Universidade de Aveiro Departamento de Química
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PRODUÇÃO DE PHB POR *ESCHERICHIA COLI*

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PRODUCTION OF PHB BY *ESCHERICHIA COLI*

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Especialização em Biotecnologia Industrial e Ambiental, realizada sob a orientação científica da Doutora Luísa Seuanes Serafim, Professora Auxiliar Convidada do Departamento de Química da Universidade de Aveiro e da Doutora Gen Larsson, Chefe da Divisão de Tecnologia de Bioprocesso, da Escola de Biotecnologia da KTH-Suécia.

Dedico este trabalho aos meus pais, irmãs e namorada pelo incansável apoio e paciência.

o júri
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Palavras-chave **Plásticos biodegradáveis, Polihidroxibutirato; *Escherichia colii* recombinante*;* genes phb; limitação de nutrientes**

Resumo

Para a comercialização de polihidroxialcanoatos (PHA), polímeros biossintéticos e biodegradáveis, têm sido feitos esforços consideráveis para reduzir os custos de produção, através do desenvolvimento de estirpes bacterianas e processos de fermentação e de recuperação mais eficientes e ainda da utilização de fontes de carbono baratas. Desde a descoberta dos genes responsáveis pela via metabólica dos PHA, muitos foram recolhidos a partir de uma vasta multiplicidade de organismos e clonados em *Escherichia coli*. A ampla variedade de microrganismos produtores de PHA corresponde a um vasto leque de condições intracelulares às quais as várias enzimas têm de se adaptar de modo a optimizar o processo. O presente trabalho teve como objectivo estudar a produção de polihidroxibutirato (PHB) a partir de *Escherichia coli* AF1000 transformada com o plasmídeo pCJY02 que contém, os genes phbA e phbB de *Cupriavidus necator* e o gene *phaC* de *Pseudomonas stutzeri* 1317.

A quantidade máxima de PHB obtida foi de 0,917 $g.L^{-1}$, o conteúdo de PHB intracelular foi de 3,65 %, a biomassa activa de 23,0 $g.L^{-1}$ e a taxa de formação de produto de 0,489 $g.g^{-1}.h^{-1}$, após cultivo em fed-batch.

Keywords **Biodegradable plastics; Polyhydroxybutyrate; recombinant *Escherichia coli*; *phb genes*; Nutrient Limitation**

Abstract

With the purpose of commercializing polyhydroxyalkanoates (PHA), a biosynthetic and biodegradable plastic, considerable effort is being dedicated to reduce the production costs through the development of bacterial strains, more efficient fermentation and recovery processes and the use of cheap carbon sources. Since the discovery of *phb* genes, many genes encoding enzymes from the PHA pathway have been cloned from different organisms to hosts like *Escherichia coli*. The broad variety of PHA-producing microbes represent a vast spectrum of different intracellular conditions to which the enzymes should be adapted in order to optimize the production of PHB. The present work is within this purpose and its goal was to study the polyhydroxybutyrate (PHB) production, by *Escherichia coli* AF1000 strain transformed with plasmid pCJY02 harboring, *phbA*, *phbB* genes from *Cupriavidus necator* and *phaC* gene from *Pseudomonas stutzeri* 1317, cultivated in minimal medium supplemented with glucose.

The maximum amount of PHB obtained was 0.917 $g.L^{-1}$, PHB content of 3.65 %, active biomass 23.0 $g.L^{-1}$ and specific production rate of 0.489 $g.g^{-1}.h^{-1}$, after test in a fed-batch reactor

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Abbreviations

CDW	Cellular dry weight
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
E.coli	*Escherichia coli*
GAO	Glycogen-accumulating organisms
Gln482	Glutamyl residue number 482
LPDE	Low-density polyethylene
MMCs	Microbial mixed cultures
Mcl-PHAs	Medium-chain-length polyhydroxyalkanoates
mRNA	Messenger ribonucleic acid
NPCM	Non-PHA cell mass
OD	Optical density
PAO	Polyphosphate accumulating organisms
PHAs	Polyhydroxyalkanoates
PHB	Poly-3-hydroxybutyrate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHB4B	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
RNase	Ribonuclease
Scl-PHAs	Short-chain-length polyhydroxyalkanoates

