3.1.

4.3.2. Growth conditions

E. coli strains were grown in LB medium (Kay et al., 2002) at 37°C with shaking (200 rpm). Kanamycin (25 mg/L) was added when needed (LB^k). Solid media were supplemented with 1.5% (w/v) agar. LB^k+Lac medium was prepared by supplementation of LB^k medium with 1% (w/v) lactose autoclaved separately.

The defined medium (MR medium), had the following composition (per liter): KH_2PO_4, 13.5g; $(NH_4)_2HPO_4$, 4.0g; citric acid monohydrate, 1.9g; 10 mL mineral solution (Lee & Chang, 1993). The mineral solution had the following composition (per liter HCl 1M): $FeSO_4.7H_2O$, 10.0g; $CaCl_2.2H_2O$, 2.0g; $ZnSO_4.7H_2O$, 2.2g; $MnSO_4.H_2O$, 0.5g; $CuSO_4.5H_2O$, 1.0g; $(NH_4)_6MO_7O_{24}.4H_2O$, 0.1g; $Na_2B_4O_7.10H_2O$, 0.02g). The pH was adjusted to 6.9 by adding NaOH pellets prior to autoclaving at 121ºC, for 20 min. Lactose 20% (w/v) and $MgSO_4.7H_2O$ 20% (w/v) solutions were prepared and autoclaved separately, and added to MR medium after cooling. MR medium was further supplemented with 1 mL/L of a proline and thiamine solution (400 mg proline and 6.740 g thiamine-HCl in 20 mL deionized water) and 1 mL/L of kanamycin (25mg/L) solution both filter sterilized.

For the fed-batch bioreactor assays, MR medium was supplemented with cheese whey (CW) to give a final concentration of about 30 g/L lactose. The CW powder used in this work was supplied by Lactogal (Portugal) and had the following composition: 78.40 wt% lactose, 13.62 wt% protein and 1.21 wt% fat. An aqueous CW solution (30 – 40%, w/v) at 121ºC for 15 minutes, followed by centrifugation at 8000 *g* for 1 h, for removal of the precipitated protein aggregates (Ahn et al. 2000). Finally, the solution was filtered through a 0.2 μm filter (SartoLab-P20 plus) to remove the remaining small protein aggregates and assure its sterility. Table 4.1 summarizes the media, carbon source and cultivation type used in each assay of this study.

Table 4.1: Media, carbon source and type of cultivation used in each assay.

Assay	Media	Carbon source	Cultivation type
Evaluation of Br^- and BrO_3^- lethal concentrations	LB^k+Lac	Lactose	Shake flask
Mutant isolation	LB^k+Lac	Lactose	Plates and shake flask
Mutant selection	LB^k+Lac MR^k+Lac	Lactose	Shake flask
Evaluation of mutant strain stability	LB^k+Lac LB^k	Lactose ---	Shake flask
Evaluation of mutant strain performance	MR^k+CW	Cheese whey	Fed-batch reactor

4.3.3. Proton suicide method

In order to evaluate the lethal effect of bromide (Br^-) and bromate (BrO_3^-) in the recombinant *E. coli* CML3-1, different equimolar concentrations of each ion were tested (0, 50, 125, 175, 200, 225 and 250 mM) in solid LB^k+Lac medium. To calculate cell viability, colony-forming units (CFU) were counted after incubation of the plates for 24 h at 37ºC. The lowest concentration of Br^-/BrO_3^-, for which no cell growth was observed, was selected and used in the subsequent experiments.

For isolation of mutants, a single colony of recombinant *E. coli* CML3-1 was inoculated into 10 mL of LB^k+Lac medium and incubated overnight at 37°C and 200 rpm. After centrifugation of the broth, the pellet was resuspended in 10 ml fresh LB^k+Lac medium supplemented with 250 mM equimolar Br^-/BrO_3^- and incubated during 96 h, as described above. Finally, the broth was centrifuged, being the pellet resuspended in 10 mL NaCl 0.9 % (w/v) and plated on solid LB^k+Lac medium.

The isolated surviving colonies were picked onto LB^k+Lac medium supplemented with 40µL/mL X-gal (5-bromo-4-chloro-indolyl-galactopyranoside (Sigma), in a dimethyl sulfoxide solution, to ensure their β-galactosidase functionality (Edwards & Taylor, 1993; Karlinsey & Hughes, 1993). The colonies showing an active β-galactosidase (blue phenotype) were subjected to a further mutagenic pressure in solid LB^k +Lac+Br^-/BrO_3^-medium.

All mutants obtained were plated on Difco™ MacConkey Agar Base medium supplemented with 1% (w/v) lactose and incubated overnight at 37ºC to evaluate their OA production capacity. The OA-producing bacteria were identified by the pink colonies formed in the presence of the neutral red indicator contained in MacConkey medium. The mutants unable

to produce OA formed yellow colonies. The yellow, and yellow with a slightly pink color, colonies were visualized with a 100 × phase-contrast microscope to select those containing higher number of intracellular P(3HB) granules.

For the isolated mutants, a denaturing gradient gel electrophoresis (DGGE) was run as described previously (Carvalho et al. 2010). The 16S DNA fragments obtained for each mutant were extracted and amplified by PCR. Basic Local Alignment tool (BLAST) was used to confirm that they were *E. coli* MG1655 mutants.

4.3.4. Selection of the best P(3HB)-producing mutant

The inocula were prepared after two adaptation steps. First, a single colony of each mutant and the original recombinant *E. coli* strain CML3-1, grown on solid LB^k+Lac, were inoculated in 20 mL LB^k+Lac or MR^k+Lac media in 100 mL shake flasks and incubated, during 16 and 32 h, respectively. Then, 20 mL of each culture were centrifuged at 8000 *g*, for 10 min and the pellet was resuspended in fresh LB^k+Lac or MR^k+Lac media (100 mL medium in 500 mL flasks) and incubated for 10 h, under the same conditions.

For each isolated mutant and the original recombinant strain CML3-1, two tests were performed, namely, in rich medium (LB^k+Lac) and in defined medium (MR^k+Lac) (Table 4.1). For both experiments, 40 mL of each inoculum were centrifuged and resuspended in fresh medium to a final volume of 200 mL in 1-L flasks and incubated under the same conditions as the inocula, for 16 h. Samples were taken at the beginning and at the end of the experiments for determination of the dry cell weight (DCW), P(3HB) content in the biomass and concentration of lactose and OA.

Mutants presenting a lower OA production and a significant P(3HB)-storing capacity were selected for the subsequent assays.

4.3.5. Evaluation of mutant stability

In order to evaluate the stability of the mutagenic pressure applied, the selected mutant strain (*E. coli* P8-X8) and the original recombinant strain (*E. coli* CML3-1) were sequentially incubated in shake flasks. The first incubation was made in LB^k+Lac (during 13.5 h), the second in LB^k during 10 h) and the third incubation again in LB^k+Lac (during 45 h). Between transfers, the broth was centrifuged and the pellet thus obtained was resuspended in fresh medium for the following incubation. Samples were taken for quantification of DCW, P(3HB) content in the biomass, lactose and OA concentration.

4.3.6. Evaluation of mutant performance in bioreactor cultivation

E. coli CML3-1 and P8-X8 inocula were both prepared in three steps: 1) a single colony taken from solid LB^k+Lac was inoculated into 20 mL of liquid MR^k+Lac medium and incubation during 32 h; 2) each culture was centrifuged, the pellet was resuspended in 100 mL of MR^k+Lac medium and incubated for 10 h; 3) 40 mL broth samples were centrifuged and resupended in 200 mL of fresh MR^k+Lac medium and further incubated for 10 h.

The bioreactor assays, with the original and the mutant strains, were carried out in 2-L bioreactors (BioStat® B-Plus, Sartorius) using 1-L working volume. MR^k medium supplemented with CW (MR^k+CW) to give an initial lactose concentration of 30 g/L was used in both assays. The temperature was controlled at 37ºC. The bioreactor was inoculated with 200 mL of each culture (CML3-1 and P8-X8) and operated in a fed-batch mode with the addition of two consecutive pulses of lactose whey (30 g/L) after exhaustion of the carbon source in each pulse, identified by un uprise in the pH value above 6.9. The pH was controlled at 6.9±0.1 by the automatic addition of NH_4OH 28% (v/v) solution, during the first pulse, and NaOH 2M, during the subsequent pulses. Lactose pulses were added when substrate was exhausted. The lactose was monitored during the bioreactor assay. The air flow rate was kept constant during the entire run (3 L/L.min) and the dissolved oxygen concentration (DOC) was controlled by automatically increasing the stirring rate from 200 to 1000 rpm. During the first two pulses, the DOC was controlled at 30% of air saturation, while during the third it was lowered to 10%. Foam formation was suppressed by the automatic addition of Antifoam A (Fluka).

Samples were periodically taken for P(3HB), DCW, lactose and OA quantification.

4.3.7. Analytical methods

The dry cell weight (DCW), P(3HB), lactose and OA quantifications were performed as described in section 3.3.6.

4.3.8. Calculations

P(3HB) content, active biomass, specific growth rate, volumetric productivity, OA volumetric productivity, storage, growth and OA yields on lactose, the specific OA yield, volumetric lactose consumption and specific lactose consumption rates were calculated as described in section 3.3.7.

4.4. Results and discussion

A genetic construct, plasmid pMAB26, was obtained to deliver integration of the P(3HB)-synthesis genes of *C. necator* into the chromosome of the bacterial strain of choice, via

a transposition-mediated mechanism. The new construct, with the *phaCAB* operon embedded in the mini-Tn5 element of plasmid pCNB5 and located under the lactose-inducible *Ptrc* promoter of this element, provided P(3HB) synthesis functions when replicating as a free plasmid (data not shown), and allowed delivery and stable integration of these genes and expression of the corresponding enzyme activities in the recipient *E. coli* strain. By using pMAB26 and the *E. coli* K-12 derivative MG1655 we easily obtained transconjugant strains able to accumulate P(3HB) granules when cultured in media containing lactose or cheese whey. One of these strains, CML3-1, was selected for further studies.

The ability of recombinant *E. coli* CML3-1 strain to produce P(3HB) using cheese whey (CW) as substrate was shown to be impaired by the production of high levels of organic acids (OA) (Chapter 3). This problem is common to high cell density aerobic fermentative processes (e.g. recombinant protein production, PHA production) using recombinant *E. coli* strains that synthesize acidic byproducts, which inhibit growth and reduce the overall capacity of the processes for the intended product (Wegan et al. 2001; Phue et al. 2005).

Aiming at improving P(3HB) production by recombinant *E. coli* CML3-1 strain, the proton suicide method was applied to generate mutants with reduced OA production capacity and, thereby, attempting to direct the strain's metabolism towards intracellular polymer accumulation.

4.4.1. Mutants isolation

The proton suicide method is based on the direct selection of mutants unable to produce OA by cultivating cells in a bromide (Br^-) and bromate (BrO_3^-) containing medium. Hence, the first step included the evaluation of the lethal concentrations of Br^- and BrO_3^- for strain CML3-1. For that purpose, different concentrations of those ions (0 – 250 mM) were tested in a LB^k+Lac agar plate assay, wherein colonies formation units (CFU) counting was used as an indicator of cell survival under the tested conditions.

The results showed that only a Br^-/BrO_3^- concentration of 250 mM was lethal for strain CML3-1 (Table 4.2). This concentration is in the range of that reported by Winkelman and Clark (1984) (200 - 300 mM) for *E. coli* K-12 strain. Thus, a concentration of 250 mM Br^-/BrO_3 was chosen for the proton suicide selection pressure.

Table 4.2: CFU counting of CML3-1 in LB^k+Lac agar plates with different bromate and bromide concentrations.

$[Br^-/BrO_3^-]$ (mM)	Growth (CFU/plate)
0	>100
50	>100
125	>100
175	95
200	83
225	75
250	0

In order to confirm that the selection pressure did not affect their β-galactosidase activity, and, consequently, their ability to use lactose as carbon source, the isolated surviving colonies were picked onto LB^k+Lac+XGal. The mutants that presented β-galactosidase activity were subjected to a second mutagenic pressure in solid LB^k+Lac medium supplemented with Br^-/BrO_3^-.

All mutants obtained from the final selection step were plated on MacConkey agar medium for the identification of the microorganisms with reduced OA production capacity, corresponding to the colonies presenting a yellow or a yellow with a slightly pink color. The wild type strain *E. coli* MG1655 formed pink colonies surrounded by a pink halo, while the original recombinant CML3-1 strain formed yellow and pink colonies (Figure 4.1). The results showed that the mutants obtained seemed to produce less OA as shown by their yellow colonies. Based on phase contrast visualization, mutants also seemed to produce more P(3HB), as shown by the higher number of granules accumulated inside the cells.

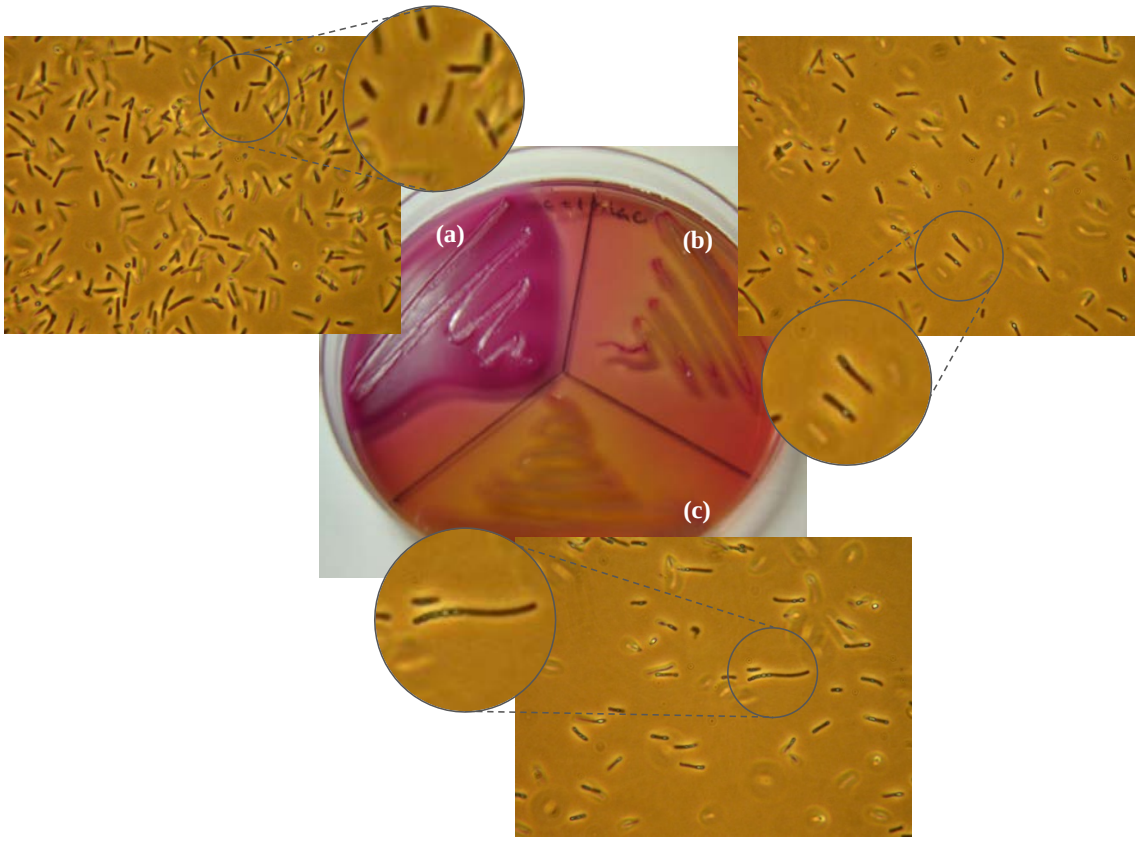


Figure 4.1: Colonies (centre image) and optical microscope photos in phase contrast (100x) of wild strain *E. coli* MG155 (a), original recombinant strain CML3-1 (b) and mutant P4-1 strain (c) grown in MacConkey agar plate supplemented with lactose. Fresh cultures were visualized with a 100 × phase-contrast objective (Olympus BX51).

Thirteen mutants were selected and isolated, which were confirmed as variants of *E. coli* MG1655 by DGGE.

4.4.2. Mutant selection

The capacity of OA and P(3HB) production by each mutant was evaluated in shake flask assays in both LB^k+Lac (Figure 4.2-a) and MR^k+Lac media (Figure 4.2-b).

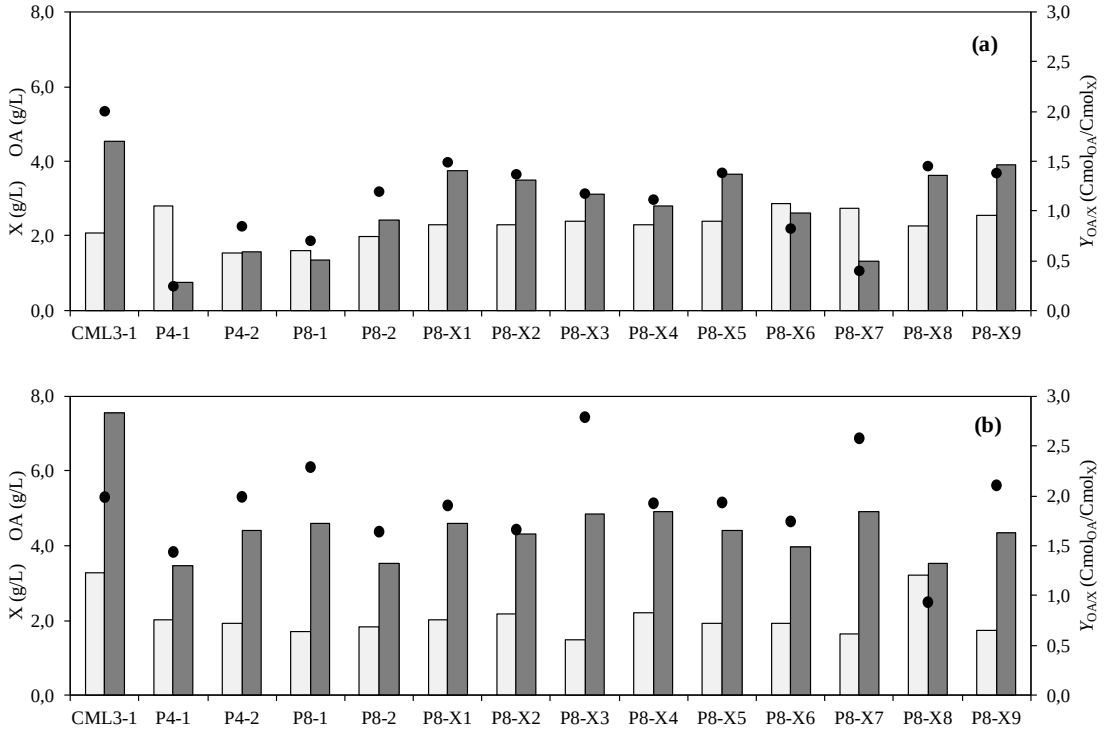


Figure 4.2: Total organic acids concentration (▭), active biomass produced (□) and the specific OA molar yield (●) for the original recombinant strain (CML3-1) and the isolated mutant strains (P4-1, P4-2, P8-1, P8-2, P8-X1, P8-X2, P8-X3, P8-X4, P8-X5, P8-X6, P8-X7, P8-X8) cultivated in (a) LB^k+Lac and (b) MR^k+Lac media, both supplemented with 1% (w/v) lactose.

Results showed that the active biomass production by the mutants grown in LB^k+Lac medium was between 1.53 and 2.86 g/L. The total amount of OA produced by each mutant, in the range 0.74 – 3.90 g/L, was lower than the original recombinant strain CML3-1 (4.54 g/L) (Figure 4.2-a). Furthermore, for all mutants, the specific OA molar yield (0.25 - 1.49 $Cmol_{OA}/Cmol_X$) was lower than that (2.01 $Cmol_{OA}/Cmol_X$) for strain CML3-1. The lowest yield was obtained for strains P4-1, P4-2, P8-1, P8-X6 and P8-X7 (< 1.0 $Cmol_{OA}/Cmol_X$) (Figure 4.2-a).

The P(3HB) content (16.0 – 22.5%) achieved by the mutant strains grown in LB^k+Lac medium was comparable to that (20.3%) of the original strain, except for strain P4-2, which was much lower (10.4%) (Figure 4.3). The results in shake flasks using LB^k+Lac medium suggest that the proton suicide method was successful in selecting mutant strains that produce less OA than the original recombinant strain, concomitant with a higher P(3HB) production, namely,

strains P4-1, P8-1, P8-X6 and P8-X7, being the latter the most promising due its high biomass production (2.74 g_X/L), low $Y_{OA/X}$ (0.40 $Cmol_{OA}/Cmol_X$) and higher P(3HB) content (22.5 %).

In parallel, shake flask assays using MR^k+Lac medium containing 1% (w/v) lactose (Figure 4.2-b) were performed in order to evaluate the capacity of the thirteen isolated mutants to grow and produce P(3HB) and OA in mineral medium.

Although all mutant strains were able to grow in MR^k+Lac medium (Figure 4.2-b), the average active biomass produced (1.48-3.22 g/L) was lower than for strain CML3-1 (3.28 g/L). Moreover, on average, cell growth was also lower than in LB^k+Lac medium (Figure 4.2-a). On the other hand, the total OA production (3.46-4.92 g/L) was lower than that of the original recombinant strain (7.55 g/L), but higher than in LB^k+Lac medium (0.74-3.90 g/L). Furthermore, the specific OA molar yield was also higher than in LB^k+Lac medium for all mutant strains. P8-X8 was the only strain that reached an active biomass concentration (3.22 g/L) similar to that of CML3-1 strain (3.28 g/L) and produced a lower amount of OA in MR^k+Lac medium (3.53 g/L) (Figure 4.2-b).

All strains, including strain CML3-1, had a lower P(3HB) content when cultivated in MR^k+Lac medium (7.7-14.9%) than in LB^k+Lac medium (10.4-22.5%) (Figure 4.3). Most of the mutant strains tested showed a higher P(3HB) content than CML3-1. Though the highest polymer content was obtained for strain P8-X3 (14.9%). This strain presented the lowest cell growth (1.48 g/L) and a high OA production (4.85 g/L), resulting in a very high specific OA molar yield (2.78 $Cmol_{OA}/Cmol_X$). The mutant strain P8-X8 presented the lowest $Y_{OA/X}$ value and had a P(3HB) content higher (12.08%) than CML3-1 (8.92%), thus confirming its potential for P(3HB) production using lactose as carbon source. Hence, strain P8-X8 was selected as the most promising mutant strain for P(3HB) production from whey and used for the subsequent tests.