Ser326	Serine residue number 326
TCA	Tricarboxylic acid cycle
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
UV	Ultra Violet
3HB	3- hydroxybutyrate
3HD	3-hydroxydecanoate
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate

1. Introduction

1.1 Background

Industrial polymers, which comprise textiles, gums, films, adhesives and coatings are constitutes of many daily use articles. These polymers are from petrochemical origin being produced in very large amounts with low production costs. Many environmental problems come from the fact that these materials are utilized only during a relatively short time span and in large amount. The majority of these highly resistant plastics are incinerated, contributing to global warming effects, or recycled, which demands a certain degree of purity of the plastics to be reutilized [1]. The rising of crude oil price and the eventual reduction of fossil fuel feedstock's, also contributes for the desire of finding new production techniques based on renewable resources and inserted into natures closed cycles [2].

Biotechnology has the potential to overcome these problems shifting the current dependence of non-renewable resources to a biomass based raw materials for the production of goods and services. Biorefinary rises as a future alternative towards more sustainable, economic and environmental industrial processes [3]. Through the conversion of biomolecules from biomass, using microorganisms as the main workers, bio-energy and bio-based materials can be obtained, following a similar concept as in the petroleum refineries [3, 4]. Among the bio-based materials, bio-polymers have growing trend due to their biosynthetic, biocompatible and biodegradable properties. Worldwide the consumption of biodegradable polymers was estimated to grow from 180 kt to 1710 kt between 2008 and 2015 [5].

Biodegradable plastics can be divided into 3 categories: chemically synthesized polymers, starch-based biodegradable plastics and polyhydroxyalkanoates (PHAs). In the case of the first one besides being susceptible to enzymatic attack, they did not match all the properties of the plastic, not being commercially available as plastic substitute. Starch-based biodegradable plastics are only partially degradable, the fragments left remain in the environment for long time [6].

The third group PHA known since 1920's, exist as naturally occurring, biodegradable biocompatible, thermoplastic polyesters, made by a wide range of microorganisms including Gram positive and Gram negative species, as also plants [2, 6].

These polymers are accumulated as granules inside cells, when nutrient supplies are imbalanced, normally due to oxygen or nitrogen limitation when an excess of carbon source is present. Accumulation of PHA does not affect the osmotic state because the soluble intermediates are polymerized into insoluble molecules, keeping the nutrients available at a relatively low maintenance cost [7]. Depending on the organism and on the physiological conditions applied to the cells, besides functioning as a carbon storage compound it can also function as energy storage compound or as a sink for reducing equivalents [8].

Combining the biological polymerization system and different monomers that can be incorporated into PHAs, new high-molecular weight materials can be produced making available new potential polymers. Once extracted from the cells, PHA molecules can be formed into films, fibers and sheets, molded into shapes and bottles, showing material properties similar to some common plastics such as polypropylene [7].

Being of natural origin, PHA can be attacked by PHA hydrolases and depolymerases secreted by a vast range of microorganisms [7]. Their activity depends on the composition, physical form and dimensions of the polymers as also the environmental conditions. PHA can be degraded into water, carbon dioxide and basic materials for photosynthetic regeneration of carbohydrates by plants, as the final products, attesting that PHAs are part of natural closed cycle of carbon, by contrast with petrol-based plastics [2].

The most studied PHA, poly-3-hydroxybutyrate (PHB) is already produced and commercialized by several companies, BIOPOL® by Metabolix [9]; Nodax™ developed by The Procter & Gamble Company; Biomer, a German company[10]; Degrapol® caprolactone [11]; Tianjin Green Bioscience, China sector [10]. Besides the reduction on prices from 12.5 US$ - 25 US$ per kg in 2003 to 2.75 US$ - 6.25 US$ in 2010 [12], is still more costly than for example polypropylene $0.185/kg [13].

The high production costs are mainly due to substrate costs and recovery methods [14, 15]. The productivity is also an important factor but it has a smaller effect on the price when compared with substrate or recovery [13, 16].