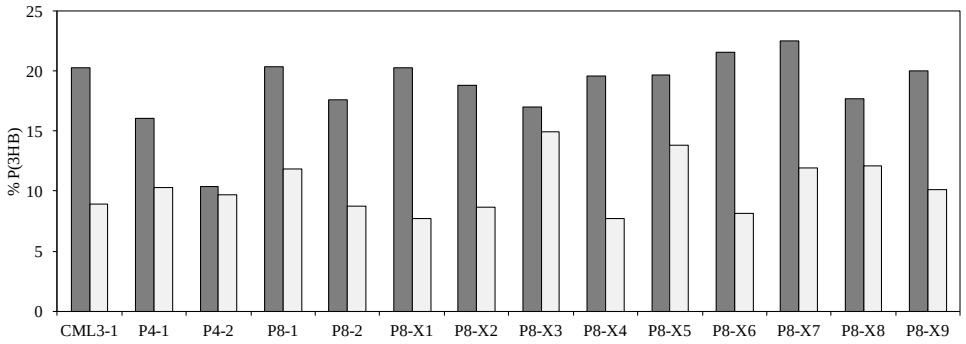


Figure 4.3: P(3HB) content in biomass produced by the original recombinant (CML3-1) strain and each mutant (P4-1, P4-2, P8-1, P8-2, P8-X1, P8-X2, P8-X3, P8-X4, P8-X5, P8-X6, P8-X7, P8-X8) cultivated in LB^k+Lac (▬) and MR^k+Lac (▭) media, both supplemented with 1% (w/v) lactose.

4.4.3. Evaluation of the stability of mutant P8-X8

In order to evaluate the stability of the mutagenic pressure applied, the mutant strain selected (P8-X8) and the original recombinant strain (CML3-1) were cultivated in three subsequent shake flasks batches (Figure 4.4). The cultivations were made in LB^k+Lac medium, except the second batch, wherein lactose was not included. LB is a rich medium containing different peptides and amino acids that could be used as carbon source when lactose was absent. Since lactose is the inducer of P(3HB), meaning that it is required for the expression of genes that encode for P(3HB) production, the objective of this strategy was to check if the culture deprived from lactose did not lose its ability to produce P(3HB) in the subsequent batch, while maintaining a lower OA production.

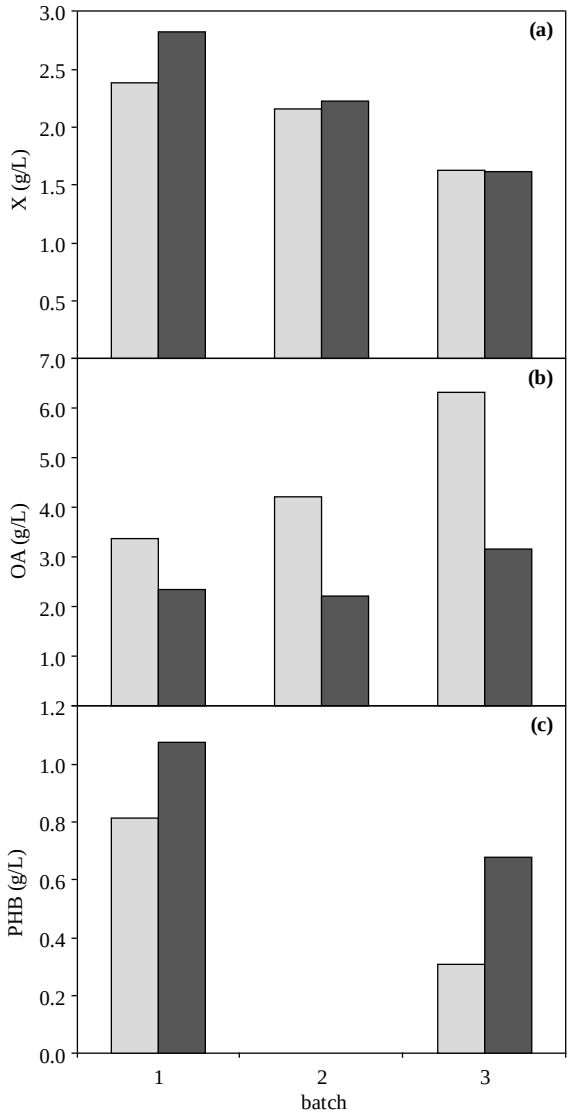


Figure 4.4: Active biomass (a), organic acid concentration (b) and P(3HB) concentration (c) in three sequential batch shake flask cultivations for CML3-1 (□) and P8-X8 mutant strains (■).

In the first two batches, cell growth was slightly higher for the mutant P8-X8 than for the original recombinant strain CML3-1, but in the subsequent batches it was identical for both strains (Figure 4.4-a). Nevertheless, cell growth was gradually reduced from 2.39 – 2.83 g/L, in the first batch, to 1.62 – 1.63 g/L, in the last batch. OA production was significantly higher for CML3-1 strain (increasing from 3.37 g/L in the first batch to 6.31 g/L in the third), while for P8-X8 strain it was kept lower (2.24 - 3.16 g/L) in all batches (Figure 4.4-b). In the first batch, both strains produced P(3HB), but polymer production was 32%, higher for strain P8-X8 (Figure 4.3-c). P(3HB) production was negligible for both strains in the second batch, due to the absence of lactose, whereas in the final batch strain P8-X8 production (0.68 g/L) was almost twice that of strain CML3-1 (0.31 g/L). These results show that the P(3HB) accumulating capacity was not lost when the mutant strain was grown on medium deprived of lactose, being higher than that of the original recombinant strain.

4.4.4. Fed-batch reactor performance of mutant P8-X8 for P(3HB) production

Given that P8-X8 showed the best balance between OA production, cell growth and P(3HB) production, this mutant strain was tested in a fed-batch bioreactor using cheese whey as carbon source. In parallel, the original strain CML3-1, cultivated under the same operational conditions, was used for comparison. The experiments were carried out with three consecutive whey pulses (Figure 4.5). Table 4.3 shows the kinetic and stoichiometric parameters obtained for both strains.

Cell growth was considerably faster for strain CML3-1 than for the mutant strain P8-X8, as shown by the specific cell growth rates obtained (0.20 h^{-1} and 0.06 h^{-1}, respectively) (Table 4.3). Nevertheless, at the end of the experiments similar active biomass concentration was reached for both strains: 28.22 g/L for CML3-1 and 33.09 g/L for P8-X8. However, strain P8-X8 took 59 h to reach the maximum P(3HB) content, while CML3-1 cultivation lasted for 25 h (Figure 4.5). On the other hand, the P(3HB) content in the biomass was 38.65% for strain P8-X8, while for CML3-1 it was 21.73%. Hence, the overall P(3HB) volumetric productivities were identical for both strains: 0.32 – 0.33 g/L h (Table 4.3).

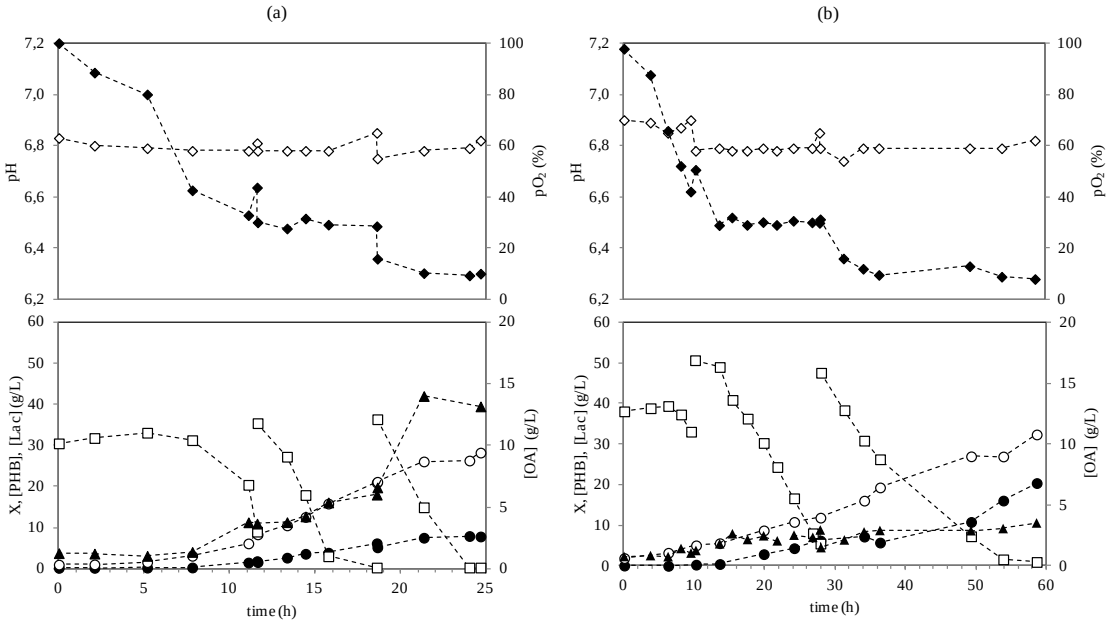


Figure 4.5: Cultivation profiles for strains CML3-1 (a) and P8-X8 (b) grown in mineral medium supplemented with cheese whey as the sole carbon source (pH (◇), dissolved oxygen concentration (◆) active biomass (○), P(3HB) concentration (●), OA concentration (▲) and lactose concentration (□).

Table 4.3: Kinetic and stoichiometric parameters obtained with the recombinant original strain CML3-1 and mutant strain P8-X8 in bioreactor cultivation using defined medium supplemented with cheese whey.

Parameter	CML3-1	P8-X8
X (g/L)	28.22	33.09
μ_{max} (h^{-1})	0.20	0.06
P(3HB) (g/L)	7.83	18.88
%P(3HB)$_{max}$	21.73	38.65
[OA] total (g/L)	13.14	3.52
$Y_{X/Lac}$ ($Cmol_X/Cmol_{lac}$)	0.32	0.39
$Y_{P(3HB)/Lac}$ ($Cmol_{P(3HB)}/Cmol_{lac}$)	0.10	0.28
$Y_{AO/Lac}$ ($Cmol_{OA}/Cmol_{lac}$)	0.11	0.04
$r_{P(3HB)}$ (g/L.h)	0.32	0.33
r_{OA} (g/L.h)	0.34	0.06
q_{Lac} ($Cmol_{lac}/Cmol_X.h$)		
1st pulse	0.19	0.03
2nd pulse	0.05	0.02
3rd pulse	0.02	0.01

In contrast with the growth yield, that was similar for strains P8-X8 and CML3-1 (0.39 and 0.32 $Cmol_X/Cmol_{Lac}$, respectively), the OA yield was significantly higher for CML3-1 (0.11 $Cmol_{OA}/Cmol_{Lac}$) than for P8-X8 (0.04 $Cmol_{OA}/Cmol_{Lac}$) (Table 4.3). Strain P8-X8 reached a

final OA production of 3.52 g/L, which was four times lower than the concentration produced by CML3-1 (13.14 g/L). Apparently, by inhibiting OA formation, strain P8-X8 had more carbon available for P(3HB) production, which is supported by the higher $Y_{P(3HB)/lac}$ of P8-X8, compared with CML3-1 (0.28 and 0.10 $Cmol_{P(3HB)}/Cmol_{Lac}$, respectively) (Table 4.3). Nonetheless, not all lactose consumed was accounted considering its use for cell growth, OA synthesis and P(3HB) for neither culture. Indeed, those processes accounted for a total yield on lactose of 0.53 $Cmol/Cmol_{Lac}$ for strain CML3-1 and 0.71 $Cmol/Cmol_{Lac}$ for strain P8-X8. The remaining lactose consumed might have been used for other unaccounted processes, such as energy for cell processes.

Although the specific lactose consumption rate was consecutively reduced throughout the three pulses for both strains, this value was consistently lower for the P8-X8 than for the original strain (Table 4.3). This is probably a consequence of the lower growth and OA production by mutant strain P8-X8. Apparently, it is energetically more favorable for CML3-1 cells to produce acids than to accumulate P(3HB). The energetic and redox unbalance generated by the fast growth observed for CML3-1 strain might have led to the activation of the anaerobic pathway as a strategy to reduce the net amount of NADH formed (Wong et al. 1999). Since P(3HB) production does not involve the consumption of NADH, it might be more energetically favorable for the culture to switch on the anaerobic metabolism, resulting on OA secretion (Park et al. 2003). On the other hand, the P8-X8 being less eficient in using the OA pathway, had to decrease the carbon uptake to reestablish the redox balance.