The most important factor in PHA production process is the organism selected to accomplish the task. In a biorefinery process the suitable microorganism has a cluster of characteristics that are requirements for an efficient process: ability to simultaneously uptake mixed sugars; quick growth to high cell densities; minimal or no by-product accumulation; and, most important, yield high levels of interesting product. However, to find in nature a wild type strain that fulfills all these requirements is quite rare. Therefore efforts have been directed to the development of tailor made industrial microorganisms suiting specific industrial requirements, through recombinant DNA technology, metabolic engineering and other bioengineering methods [17].

Many authors present a vast range of different microorganism from different species that are capable to naturally produce PHA [6, 18]. However Natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time and relatively low optimal growth temperature, are often hard to lyse and contain pathways for PHA degradation [14, 19]. *Escherichia coli,* even though is not a native producer of PHAs, presents a group of characteristics that make this specie one of the most suitable hosts for PHA production. Besides being the best known bacteria, it has proven to be suitable as a heterologous expression host for foreign genes that can be easily manipulated and improved (metabolically engineer strains). Moreover high-cell-density cultivation strategies for numerous *E. coli* strains are well established. Finally, *E. coli*, like any non-native PHA production host, also does not have any PHA regulatory systems in place or enzymes that degrade PHAs [6, 20].

To be economically feasible, a biotechnological PHA production system using *E.coli,* requires the transfer of PHA synthase structural genes, expression of an enzymatically active PHA synthase protein, and engineered pathways that can provide this key enzyme with suitable substrates at sufficient concentrations. Since the discovery of *phb* genes encoding the enzymes needed for the PHA pathway (*phbA* - biosynthetic beta-Ketoacyl-CoA thiolase; *phbB* - NADPH dependent Acetoacetyl-CoA reductase;

phbC - PHB polymerase), they have been cloned from and to different organisms. The broad variety of PHA-producing microbes would represent a vast spectrum of intracellular conditions to which the enzymes responsible for the PHA pathway would have to be adapted [3, 14, 19].

1.2 Objective of this work

The objective of this work consisted in the study and maximization of the production of poly-3-hydroxybutyrate (PHB) using a *Escherichia coli* AF1000, transformed with plasmid pCJY02 harboring *phbA* and *phbB* genes from *Cupriavidus necator* and *phaC* gene from *Pseudomonas stutzeri* 1317 in minimal medium supplemented with glucose.

2 State of the art

2.1 Chemical structure and physical properties

The diversity of PHA polymer family comes from variations in the composition and length of the side chains, as their ability to change reactive substitutes. The overall structure is formed by primarily linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers. The carboxyl group of one monomer forms an ester bond with the hydroxyl group the neighbor monomer (Figure 1) [21].

PHA can be divided in two different classes, short-chain-length PHAs (scl-PHAs) and medium-chain-length PHAs (mcl-PHAs). Scl-PHAs can be originated by simple sugars or volatile fatty acids or co-feeding of both types of substrates, such as glucose and valerate. The co-feeding may result in the formation of copolymers containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers along with 3-hydroxybutyrate (3HB). Mcl-PHAs are composed of C6 to C16 3-hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically, the composition of the resulting PHA depends on the growth substrate used [21, 22]. Most of microbes synthesize either scl-PHAs containing primarily 3HB units or mcl-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major co-monomers [22]. Poly(3-hydroxybutyrate), P(3HB), is the most common type of PHA and extensively studied, triggering the commercial interest in this class of polymers, not only because of its physical properties but also due to possible use of agricultural waste products as carbon sources for fermentative production [21, 22].

Random copolymers of PHB can be formed when mixed substrates are used, such as a mix of glucose and valerate. When substrates are alternated overtime, it is possible to obtain PHA block copolymers synthesized by bacteria, examples are poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) or poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (PHB4B) [21].

The type and composition of the monomers determines physical properties of PHAs. In contrast to scl-PHAs, mcl-PHAs have much lower crystallinity and higher elasticity, providing their applications as biodegradable rubber and elasteomer. Random

copolymers of 3HB and little amount of MCL monomers are found to be flexible and tough materials comparable to low-density polyethylene (LPDE), which are suitable for commercial applications. The material properties of PHAs consisting of different monomers are summarized in table 1 [21, 23, 24].