4.5. Conclusion

The proton suicide method was successfully used to generate a mutant *E. coli* strain, named P8-X8, with reduced OA production, concomitant with a higher P(3HB) accumulating capacity comparing to the original recombinant strain CML3-1. The mutant strain was able to use cheese whey as the sole carbon source for P(3HB) synthesis, with a polymer productivity similar to the recombinant strain and a considerably higher yield on lactose. Future work will be focused on the optimization of P(3HB) production by the P8-X8 strain.

Chapter 5

This chapter was adapted from the manuscript: Pais J, Gameiro T., Freitas F., Serafim L., Ramos A., Reis A.M.R.; Medium optimization for polyhydroxyalkanoates production by *Haloferax mediterranei* from cheese whey. (in preparation)

5. Medium optimization for polyhydroxyalkanoates production by *Haloferax mediterranei* from cheese whey

5.1. Summary

This study aimed at optimizing the composition of the highly saline medium (HS) to improve the conversion of chemically hydrolyzed cheese whey into polyhydroxyalkanoates (PHAs) by *Haloferax mediterranei*. The results showed that supplementation of HS with 10 mL of a micronutrients solution (MS) improved PHA productivity from 0.28 g/L.day to 0.97 g/L.day, using glucose and galactose as carbon sources. The use of hydrolyzed cheese whey as substrate further improved the process productivity to 1.97 g/L.day. Bioreactor cultivation of *H. mediterranei* with the optimized medium resulted in the production of a final active biomass concentration of 8.84 g/L with a polymer content of 52.56%, corresponding to a volumetric productivity of 4.04g/L.day. The culture produced a P(3HB-co-3HV) copolymer with 0.83% of HV, extracted by osmotic pressure, and it presented a molecular weight of 4.4×10^5 with a polydispersity of 1.5 and a crystallinity of 35.45%.

5.2. Introduction

Haloferax mediterranei is an extremely halophilic organism belonging to the family Halobacteriaceae in the domain Archaea. It was first isolated from seawater evaporation ponds near Alicante, Spain (Oren, 2002). *H. mediterranei* grows faster than most other members of Halobacteriaceae and exhibits remarkable metabolic efficiency and genome stability at high saline concentrations. Therefore, it has served as a good model for haloarchaeal physiology and metabolism studies for several decades (Oren, 2002).

H. mediterranei has been investigated as a PHAs producer (Rodriguez-Valera et al. 1983; Fernandez et al. 1986; Lillo & Rodriguez-Valera, 1990; Chen et al. 2006; Don et al. 2006; Koller et al. 2007a, 2007b, 2008; Bhattacharya et al. 2012). In particular, *H. mediterranei* is capable of accumulating poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), from many cheap carbon sources, such as starch (Lillo & Rodriguez-Valera, 1990), cheese whey (Koller et al. 2007a), extruded rice bran and extruded cornstarch (Huang et al., 2006), and vinasse (Bahattacharya et al. 2012), without medium supplementation with precursors (Koller et al. 2007a).

H. mediterranei requires high salt concentrations (NaCl concentration above 22%, w/v) for optimum cell growth (Quillaguamán et al. 2010). At such high salt concentration, the growth of non-halophilic microorganisms is prevented, thus allowing for the process to be carried out without strict sterile conditions, with the concomitant reduction of the costs of processes based on this culture (Quillaguamán et al. 2010). On the other hand, salts can corrode the stainless steel used in large-scale fermentors. In order to prevent corrosion, Hezayen et al. (2000) have tested in bench-scale cultivations, bioreactors made of polyetherether ketone, glass, and silicium nitrite ceramics for the production of P(3HB) by halophilic Archaea strain 56 (*Halopiger aswanensis*) (Hezayen et al. 2000).

A further advantage of the process for PHA production by halophilic Archaea is the ease of recovery of the polymer by hypo osmotic shock of the cells on treatment with salt-deficient water (Choi & Lee, 1999). This feature can advantageously be used for PHA recovery from cells on treatment with salt-deficient water (hypo osmotic shock) (Choi & Lee, 1999). Therefore, the downstream processing, which usually involves disruption of cell membrane, polymer separation from cell debris and, finally, polymer purification, is significantly simplified, thus contributing to the reduction of the total production costs, that otherwise can account up to 40% of the total production costs (Quillaguamán et al. 2010; Choi & Lee, 1999).

Though their interesting properties, PHAs are still not economically competitive, as their selling price is considerably higher than that of conventional synthetic plastics (Verlinden et al. 2007; Solaiman et al. 2006). *H. mediterranei* has been reported to be unable to use lactose as carbon source (Anton et. al, 1988), requiring a preliminary hydrolysis step (enzymatic or chemical) to make the constituent monosaccharaides (glucose and galactose) available for the culture. The conversion of enzymatically hydrolyzed cheese whey into P(3HB-co-3HV) by *H. mediterranei* was demonstrated by Koller et al. (2007a, 2007b, 2008). The polymer content in the biomass was 72.8 %, the volumetric productivity was 0.11g/L.h and the yield of polymer was 0.29 g_{PHA}/g_{Sugar} (Koller et al. 2007a).

Different media composition has been used to cultivate *H. mediterranei* with different substrates (Table 5.2). Lillo and Rodriguez-Valera (1990) reported P(3HB) production from glucose and starch using KH_2PO_4 as a phosphorus source. They had tested different KH_2PO_4 concentrations and concluded that 37.5 mg/L of KH_2PO_4 resulted in higher polymer production. Under those conditions, biomass with a 60% of PHA content, which represents 6 g/L of P(3HB) and a storage yield of 0.33 g/g, were obtained (Lillo & Rodriguez-Valera, 1990). The authors showed that lower phosphate concentrations gave poorer growth and a corresponding decrease in P(3HB) production.

To cope with the high salt concentration in their habitats, halophilic Archaea accumulate mainly KCl in equivalent concentration to that of NaCl in the extracellular

environment as strategy to keep NaCl outside the cell (Quillaguamán et al. 2010). Chen and co-workers (2006) used corn starch as carbon source in a medium without KCl and they obtained 20g/L of P(3HB-co-3HV), which represented 50.8% of PHA content. Lillo and Rodriguez-Valera (1990) used the same carbon source and they supplemented the medium broth with 0.5 g/L of KCl and only obtained 6.45 g/L of P(3HB). Despite, the effect of KCl on PHA production was not described in literature, regarding the results obtained it seems that the absence of KCl could improve the polymer storage.

Koller and co-workers (2007a) described the production of P(3HB-co-3HV) from hydrolyzed cheese whey, using a highly saline medium (HS) supplemented with a micronutrients solution (MS) (Koller et al. 2007a). Under such conditions, a polymer content of 72.8% was reached, being the final polymer production 12.2 g/L of PHA, with a storage yield of 0.29g/g (Koller et al. 2007a). On the other hand, Huang et al. (2006), using a medium without MS and with extruded rice bran and extruded cornstarch as carbon sources, obtained a cell concentration of 140 g/L and a PHA concentration of 77.8 g/L in a repeated fed-batch fermentation.

To the best of our knowledge, no systematic study has been made to assess the impact of different media composition on *H. mediterranei* growth and PHA production from cheese whey. In this work, three sets of shake flask experiments were performed to evaluate PHA production by *H. mediterranei* grown on different media supplemented with glucose and galactose as the carbon sources. The ability of *H. mediterranei* in using hydrolyzed cheese whey for PHA production was also tested in shake flask and bioreactor assays. The biopolymer obtained in bioreactor assays was characterized in terms of average molecular weight and thermal properties.

5.3. Materials and Methods

5.3.1. Microorganism and Media

Haloferax mediterranei ATCC 33500 was used in all experiments. *H. mediterranei* was cultivated in a highly saline (HS) medium described by Koller and co-workers (2007), with the following composition (per liter): KCl, 4g; yeast extract, 5g; $NaHCO_3$, 0.2g; NaBr, 0.5g; NaCl, 156g; $CaCl_2.2H_2O$, 1g; $MgSO_4.7H_2O$, 20g; $MgCl_2.6H_2O$, 13g. $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and $MgCl_2.6H_2O$ solutions were prepared and autoclaved separately in order to avoid salts precipitation and added to the basal medium after cooling. The micronutrients solution (MS), used in experiments B, E to L and in bioreactor assay, had the following composition (per liter): $ZnSO_4.7H_2O$ 0.1g, $MnCl_2.4H_2O$ 0.03g, H_3BO_3 0.3g, $CaCl_2.6H_2O$ 0.2g, $CuSO_4$ 0.006g, $Na_2MoO_4.H_2O$ 0.03g. HS medium was supplemented with the carbon source to a concentration

of glucose and galactose of 8 g/L, each (Table 5.1). Glucose, galactose solutions were prepared and autoclaved separately at 121°C for 15 minutes. For the preparation of solid medium, 15g agar were added to HS medium prior to autoclaving (120ºC, 20 minutes).

5.3.2. Lactose and cheese whey hydrolysis

H. mediterranei does not use lactose as carbon source but uses its monosaccharides (Koller et al., 2007b) and since this sugar is the main component of cheese whey, a chemical hydrolysis was performed in order to convert lactose into glucose and galactose. For this propose a chemical hydrolysis of 208 g/L of lactose at 100ºC was applied Different concentrations of HCl (0.4, 0.7 and 1 M) during different time periods (30, 60, 90 minutes) were tested. The method that presented a higher degree of conversion without monomers degradation was chosen to hydrolyze cheese whey. For the experiments with cheese whey, two different hydrolyzates were used: 1) hydrolyzed crude cheese whey, was prepared by heating a cheese whey solution (208g whey powder dissolved in 1 L of HCl solution) at 100ºC, under stirring; 2) hydrolyzed autoclaved cheese whey was prepared by autoclaving a cheese whey solution (208g whey powder dissolved in 1L of deionized water) at 121°C for 15 minutes, following its centrifugation (1 hour at 8000 rpm) for removal of the precipitated proteins. The clarified solution, obtained after precipitated protein has been removed, was hydrolyzed using the conditions mentioned above. After cooling, both hydrolyzates were neutralized by the addition of NaOH pellets and centrifuged for 1 hour at 8000 rpm for the removal of suspended particles. The precipitated proteins obtained by centrifugation of the autoclaved cheese whey solution were lyophilized and hydrolyzed with HCl, as described above.

5.3.3. Inocula preparation

For each experiment, *H. mediterranei* was plated on solid HS medium supplemented with glucose and galactose (8 g/L, each) and incubated during 72h at 37°C. A single colony was used to inoculate 100 mL of liquid HS medium supplemented with glucose and galactose (8 g/L, each), in 250 mL-Flask and incubated during 72 hours at 37°C, in an orbital shaker at 200 rpm.

5.3.4. Shake flask experiments

Three sets of experiments were performed, wherein media with different composition were tested and compared with the standard HS medium (experiment A) (Table 5.1). For the first set, HS medium was modified as follows: B, supplementation with 1 mL of a micronutrients solution (MS); C, suppression of KCl from the medium; D, supplementation with KH_2PO_4 (37.5 mg/L). In the second set of experiments, experiments E – H, HS medium

was supplemented with increasing volumes (1, 2, 5 and 10 mL) of MS. Finally, in the third set, cultivation on HS medium supplemented with 10 mL of MS (I) was compared with the cultivation on HS medium supplemented with whey protein hydrolysate (J), hydrolysed crude cheese whey (K) and hydrolysed autoclaved cheese whey (L).

For each experiment, 80 mL of the inoculum prepared as described above were centrifuged and ressuspended in 400 mL fresh HS medium supplemented with the appropriate carbon source. All experiments were performed in 1-L flasks incubated in an orbital shaker (200 rpm), at 37ºC for 120 hours. Samples were periodically taken during the experiments for quantification of biomass, glucose galactose, and PHAs.