Table 1 - Properties of PHAs and polypropylene (PP) (Tsuge 2002)

Parameter	**PHB**	**PHBV**	**PHB4B**	**PHBHx**	**PP**
Melting temperature (oC)	177	145	150	127	176
Glass Transition temperature (oC)	2	-1	-7	-1	-10
Crystallinity (%)	60	56	45	34	50-70
Tensile strength (MPa)	43	20	26	21	38
Extension to break (%)	5	50	444	400	400

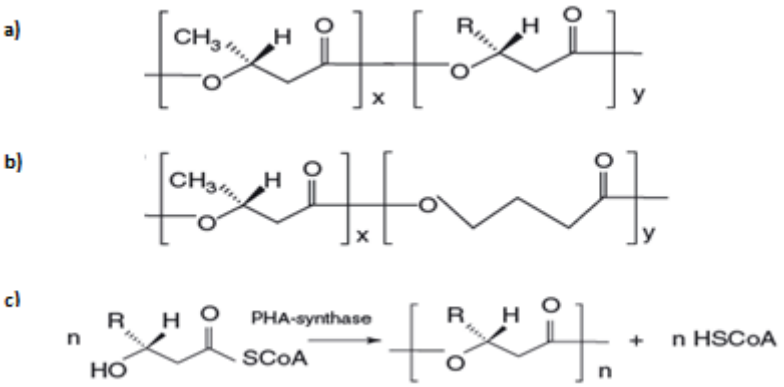

Figure1-(a) Polyhydroxybutyrate copolymers; (b) Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (PHB4B); (c) Synthesis of PHAs in bacteria using hydroxyacyl-CoA thioesters as precursor. (Verlinden et al, 2007)

2.2 Biosynthetic pathways

In the bacterial cell, carbon substrates are metabolized through many different pathways. The three most-studied PHA metabolic pathways are shown in Figure 2 [6]. Sugars such as glucose and fructose are mostly processed via pathway I, yielding PHB homopolymer. If fatty acids or sugars are metabolized by pathway II, III or other pathways, copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or poly(3-hydroxybutyrate-co-3-hydroxypropionate) are produced [14, 19, 21]. Under PHB-accumulating conditions in *E.coli*, when glucose is the carbon source, Entner-Duodoroff pathway (Figure 3) is the main metabolic pathway active, because it allows the production of one mole of NADPH, NADH, and ATP from one mole of glucose. Other possible pathways are Embden–Myerhoff pathway and pentose phosphate pathway. In the case of the first one, only NADH and ATP is produced lacking the formation of NADPH, which is required for P(3HB) synthesis; the second case, besides generation of the NADPH, is mainly used for nucleic acids and amino acids production [25, 26].

The first enzyme involved in PHB formation is encoded by the *phbA* gene and it is a biosynthetic beta-Ketoacyl-CoA thiolase, member of a family of enzymes involved in the thiolytic cleavage of substrates into acyl-CoA and acetyl-CoA. This enzyme in particular, has a specific action range of chain length, from C3 to C5 and is specific for acetoacetyl-CoA [22], and is also responsible for the condensation of two acetyl-CoA molecules, producing acetoacetyl-CoA [19, 27]. The *phbB* gene, encode a NADPH dependent Acetoacetyl-CoA reductase and catalyzes the second step in the PHB pathway, converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The reactions catalyzed by thiolase and reductase provide the monomer for head-to-tail polymerization of the monomer to PHB, catalyzed by PHB polymerase encoded by *phbC* gene [6, 28].Acetyl-CoA is necessary for other inherent metabolic pathways (TCA cycle, Fatty acid biosynthesis, amino acid synthesis), the decreasing in its availability is prejudicial for PHB biosynthesis and this competition leads to a necessary adjustment of the cellular metabolic network [19, 23, 29].

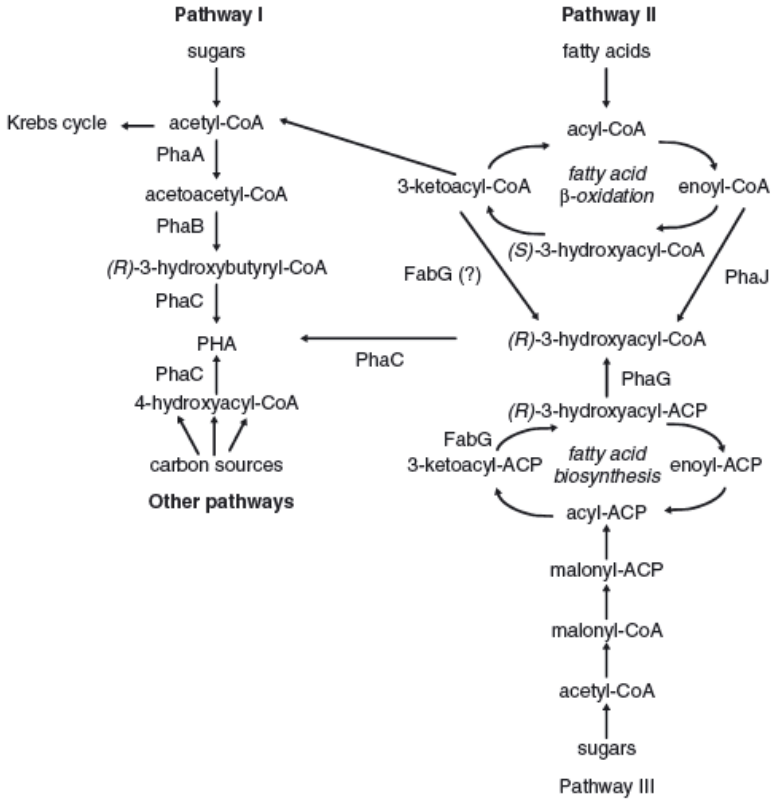

Figure 2 - Metabolic pathways supplying monomers for PHA synthesis (Tsuge et al, 2002).

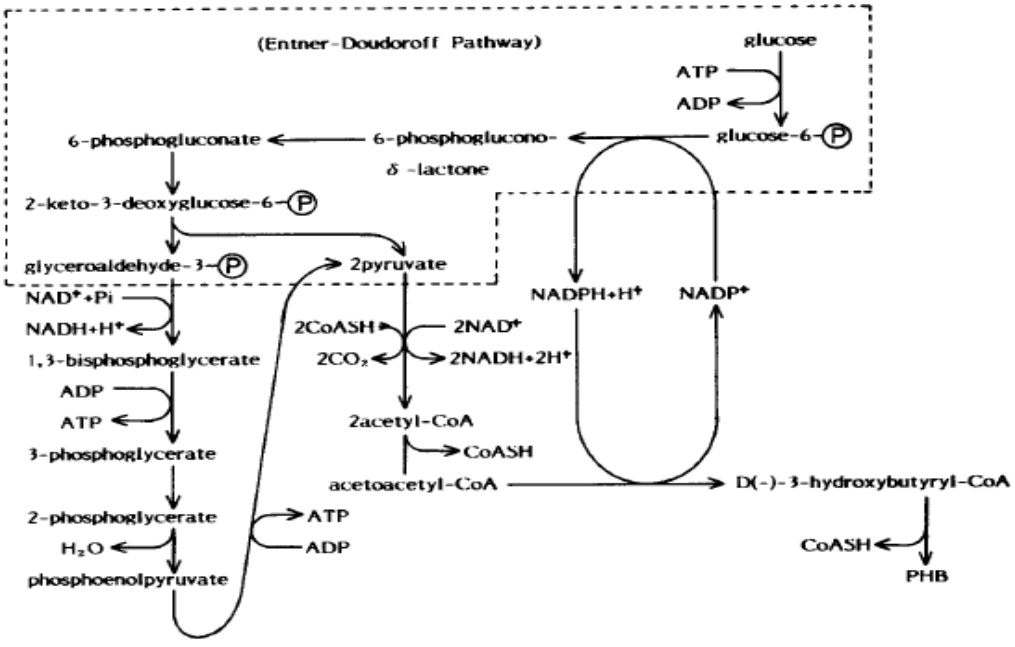

Figure 3 - Entner-Duodoroff pathway. (Yamane, 1993)