5.3.5. Batch bioreactor cultivation

Cultivation of *H. mediterranei* was performed in a BioStat® B-Plus bioreactor (Sartorius, Germany) with a working volume of 2 L, in HS medium supplemented with hydrolysed crude cheese whey containing approximately 8 g/L of glucose and 8 g/L of galactose. The temperature was controlled at 40ºC. The pH was controlled at 7.2 by the automatic addition of NaOH 2M and HCl 2M solutions. A constant air flow rate of 1 vvm was kept during the assay. The dissolved oxygen concentration pO_2 (%) was controlled at 20% by the automatic variation of the stirring speed (200 - 800 rpm). Foaming was suppressed by the automatic addition of Antifoam A (Sigma).

Samples were periodically taken for quantification of biomass, glucose, galactose, polymer and supernatant protein.

5.3.6. Analytical methods

5.3.6.1. Biomass concentration

Cell growth was monitored by measuring the optical density of at 520 nm (OD_{520nm}) with a spectrophotometer (Elios α, ThermoSpectronic). The cell dry weight (DCW) was estimated considering that one unit of OD_{520} nm is equivalent to 0.42 g/L DCW (Huang et al., 2006).

5.3.6.2. PHAs quantification

PHA quantification was performed as described in section 3.3.6, using a calibration curve with HB and HV monomers.

5.3.6.3. Glucose and galactose quantification

The cell-free supernatant obtained by centrifugation of the broth samples (8000 rpm, 10 min) was used for the quantification of glucose and galactose by high-performance liquid chromatography (HPLC). The analysis was performed using an Aminex HPX-87H (Biorad) column, coupled to a Refractive Index (RI) detector. The mobile phase was H_2SO_4 0.01 N, with an elution rate of 0.6 mL/min and an operating temperature of 50 °C. Glucose and galactose (Sigma Aldrich) standard solutions (0.125 - 1.0 g/L) were used.

5.3.6.4. Protein quantification

The Lowry method (Lowry et al. 1951) was applied to quantify the protein concentration in the supernatant. Bovine serum albumin (BSA) was used as standard for method calibration in concentrations up to 100 mg/L.

5.3.7. Polymer extraction

Polymer extraction was performed by hypo osmotic shock, as described by Escalona et al. (1996). The culture broth was centrifuged for 15 minutes at 6000 rpm. The supernatant was discarded and the cell pellet was washed with NaCl 10 % (w/v). The pellet obtained was repeatedly washed with deionized water (with the proportion 1g DCW/20mL water) until a whitish pellet was obtained. After several centrifugations, the cell debris, that remained floating at the surface, were discarded with the supernatant. The pellet was dried at 70°C until constant weight.

5.3.8. Characterization of PHAs

5.3.8.1. Molecular Weight Determination

Number average molecular weight (Mn) and weight average molecular weight (Mw), as well as the polydispersity index (Mw/Mn), PDI, were obtained by size exclusion chromatography (SEC) in a Waters apparatus equipped with a series of three Waters Ultrastyragel columns, with porosities of 10^3, 10^4 and 10^5A°. The analysis was performed at 30ºC, with chloroform as eluent, at a flow rate of 1.0 mL/min. Absolute values of Mw and Mn were determined. Universal calibration was performed and the calibration curve was generated with monodisperse polystyrene (PS) standards (in the range 2×10^3 to 4×10^6; Waters, Minneapolis, USA and Polymer Laboratories, Shropshire, UK).

The calibration curve was correlated with PHA using the Mark-Houwink-Sakurada relationship,

$$[\eta] = K M^{a},$$

where [η] is the viscosity number limit and K and a are the Mark-Houwink constants for each polymer/solvent/temperature system .

The values of these constants used for the pairs P(3HB)/chloroform and PS/chloroform were K=0.0118 mL/g, a=0.794, and K=0.0049 mL/g, a=0.78, respectively. Sample injection volumes of 150 μL were used.

5.3.8.2. Transition temperature determination

The melting point (Tm), the glass transition temperature (Tg), and the melting enthalpy were determined by differential scanning calorimetry (DSC) in an apparatus equipped with a cryogenic system, Setaram DSC 131. Samples preparation was performed according Serafim et al. (2008). The melting enthalpy was determined from the obtained thermogram and allowed for the calculation of the degree of crystallinity. It was assumed for all the PHA analyzed, that PHA with 100% of crystallinity presents a melting enthalpy of 132 J/g. (Miguel et al. 2001).

5.3.9. Calculations

P(3HB-co-HV) content, active biomass, specific growth rate, volumetric productivity, storage, growth yields on sugars (glucose and galactose), volumetric sugars consumption and specific sugars consumption were calculated as described in section 3.3.7.

5.4. Results and discussion

5.4.1. Effect of micronutrients on *H. mediterranei* growth and PHA production

Highly saline medium (HS) supplemented with different micronutrients, was used for the cultivation of *H. mediterranei* aiming at assessing their impact on PHA production. Four experiments were performed: A) highly saline media (HS) was used as control; B) HS medium supplemented with 1 mL of micronutrients solution (MS); C) HS medium without KCl; and D) HS medium supplemented with K_2HPO_4 (Table 5.1). In all experiments, equimolar amounts of commercial glucose and galactose were used as model substrates. Results are presented in Figure 5.1 and Table 5.1.

Table 5.1: Parameters obtained for *H. mediterranei* cultivated in shake flasks with different media composition for the different sets of experiments.

Exp.	Medium composition	Carbon source	ΔX (g/L)	PHA_{max} (%)	ΔPHA (g/L)	%HV	Δglucose (g/L)	Δgalactose (g/L)	$Y_{X/sugar}$ (g_X/g_{sugar})	$Y_{PHA/sugar}$ (g_{PHA}/g_{sugar})	r_{PHA} (g/L.day)
A	HS	glucose + galactose	3.58	27	1.40	10	1.70	0.00	2.11	0.82	0.28
B	HS+1mL MS		6.05	33	2.07	11	5.57	0.00	1.09	0.37	0.41
C	HS without KCl		1.28	12	0.19	14	1.51	0.00	0.85	0.12	0.04
D	HS+K_2HPO_4		3.44	21	0.53	5	2.73	0.00	1.26	0.19	0.11
E	HS+1mL MS	glucose + galactose	5.46	46	3.63	34	6.10	2.07	0.67	0.44	0.73
F	HS+2mL MS		6.61	42	3.67	39	6.27	2.86	0.72	0.40	0.73
G	HS+5mL MS		4.90	40	4.10	52	6.75	2.69	0.52	0.43	0.82
H	HS+10mL MS		6.11	41	4.85	56	6.07	3.40	0.65	0.51	0.97
I	HS+10mL MS	Glucose + galactose	6.79	46	6.66	23	7.93	2.19	0.67	0.66	1.33
J	HS+10mL MS+ hydrolyzed whey protein		3.90	59	7.31	13	7.12	1.08	0.48	0.89	1.46
K	HS+10 mL MS	hydrolyzed crude whey	3.84	65	9.86	13	5.98	3.04	0.43	1.09	1.97
L	HS+10 mL MS	hydrolized autoclaved whey	6.04	43	5.34	17	6.55	3.90	0.58	0.51	1.09

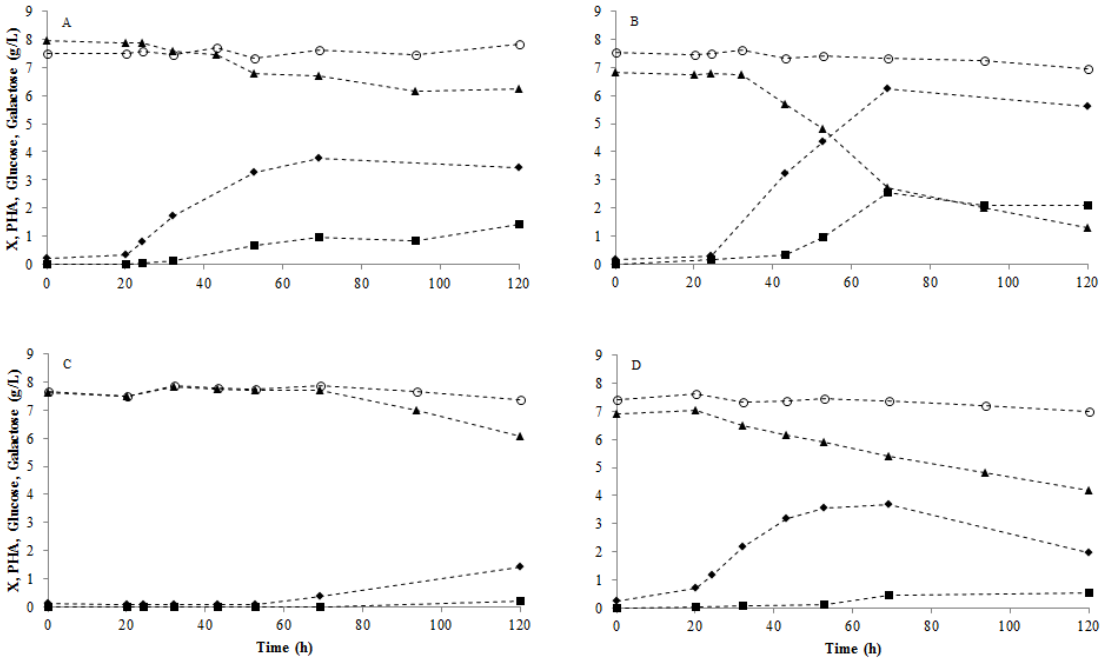


Figure 5.1: Profiles of active biomass (◆), PHA (■), glucose (▲) and galactose (○) concentration obtained in experiments with different media composition (experiment A - HS medium; experiment B – HS medium+1 mL MS; experiment C – HS medium without KCl; experiment D – HS medium with KH_2PO_4).

In experiment A (Figure 5.1-A), an active biomass production of 3.58 g/L was achieved after 69 hours of cultivation (Table 5.1). Glucose started to be consumed only after 20 hours of incubation. During this test, growth stopped after 1.70 g/L glucose was consumed, while no galactose was taken up. These results suggest that *H. mediterranei* has preference for glucose over galactose.

PHA was stored as a copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (P(3HB-co-3HV)) and its production occurred concomitantly with cell growth, reaching a final cell content of 27 % (w/w), corresponding to a polymer concentration of 1.40 g/L (Table 5.1). Both HB and HV monomers were produced simultaneously and the polymer reached a final HV content of 10% (mol/mol). The volumetric productivity and the yield were 0.28 gPHA/L.day and $0.82 g_{PHA}/g_{sugars}$, respectively (Table 5.1).

In test B (Figure 5.1-B), wherein HS was supplemented with micronutrients solution, cell growth was higher (6.05 g/L) than in assay A (Table 5.1). This was concomitant with the higher glucose consumption observed (5.57 g/L). There was also a slight increase in the polymer cell content (33%), as well as in the concentration of PHAs produced (2.07 g/L) in comparison with experiment A (Table 5.1).

As observed for experiment A, in experiment B, glucose was firstly consumed, confirming the preference of *H. mediterranei* for glucose as carbon source. Despite the slightly variation in galactose profile, its consumption was very low, almost 0 g/L. The polymer

obtained in experiment B had an HV content (11% mol/mol) similar to experiment A (10% mol/mol) (Table 5.1).

The results of experiments A and B showed that both biomass and polymer production can be increased by the addition of MS. Nevertheless, the values obtained were considerably lower than those obtained by Koller et al. (2007a) in a bioreactor under controlled conditions using a similar medium supplemented with the same micronutrients solution (Table 5.2). The polymer volumetric productivity increased from 0.28 g/L.day (experiment A) to 0.41 g/L.day (experiment B) (Table 5.1), while the yields of biomass and PHA on consumed sugars were lower (1.09 g_X/g_{sugar} and 0.37 g_{PHA}/g_{sugar}, respectively), than in assay A (2.11 g_X/g_{sugar} and 0.82g_{PHA}/g_{sugar}). These results could suggest that the addition of MS favored the growth and polymer production. However, regarding the yields on sugars, glucose could be deviating for another product not yet identified.

In experiment C, *H. mediterranei* was cultivated in HS medium wherein KCl was suppressed. The choice of KCl as a nutrient to be tested was based on results reported by Chen et al., 2006 that used a medium that did not contain KCl, in contrast with other studies with the same strain that included that component (Lillo & Rodriguez-Valera, 1990).

The results showed that growth and PHAs production were impaired when the KCl was not present in HS medium (Figure 5.1-C). Despite the amount of glucose consumed in assay C (1.51 g/L) being similar to that of assay A (1.70 g/L), in the former lower amounts of biomass and PHA were produced (1.28 g/L *vs* 0.19 g/L, respectively). The polymer cell content was 12%, corresponding to a volumetric productivity of 0.04 g/L.day (Table 5.1). These results suggest that the absence of KCl severely affected cell growth and production of PHA, and are in contrast with Chen et al. (2006) that reported a production of 20g/L of P(3HB-co-3HV) and a productivity of 0.27 g/L.day, for a polymer cell content of 50.8%. However, Chen et al. (2006) used cornstarch as substrate.

Chen and co-workers (2006) used corn starch as carbon source in a medium without KCl and obtained 20g/L of P(3HB-co-3HV), which represented 50.8% of DCW. Lillo and Rodriguez-Valera (1990) used 0.5 g/L of KCl and only obtained 6.45 g/L of P(3HB).

To cope with the high salt concentration in their habitats, halophilic *Archaea* accumulate mainly KCl (in inorganic cations form - K+) in equivalent concentration to that of NaCl in the extracellular environment as strategy to compensate the osmotic pressure (Quillaguamán et al. 2010; Roberts, 2005). These findings could suggest that the absence of KCl could improve the polymer storage, once KCl is accumulated intracellularly and it could compete with polymer storage.

Table 5.2: Comparison of results obtained in this work with the results obtained in the literature. Results obtained with *H. mediterranei* cultivation using different carbon, nitrogen e phosphorous source and parameters obtained with the *H. mediterranei* in bioreactor using HS medium supplemented with 10 mL of Micronutrients Solution (MS) and hydrolyzed crude whey.

Carbon source	**Phosphorus and nitrogen source**	**Product**	**CDW (g/L)**	**μ (h^{-1})**	**[PHA] (g/L)**	**[PHA] %**	**$Y_{x/sugar}$ (g_X/g_{sugar})**	**$Y_{PHA/sugar}$ (g_{PHA}/g_{sugar})**	**$r_{P(3HB\text{-}co\text{-}3HV)}$ (g/L.day)**	
Cheese whey	YE	P(3HB-co-3HV)	14.11	0.11	7.91	53	0.68	0.61	4.04	present work
Glucose and starch	KH_2PO_4 and NH_4Cl	P(3HB)	-	0.04	6	60	-	0.33	-	Lillo & Rodriguez-Valera, 1990
Cheese whey	YE	P(3HB-co-3HV)	4.6	0.10	12.2	72.8	-	0.29	2.64	Koller et al., 2007a
Extruded rice bran and extruded cornstarch	YE	P(3HB-co-3HV)	140	0.11	77.8	55.6	-	-	-	Huang et al., 2006
Cornstarch	YE	P(3HB)	39.4	0.09	20	50.8	-	-	-	Chen et al., 2006

Supplementation of HS medium with KH_2PO_4, in test D, showed no significant effect on cell growth since biomass concentration was similar to the obtained in test A (3.44 g/L and 3.58 g/L, respectively) (Table 5.1), even with a higher amount of glucose being consumed (Figure 5.1-D). On the other hand, the presence of K_2HPO_4 seemed to be detrimental for PHAs storage, which resulted in polymer cell content (21 %) lower than in experiment A (27 %). This effect of K_2HPO_4 on polymer content was not in accordance with what was reported by Lillo and Rodriguez-Valera (1990). These authors observed that the P(3HB) production was higher (3.09 g/L of P(3HB)) when KH_2PO_4 concentration was 37.5 mg/L. Concentrations above 37.5 mg/L (75-300 mg/L) and below 3.7.5 mg/L (9.38 mg/L) resulted in decrease on PHA production (1.70-0.73 g/L and 1.76 g/L of P(3HB), respectively). It was also hypothesized by us, that phosphate availability may have stimulated the synthesis of other cellular products, such as biomass or exopolysaccharides (Antón et al. 1988). In fact, in experiment D, an increase of broth's viscosity was observed. However, it is important to note, that not all EPS provide viscosity to the medium broth and also there are other products that could confer viscosity to the medium.

The results obtained in the first set of experiments showed that cell growth and polymer storage could be improved by supplementation of HS medium with the micronutrients solution.

5.4.2. Effect of increasing micronutrients concentration

In order to assess if further increasing of MS concentration could favor PHA production by *H. mediterranei*, a second set of experiments was ran, where 1, 2, 5 and 10 mL of MS were added to the medium (experiments E, F, G and H). Experiment E replicated experiment B, except for the fact that the inoculum was already prepared in HS medium supplemented with 1 mL of MS. Figure 5.2 and Table 5.1 show the results obtained in this second set of experiments.

The growth profile was similar for the four experiments without a visible lag phase and the maximum biomass concentration of 4.90 – 6.61 g/L was reached at 40h, suggesting that increasing the amount of micronutrients has not a significant impact on cell growth. Although the polymer content in the biomass decreased with increasing MS concentration, the overall PHA production increased (4.10 to 4.85 g/L) (Table 5.1). On the other hand, MS concentration had also a strong impact on polymer composition: an HV content of 34 to 56% (mol/mol) was obtained when MS was added. This higher HV production could be associated with galactose consumption, since it started to be produced when glucose was exhausted while HB production stayed practically constant. Contrary to what was observed in experiment A and B, where galactose was not consumed and the growth only stopped in the end of incubation, in experiments E, F, G and H the growth stopped when glucose was exhausted within around 40h of cultivation (Figure 5.2). Moreover, after glucose depletion, galactose was also consumed,

which was not observed previously. The higher consumption of both sugars in this set of assays might be associated to the higher concentration of MS available for the bacteria metabolism.

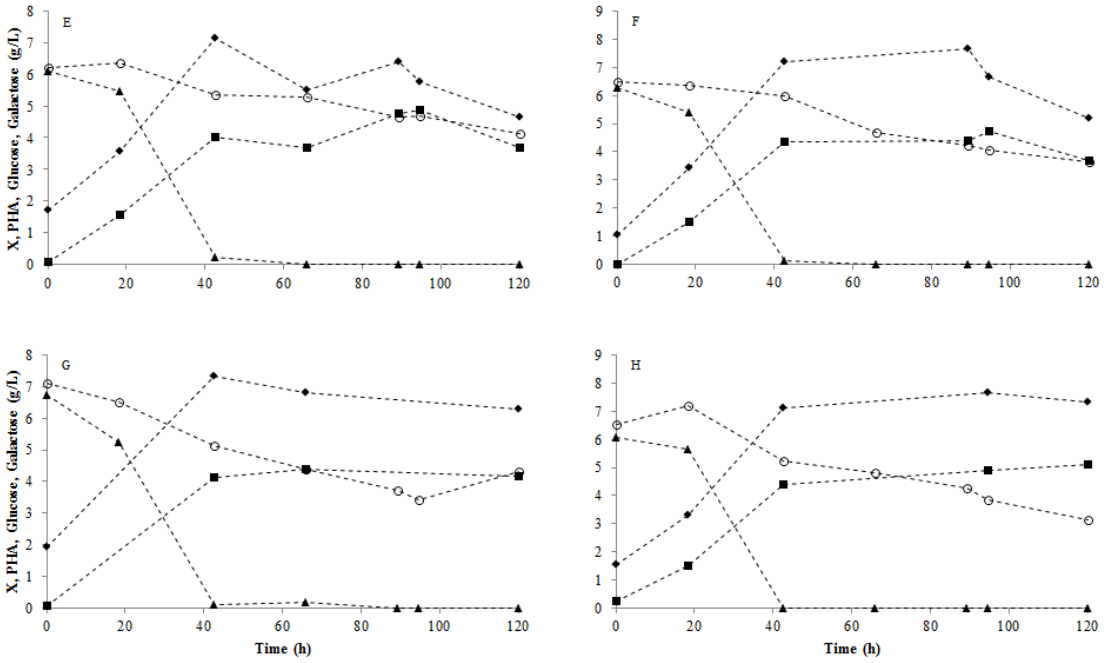


Figure 5.2: Profiles of active biomass (◆), PHA (■), glucose (▲) and galactose (○) concentration obtained in experiments with different media composition (experiment E - HS medium+1 mL MS; experiment F - HS medium+2 mL MS; experiment G - HS medium+5 mL MS; experiment H - HS medium+10 mL MS).

These results may suggest that the availability in the micronutrients (Zn^{2+}, Mn^{2+}, Cu^{2+} and Mo^{2+}) supplied in MS could play an important role on *H. mediterranei* metabolism. The impact of such cations in Archaea is not well known. However, these elements have an important role in several metabolic routes of bacteria. For example, Zn^{2+} is present in alcohol dehydrogenase, alkaline phosphatase, aldolase RNA and DNA polymerase (Goltschalk, 1983).

When MS solution was added to the inocula used for the assays E to H, galactose consumption was observed. Furthermore, in the experiments E to H, where each inocula medium was supplemented with MS, the PHA content was higher than in those experiments where the inocula media were not supplemented with MS solution (Experiments A to D). These results may suggest that components of MS media are important in driving galactose to HV production. In this set of experiments the HV content obtained was higher than those reported by Koller and co-workers (2007a) where a co-polymer with 6% of HV was obtained.

The results obtained in this set of experiments showed that P(3HB-co-3HV) production could be improved by the addition of 10 mL of MS. Under such condition, glucose and galactose consumption was improved, which is important for the development of a process for

the conversion of hydrolyzed cheese whey into PHAs. For this reason, the HS medium supplemented with 10 mL of MS was used in the subsequent assays.

5.4.3. Cultivation of *H. mediterranei* on medium supplemented with cheese whey hydrolyzates

In the following set of assays, cheese whey was tested as substrate for cultivation of *H. mediterranei* and production of PHA. Since *H. mediterranei* is unable to use lactose, cheese whey needed to be hydrolyzed into glucose and galactose.

5.4.3.1. Cheese whey hydrolysis

Chemical hydrolysis of cheese whey using HCl was the selected method for this study. This method is cheaper than the enzymatic and can contribute to increase the medium salinity. In order to identify the best condition for cheese whey hydrolysis, different concentrations of HCl (0.4, 0.7, 1 M) and different times of hydrolysis at 100ºC were tested. Using 1M of HCl at 100ºC during 90 minutes the lactose hydrolysis was almost complete (96%) and no degradation of glucose and galactose was observed. (Figure 5.3)

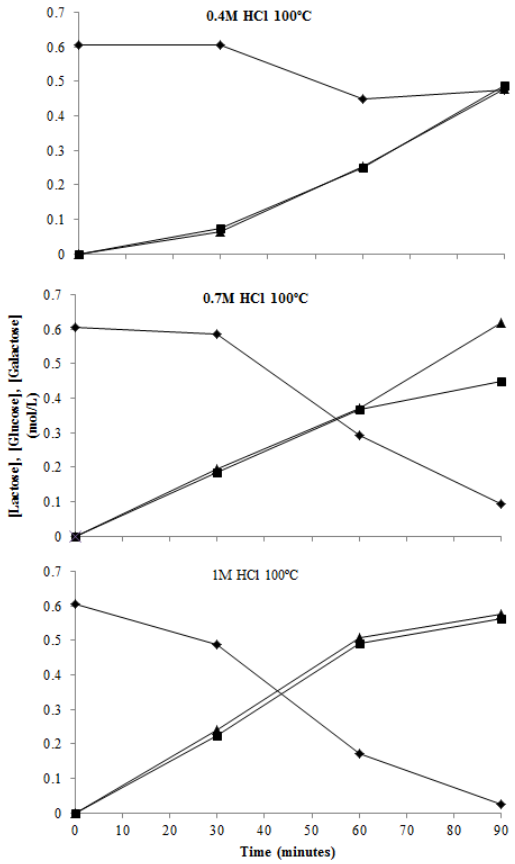


Figure 5.3: Molar concentration of lactose (◆), glucose (■) and galactose (▲) during the time of hydrolysis performed at 100ºC using HCl different concentrations: 0.4, 0.7 and 1M.