Other important factor is the presence of Acetyl-CoA the main precursor for PHB production, therefore the carbon substrate used will determine which pathway will originate the precursor and how much is available for PHA production. Since acetyl-CoA is necessary for other inherent metabolic pathways (TCA cycle, Fatty acid biosynthesis, amino acid synthesis), high amounts have to be present in order to fulfill all metabolic needs of the cell, therefore an excess of carbon source is required [19, 23, 29]. In the case that glucose is the main carbon source, high amounts, will lead to the excretion of partially oxidized metabolites mostly in the form of acetic acid. This phenomenon is called overflow metabolism and occurs if the glucose concentration exceeds a critical value (20-30 $mg.L^{-1}$) [30, 31]. It is known that a high concentration of acetate reduces growth rate, biomass yield and maximum attainable cell densities in high cell density cultures, having more detrimental effect on recombinant cells than on non-recombinant cells, with significant reduction in recombinant protein production [12, 32, 33]. The reason for the overflow metabolism is not clear but it may be a result of an imbalance between the glycolysis and the TCA-cycle or of saturation of the TCA-cycle or the electron transport chain. The main route for acetate production is from Acetyl-CoA, generating ATP. The other route for acetate production is directly from pyruvate, but it plays a minor role [30].

2.3 PHAs-producing organisms

Many authors present a vast range of different organism from different species that are capable to produce PHA [6, 22]. However all of them have different characteristics that should be taken in consideration in the development of an industrial process for PHA production. Since the discovery of *phb* genes, many genes encoding PHA pathway enzymes have been cloned from and to different organisms.

2.3.1 Higher organisms

Efforts have been made to produce P(3HB) in plants due its great potential to produced high amounts of PHA with reasonable amount of acres occupied but only if the 30% of the seed oils were substituted by PHA [15]. An intriguing development is the

potential for transgenic P(3HB) to play a role in engineering new characteristics into existing materials such as cotton [15]. Obviously, the limits of transgenic PHA production are unpredictable. Stable expression of the *phb* genes has been achieved and the P(3HB) produced is chemically identical to the bacterial products with respect to the thermal properties and molecular weight with broader range indicated that plants can make P(3HB) of sufficient quality for industrial processing. Some examples of species that have been studied are *Arabidopsis thaliana*; *Gossypium hirsutum* (cotton); *Zea mays* (corn) [22]. The compartmentalization of plants metabolism requires *phb* genes to be targeted to the compartment of the plant cells where the concentration of acetyl-CoA is the highest but only in such a way that growth of the plant is not restricted [22]. Nawrath et al., 1994, target a specific subcellular compartment, the plastid, where biosynthesis of triglycerides from acetyl-CoA normally occurs. The amount of homopolymer stored within plastids was up to 14% of the dry mass of the plants, with few deleterious effects on the growth or fertility of the hosts. The obtained granules had similar size and appearance to those of bacterial PHA inclusions [1].

The cost of PHAs produced in plants might be lowered enough to make them competitive with conventional plastics if production on an agricultural scale is feasible. This will depend on the progress obtained with further increase of accumulation levels; the reduction of deleterious effects on crop value; induction of PHAs synthesis other than P(3HB); extraction at reasonable costs. However one of the biggest obstacle is the tendency of arable land to become a precious commodity [1].

Studies with animal cells using a baculovirus system revealed that expression of P(3HB) polymerase is possible and P(3HB) granules in the insect cells were visualized by immunofluorescence. However only 1 mg of P(3HB) was isolated from 1 liter of cells, corresponding to 0.16% of the cell dry weight [1, 34].

Low values were also obtained in yeast by expressing only part of the biosynthetic pathway, since native β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase enzymes are present. P(3HB) was accumulated to only 0.5% of the cell dry weigh probably due to insufficient activity of the endogenous enzymes [1, 34].

2.3.2 Bacteria

Natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time and relatively low optimal growth temperature, are often hard to lyse and contain pathways for PHA degradation [22].