For HCl concentrations of 0.4 and 0.7 M lactose was not fully hydrolyzed after 90 minutes. Considering these results, the hydrolysis with 1M of HCl during 90 minutes was the method chosen in experiments J (for the hydrolysis of whey protein), K and L.

5.4.3.2. Experiments with hydrolyzed cheese whey

Considering the previous results, experiments I to L were performed with HS medium supplemented with 10 mL of MS, but different substrates were used in each assay (Table 5.1): for experiment K hydrolyzed crude whey and for experiment L hydrolyzed autoclaved whey was used aiming at selecting the best substrate for a cell growth and PHA production. Commercial glucose and galactose were used as carbon sources in experiments J. In this experiment, HS medium was supplemented with hydrolyzed whey protein in order to evaluate the ability of the culture to use the aminoacids and small peptides resulting from whey protein hydrolysis as carbon and/or nitrogen sources. Experiment I was the control assay, being commercial glucose and galactose used as the carbon source without further supplementation. Results obtained were shown in Table 5.1 and Figure 5.4.

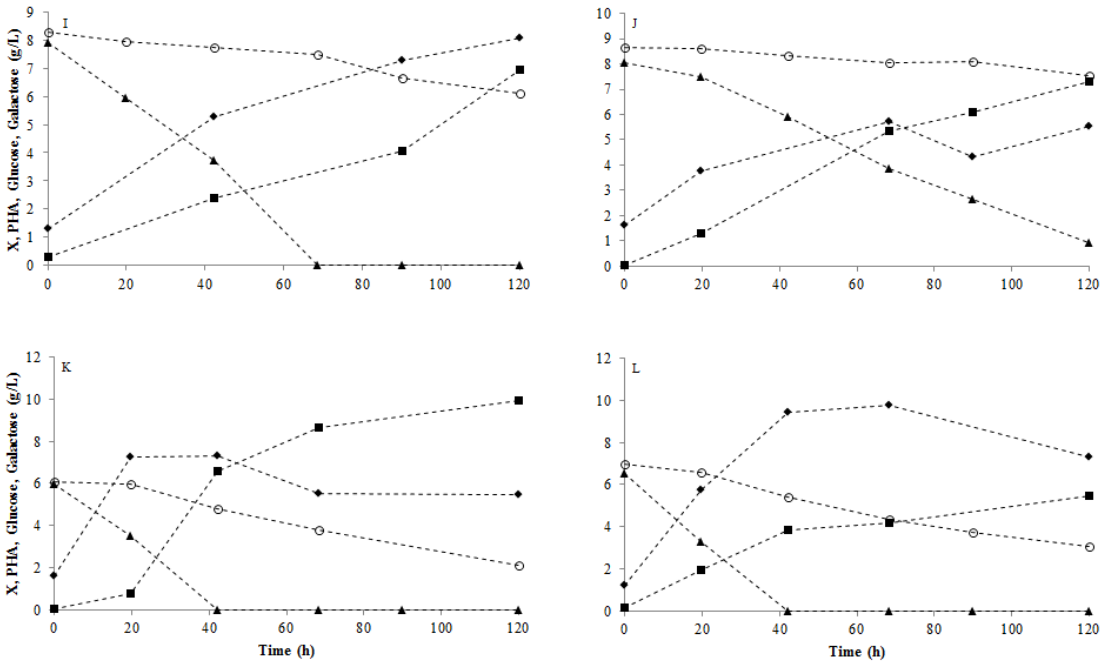


Figure 5.4: Profiles of active biomass (◆), PHA (■), glucose (▲), and galactose (○) concentration obtained in experiments with different media composition (I - HS medium+10 mL MS; J - HS medium+10 mL MS supplemented with hydrolyzed whey protein; K - HS medium+10 mL MS supplemented with hydrolyzed crude whey; L - HS medium+10 mL MS supplemented with hydrolyzed autoclaved whey).

The use of hydrolyzed crude cheese whey (assay K) led to an overall decrease in the production of active biomass (3.84 g/L) and an increase in PHA production (9.92 g/L), due to the higher PHA content in the biomass (65%), comparing with experiments I and L, where the substrate were commercial glucose/galactose and hydrolyzed autoclaved whey, respectively (Table 5.1).

Hydrolyzed autoclaved cheese whey (assay L), which contained a lower protein content (8.89 g/L), resulted in a higher biomass production (6.04 g/L), but lower PHA production (5.45 g/L) (Table 5.1). In this assay, thought the high galactose consumption observed (3.90 g/L), it did not revert into the PHA production. Given that medium broth viscosity increases during the experiment, it can be hypothesize that an extracellular polysaccharide might be formed from galactose, as already reported by Antón et al. (1988). Both, the biomass and PHA yields on sugar were lower (0.58 g_X/g_{sugar} and 0.52 g_{PHA}/g_{sugar} respectively) than in assay I (0.67 g_X/g_{sugar} and 0.66 g_{PHA}/g_{sugar} respectively). Considering the obtained values of yields and productivity for assays K and L, hydrolyzed crude cheese whey seems to be a better carbon source than hydrolyzed autoclaved cheese whey: 0.08 g/L.h *vs* 0.05g/L.h, respectively.

As it was observed in the first set of experiments (A-D), in assays I, J, K and L the PHA production was concomitant with the glucose consumption. Furthermore, a higher concentration of biomass was achieved when hydrolyzed autoclaved whey was used, which might be due to the less polymer produced: indeed, in assay L (hydrolyzed autoclaved whey as substrate) the PHA accumulated was 43% of DCW, while using hydrolyzed crude whey this value was 65% of DCW. The monomeric composition of the biopolymer obtained with hydrolyzed crude whey and hydrolyzed autoclaved was different: in assay K contained 13% (molar) of HV while in assay L contained 17% (molar) of HV.

The HS medium supplemented with 10 ml of MS and using hydrolyzed crude cheese whey as carbon source showed to be the best medium for P(3HB-co-3HV) production, considering that it achieved the highest volumetric productivity (1.97 g/L.day) and the highest polymer content (65%) of this study.

Experiments H and I were replicas, except for the fact that the inoculum of the later was prepared using HS medium supplemented with 10 mL of MS. This may explain the higher cell growth and PHA accumulation observed in experiment I. Indeed, in that assay the culture continued to grow and synthesize PHA until the end of the run (Figure 5.4-A), resulting in higher biomass and PHA production, as well as in higher volumetric productivity (1.33 g/L.day) (Table 5.1). Moreover, the HV content of the polymer was considerably reduced (23% molar) when compared to experiment H (56% molar).

The addition of hydrolyzed whey protein in experiment J did not contribute to improve growth (lower active biomass was achieved: 3.90 g/L), while PHA production and volumetric

productivity were increased (Table 5.1). The yield of biomass per sugar was higher in assay I ($0.67 g_X/g_{sugar}$) than in assay J ($0.48\ g_X/g_{sugar}$). On the contrary, the yield of PHA per sugars was higher in assay J ($0.89\ g_{PHA}/g_{sugar}$) than in assay I ($0.66\ g_{PHA}/g_{sugar}$). On the other hand, the HV content was only 13% (molar).

5.4.4. PHA production by *H. mediterranei* with cheese whey hydrolyzate in bioreactor

5.4.4.1. Bioreactor assay

Cultivation of *H. mediterranei* in a bioreactor was performed using hydrolyzed crude cheese whey supplemented with 10 mL MS. The biomass, sugars, polymer and supernatant protein concentration profiles during the bioreactor operation were represented in Figure 5.5. The parameters obtained with this assay were presented in Table 5.2.

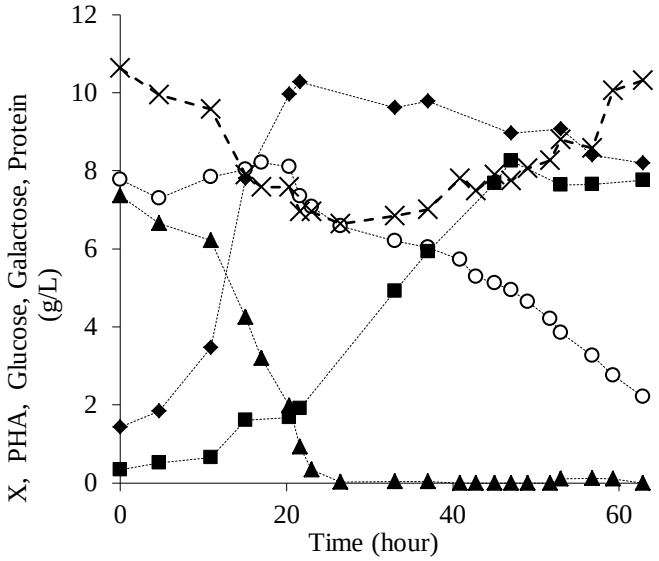


Figure 5.5: Active biomass (◆), PHA (■), glucose (▲), galactose (○) and supernatant protein (×) during the bioreactor operation using HS medium supplemented with 10 mL of MS and hydrolyzed crude whey.

In this assay, no lag phase was observed (Figure 5.5) and the culture grew at a specific growth rate of 0.11 h^{-1} (Table 5.2). Growth stopped after 22 hours of cultivation, and the active biomass produced was 8.4 g/L. Growth rate was similar (0.11 h^{-1}) to that obtained by Koller et al. (2006 and 2007a) for *H. mediterranei* cultivation on similar medium, 0.10 h^{-1}, the active biomass was doubled (4.6 g/L) (Koller et al. 2007a).

Glucose was the preferred substrate, being consumed mainly for growth. When the glucose was almost depleted (1.68 g/L) and the growth was ceased, galactose started to be consumed. This behaviour agrees with what was reported by Koller and co-workers (2007a).

The total amount of galactose consumed was 5.71 g/L. The amount of PHA produced, until glucose was depleted, was 1.57 g/L. During galactose consumption, 6.34 g/L of PHA were produced. Glucose was consumed faster than galactose, with specific consumption rates of 0.05 h^{-1} and 0.02 h^{-1}, respectively.

After growth stopped and all glucose was depleted, $\%pO_2$ slightly increased from 21.58% to 30.8%. This could be interpreted as a signal of glucose depletion and switch to galactose consumption. Furthermore, until this moment, pH which was controlled with NaOH, from this moment it begun to be controlled by HCl addition. This sudden increasing of alkalinity happened at the same time that glucose was depleted, thus, it could be associated to the end of glucose catabolism. These changes on pH can be used to design and implement feeding control strategies, with benefits in bioreactor operation and optimization.

The P(3HB-co-3HV) production showed to be partially growth associated, reaching a higher production rate after growth have stopped. Indeed, the P(3HB-co-3HV) production rate was significantly higher during the stationary phase (0.25 g/L.h) than during the growth phase (0.08 g/L.h). Polymer production stopped after 47 hours of cultivation, achieving 7.91 g/L and a PHA cell content of 53 % of DCW. However, galactose still continued to be consumed, which could be associated to the synthesis of an extracellular proteic material since the amount of protein in the supernatant continued to increase until the end of the experiment (Figure 5.5). Contrary to what was observed in the assays in shake flask, a low amount of HV content 1.5% (molar) was produced. Furthermore, in bioreactor a high amount of biomass was produced and a higher viscosity was observed, comparing with shake flask. The galactose may have been also consumed for exopolysaccharide instead PHV, that was very lower comparing with the values obtained in shake flask: %HV in the polymer was situated between 5 and 56% (Table 5.1), in the bioreactor assay the maxim %HV was only 1.5%.