Other microorganisms have been studied in order to overcome the unattractive characteristics of the natural producers or to discover other features that could improve the efficiency of recombinant PHA producing strains and industrial process; some examples are present in table 2.

Table 2- Examples of polyhydroxyalkanoate (PHA)-accumulating microorganisms (Koller et al, 2010).

Acidovorax	*Gloeothece*
Aeromonas	*Haloarcula*
Alcaligenes	*Hydrogenophag*
Bacillus	*Methylobacterium*
Cupriavidus	*Oscillatoria*
Comamonas	*Pseudomonas*
Erwinia	*Ralstonia*
Gloeocapsa	*Vibrio*

2.3.3 Pure and mixed bacterial cultures.

The use of pure or mixed bacterial cultures is related with process chosen for the PHA production. Pure cultures allow a better control of the process through the use metabolic engineered strains and different specific substrates in order to improve productivity or the molecular design the final product [35]. Mixed cultures often have an unknown composition and are used for the PHA production from waste materials with unknown or variable composition. During this section both processes will reviewed and explained.

2.3.3.1 Bacterial mixed-cultures

It is possible to produce PHA from waste materials recurring to open microbial mixed cultures (MMCs). MMCs are microbial populations, often with unknown compositions, selected by the operational conditions imposed on the biological system resulting on polymer accumulation not induced by nutrient limitation. This system reduces bioreactor and operation costs, including sterilization, and is suitable for the use of agro industrial wastes with unknown or variable composition [35]. MMCs allow the use of already existing wastewater treatment plants to produce PHA but require long operation periods, on the opposite of some existing processes [13, 36].

Two types of microorganisms are capable of anaerobic storage of carbon source in mixed culture, polyphosphate accumulating organisms (PAO) and the glycogen-accumulating organisms (GAO).

PAOs and GAOs proliferate in systems where the substrate is present regularly while an electron acceptor is absent. The PAOs competitive advantage is based on their capacity to utilize the energy stored as polyphosphates to store exogenous substrate in the form of PHA when there is no electron acceptor (oxygen or nitrate) available for energy generation. GAOs rely on substrates which can be fermented (e.g., glucose), and they store the fermentation products inside the cell. These organisms can also use internal stored glycogen for fermentation to PHB. The energy released in the glycolysis process is subsequently used to accumulate fermentation products (e.g., acetate) in the form of PHB [13].

Even though this process is capable of enrichment of PHA accumulating microorganisms, there is no insurance that anaerobic‐ aerobic operation of the activated sludge process is the most efficient method therefore other processes were developed in order to improve the process.

Microaerophilic‐ aerobic process is based in the addition of a limited amount of oxygen to the anaerobic zone of anaerobic‐ aerobic operation, allowing the microorganisms up take organic substrates by obtaining energy through oxidative degradation of some part of the organic substrates. If oxygen supplied is sufficient, the microorganism may be able to get enough energy for assimilative activities such as the

production of protein, glycogen and other cellular components simultaneously with taking up organic substrates. On the other hand, if the supply of oxygen is adequately controlled, the assimilative activity will be suppressed while letting the microorganism accumulate PHA. This method allows selections of PHA accumulators regardless of the ability of microorganisms to accumulate poly-P or glycogen, and the selected PHA accumulators will have a lower tendency to accumulate glycogen [13, 35].

Another used process is based in the fact that activated sludge processes are highly dynamic with respect to the feed regime. Periods of external substrate availability (feast period) and no external substrate availability (famine period) generates an unbalanced growth of the biomass and the storage phenomena usually dominates over growth because it requires less adaptation than growth [21]. However under conditions in which substrate is present continuously for a long time, physiological adaptation occurs, and growth becomes more important [13, 35].

2.3.3.2 Bacterial pure cultures

PHA has been industrially produced by pure cultures including *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans*, recombinant *Alcaligenes eutrophus* among others. With the use of pure cultures is possible to obtain different material properties of the biopolyester by fine-tuning of the composition of the PHA through the use of different substrates or using recombinant strains with specific metabolic pathways [2, 35].