The yield of polymer production was $0.61 g_{PHA}/g_{sugar}$, which is the double of that (0.33 $g_{PHA}/g_{sugar)}$ obtained by Koller and co-workers (2007a). Furthermore, the amount of P(3HB-co-3HV) content (53% molar) was lower than that reported by Koller et al. (2007a) (72.8% molar), while the volumetric productivity was almost the double, 4.04 g/L.day and 2.64 g/L.day, respectively. Koller et al. (2007a) used as cheese whey hydrolysed by enzymatic method, previously ultrafiltrated for protein removal, while in the present work, the chemical hydrolyzed cheese whey still contains peptides and aminoacids. The latter could have been used as substrates and thus contributed to the higher value of productivity.

The P(3HB-co-3HV) content is associated with the experiment's duration and with the amount of carbon source made available to the culture. This study was performed during 64 h, while Koller study (Koller et al., 2007b) lasted for 120 h. While Koller et al. (2007a) used 10 g/L of sugars added in several pulses, in this work 8 g/L were spiked in one pulse and galactose

was not totally consumed. Assuming that in the Koller work all the sugars were consumed, the lower P(3HB-co-3HV) content obtained in our study may be justified by the lower availability of carbon source.

5.4.4.2. Polymer Characterization

The polymer was extracted from the biomass by osmotic chock using distilled water. The polymer obtained presented a molecular weight of 4.4×10^5 with a polydispersity of 1.5 and a crystallinity of 35.45%. For the same strain, the molecular weight was similar to that presented by Don et al. (2006) and Koller et al. (2007b) (5.7×10^5 and 9.9×10^5, respectively), that also used hydrolyzed cheese whey as carbon source. Koller et al. (2007b) extracted and purified the polymer with chloroform.

The lower crystallinity, comparing to the value reported by Verlinden et al. (2007) (60%), could indicate a better processability of PHA, once the polymeric chain is composed with two different monomers (HB and HV), what confers a lower rigidity of polymeric structure. On the other hand, and taking into account that the HV content on the polymer obtained in the bioreactor assay was very low, the low crystallinity could be associated to the low purity of the biopolymer extracted by osmotic pressure using distilled water.

Contrary to what was reported in literature, only one melting endotherm was observed (151.1 ºC), near the values reported by Koller et al. (2007b). However, Koller et al. (2007b) observed two melting temperatures (150.8 and 158.9 ºC), which was probably associated with HV distribution in blocks inside P(3HB) matrix or with the presence of P(3HB) and PHV blends. In this study, only one melting temperature was detected, probably, due the fact that the HV monomers production was very low (1.5%).

5.5. Conclusions

The medium HS supplementation with MS had improved the P(3HB-co-3HV) production. The use of chemical hydrolyzed cheese whey and HS medium supplemented with MS allowed to improve the P(3HB-co-3HV) production and productivity relatively to Koller´s work, where it cheese whey was also used. The yield of polymer production was $0.61 g_{PHA}/g_{sugar}$, which is the double of that obtained by Koller and co-workers (2007a) ($0.33\ g_{PHA}/g_{sugar}$ of yield of P(3HB-co-3HV)) and the volumetric productivity was almost the double, 4.04 g/L.day and 2.64 g/L.day, respectively.

The polymer obtained (P(3HB-co-3HV)) was easily extracted by osmotic chock and presented a molecular weight of 4.4×10^5 with a polydispersity of 1.5 and a crystallinity of 35.45%.

The results indicate that HS medium supplemented with 10 mL of MS and hydrolyzed crude cheese whey was the best medium to the conversion of whey lactose into P(3HB-co-3HV) by *Haloferax mediterranei*. It was also showed that chemical hydrolysis of whey can substitute the expensive enzymatic hydrolysis.

Chapter 6

6. Conclusions and Future work

6.1. Conclusions

The objective of this work was the use of a by-product of cheese industry, cheese whey, as carbon substrate, in order to reduce the global costs of PHA production. Due to these high costs PHAs are not economically attractive, to compete with oil-based plastics. In order to accomplish this aim, bacterial strains were used to produce PHAs from cheese whey, a new recombinant *Escherichia coli*, harboring *Cupriavidus necator* P(3HB) synthesis genes able to use sweet cheese whey, with low salt content, and *Haloferax mediterranei* able to use salty whey, to produce P(3HB-co-3HV).

Five new recombinant *E. coli* strains were tested and strain CML3-1 was selected due to its better performance in terms of biomass and P(3HB) production (1.92 and 2.14 g/L of biomass and P(3HB), respectively, in shake flask assays). Then different media were tested (LB and MR) supplemented with lactose or cheese whey. MR medium showed to be a better medium than LB for the recombinant strain selected, since the results obtained showed that growth seemed to be limited with LB medium and it was not possible to achieve high cell density. However, in both media organic acids were produced as a result of carbon source deviation decreasing the storage yield.

The previous experiments were performed without oxygen limitation, being the dissolved oxygen concentration maintained at 60%. In order to increase the P(3HB) production, an oxygen limitation strategy (30%) was implemented and the same pulse feeding strategy was used. The reduction of pO_2 improved P(3HB) production: at 60%, only 10.72 g/L of P(3HB) were produced, while at 30% more than the double of P(3HB) was attained 28.68 g/L. However, with this strategy, OA production also increased.

In order to decrease the amount of OA produced, three fed-batch strategies were tested: a continuous feeding mode, during which the lactose concentration was maintained above 20 g/L and pulse-wise feeding, keeping the lactose concentration either between 0 and 10 g/L or between 10 and 20 g/L. Despite, the feeding in continuous mode had contributed for a higher P(3HB) production (38.55 g/L), it also contributed for a very high level of OA concentration (115.76 g/L). The pulse-wise feeding, maintaining the lactose concentration between 10 and 20 g/L, contributed for a lower OA production. However, P(3HB) production was also lowered to 25.56 g/L.

Based on the experimental results, the continuous feeding mode seemed to be the best way to achieve high P(3HB) production, however, it was mandatory to decrease de OA production in order to drive more lactose for biopolymer production.

To block or to inhibit the metabolic routes of OA production could be the strategy to increase the P(3HB) production. One way to decrease de OA production, without to knock out the responsible genes was to obtain an *E. coli* mutant strain with a reduced OA-producing capacity, aiming at driving bacterial metabolism towards PHAs synthesis. Thus, the proton suicide method was applied and it successfully generated a mutant *E. coli* strain, named P8-X8, with reduced OA production, concomitant with a higher P(3HB) accumulating capacity, comparing to the original recombinant strain CML3-1. Strain P8-X8 reached a final OA production of 3.52 g/L, which was four times lower than the concentration produced by CML3-1 (13.14 g/L). Apparently, by inhibiting OA formation, strain P8-X8 had more carbon available for P(3HB) production, which is supported by the higher $Y_{P(3HB)/lac}$ of P8-X8, compared with CML3-1 (0.28 and 0.10 $Cmol_{P(3HB)}/Cmol_{lac}$, respectively).

Haloferax mediterranei was used in highly saline medium for the conversion of chemically hydrolyzed cheese whey into polyhydroxyalkanoates (PHAs). This strain produced P(3HB-co-3HV) from salty cheese whey. Medium optimization studies revealed that medium HS supplemented with MS improved the co-polymer production. To convert whey lactose into glucose and galactose, chemical hydrolysis with HCl 1 M during 90 minutes was used. Furthermore, HS medium supplemented with MS solution and hydrolyzed crude whey led to an improvement of P(3HB-co-3HV) production relatively to the work already developed: the yield of polymer production was $0.61 g_{PHA}/g_{sugar}$, which was the double of that obtained by Koller and co-workers (2007a) (0.33 g_{PHA}/g_{sugar} of yield of P(3HB-co-3HV)) and the volumetric productivity was almost the double, 0.17 g/L.h and 0.09 g/L.h, respectively. The polymer obtained P(3HB-co-3HV) was easily extracted by osmotic chock and presented a molecular weight of 4.4×10^5 with a polydispersity of 1.5 and a crystallinity of 35.45%.

6.2. Future work

Optimization of P(3HB) production by P8-X8 *E. coli* mutant strain and optimization of P(3HB-co-3HV) production by *H. mediterrane* deserves further work.

To optimize the P(3HB) production, the oxygen limitation should be studied. With OA metabolic routes inhibited, a stronger oxygen limitation could be applied, in order to achieve a higher P(3HB) production. Reducing the dissolved oxygen concentration (below 30%) might contribute for improvement of polymer production. The starting time to impose oxygen limitation should also be studied, once it is determinant to achieve high polymer production.

An automatic continuous feeding strategy may be developed, based online acquisition of culture optical density.

The effect of ammonia limitation should also be studied since it is usually used for induction of P(3HB) production. The impact of nitrogen concentration, as well as feeding strategy, on PHA production should be evaluated. Furthermore, the use of different nitrogen sources as a way to decrease the operating cost should also be studied.

The use of high efficiency reactor configurations, such as cell recycle membrane system, should be implemented as a strategy to increase the PHA productivity. This strategy was evaluated by Ahn et al. (2001) to solve the problems associated with the volumetric limitation of bioreactor caused by the low amount of lactose in the feeding solution.

The downstream process may also be improved. Some adjustments should be made in genetic modification of *E. coli*, in order to allow the cells disruption, to facilitate the polymer extraction. As reported by Martinez et al. (2011), the autolytic cell disruption system is based on two simultaneous strategies: the coordinated action of two proteins from pneumococcai bacteriophage Ej-1, an endolysin and holin, and the mutation of the tolB gene, which exhibits alterations in outer membrane integrity that induce lysis hypersensitivity. On the other hand, new green solvents could be used to extract and to purify the polymer.

Production of P(3HB-co-3HV) by *H. mediterranei* using different glucose and galactose concentrations may be tested in order to achieve higher growth and storage performances. Also, different feeding strategies, namely pulse wise feeding or continuous mode, maintaining the optimal sugars concentration, should be tested.

The medium salinity is an important factor for this strain, thus its impact, on growth of *H. mediterranei* and PHA production, should be studied. D'Souza et al. (1997) reported that the Haloarchaea requires 200–250 g/L NaCl for optimal growth. Based on this information it will be possible to supplement the medium broth with a concentrated saline solution. According to Huang et al. (2006) the conductivity should have been maintained within the range of 3–4 S/m, which represents approximately the total salt mixture concentration of 173–230 g/L. Automatic control of salts concentration during the bioreactor operation, may be achieved using a conductivity sensor.

Being the production of biopolymers a green and sustainable process, the medium broth with high salinity has to be recycled. The recycling of the highly saline side streams has to be tested and optimized. Additionally, high salinity imposes special specifications on the bioreactor material, equipment and the probes. A novel corrosion-resistant bioreactor composed of polyetherether ketone, tech glass and silicium nitrite ceramics was constructed and applied for the cultivation of halophilic archaea. (Hezayen et al., 2000) A similar bioreactor should have be constructed to optimize the PHA production and to scale up the process.

The polymer extraction has to be optimized in order to achieve a higher polymer purity and yield of extraction. A high amount of water used for the osmotic shock is needed, so it is important to reuse this water in order to maintain the process sustainability.

The production of an exopolysaccharide (EPS), which it was released into the medium, causing an increase of viscosity, is a disadvantage in using *H. mediterranei* for PHA production. The metabolic routes responsible for the EPS formation should have to be study, as well, the responsible genes for this production. Knowing these metabolic routes and the genes involved, it could be possible to inhibit or block EPS formation, being possible to obtain a higher P(3HB-co-3HV) production.

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