Batch and fed-batch fermentations are widely used in the industrial fermentation processes. The batch technique besides being the easiest cultivation method, its industrial usefulness is limited due to oxygen limitation, formation of by products and lack of control over the cell growth [30, 37]. Fed-batch cultivation is more efficient than batch cultivation in terms of achieving high product and cell concentration because the medium composition can be controlled by substrate inhibition. Therefore, high initial concentration of substrates fed can be avoided [19, 23, 29].

The fed-batch technology is a common industrial method for recombinant protein production. This technique enables to achieve high cells concentration allowing high

PHA productivity, through control of the amount of substrate that is feed to the culture. The substrate limitation offers a tool for reaction rate control, avoiding engineering limitations with respect to cooling and oxygen transfers, also permits some metabolic control by which overflow metabolism, resulting in formation of acetic acid, can be avoided. Fed-batch cultivations may be started as batch cultures and when the initial substrate is depleted, the feed of substrate can be started. In the industry, it is common to start the feed of substrate directly after the inoculation of the reactor [30, 31].

Based on the operational conditions required PHA-producing bacteria can be classified divided in two major, the first for those requiring the limitation of an essential nutrient (nitogen, phosphorous, magnesium or sulphur) and an excess of carbon source, examples are *A. eutrophus*, *Protomonas extorquens*, and *Protomonas oleovorans*; the second for those do not require nutrient limitation and can accumulate the polymer during growth (*Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*) [6].

Fed-batch culture of bacteria belonging to the first group is based in a two-step cultivation method. A desired concentration of biomass is obtained without nutrient limitation in the first stage. In the second stage, an essential nutrient is kept in limiting concentration allowing for an efficient PHA synthesis. During this stage, the residual cell concentration (defined as the cell concentration minus the PHA concentration) remains almost constant and the cell concentration increases only because of the intracellular accumulation of PHA. For the cultivation of these bacteria, a mixture of carbon source and a nutrient to be limited should be fed at an optimal ratio to produce PHA with high productivity [6, 38].

For the fed-batch culture of bacteria belonging to the none natural producers, since PHA synthesis is not dependent on nutrient limitation in these bacteria, cell growth and PHA accumulation need to be balanced to avoid incomplete accumulation of PHA or premature termination of fermentation. Complex nitrogen sources can be supplemented to enhance cell growth as well as polymer accumulation [6, 21, 39].

2.4 PHA production in recombinant *E. coli*

Even though *Escherichia coli* is not a native producer of PHAs, this bacterium bears a group of characteristics that make one of the most suitable hosts for PHA production. Besides being the best known bacteria, it has proven to be suitable as a heterologous expression host for foreign genes, that can be easily manipulated and improved (metabolically engineer strains); high-cell-density cultivation strategies for numerous *E. coli* strains are well established; *E. coli*, like any non-native PHA production host, also does not have any PHA regulatory systems in place or enzymes that degrade PHAs [6, 20].

A biotechnological PHA production system to be economically feasible requires transfer of a PHA synthase structural gene, expression of an enzymatically active PHA synthase protein, engineered pathways that can provide this key enzyme with suitable substrates at sufficient concentrations. To reproduce this metabolic pathway in *E. coli* three key genes *phbA*, *phbB*, *phbC* which encode b-ketothiolase, acetoacetyl-CoA reductase and PHB synthase respectively, have to be functionally expressed in the host in order to produce 3-hydroxyacyl-CoA with short side chain [28].

Since the insert genes on *E.coli* are not naturally present, they put on the cell a heavy metabolic stress that leads to delay of cell growth, loss of the plasmid and consequent loss of the PHB-producing phenotype [40]. Some genes can be inactivated or stimulated in order to improve the yields and productivity. Li et al (2007), presents some examples of gene that were modified resulting in the improvement of PHB production such as: inactivation of the *pta* gene, which encodes a phosphotransacetylase, lead *E. coli* to accumulate more PHB than wild type *E. coli*; the inactivation of phosphoglucose isomerase (*pgi*) gene originate more NADPH from pentose phosphate pathway (PP), and eventually enhance the PHB production; also increasing, glucose-6-phosphate dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) would increase NADPH available for PHB production; to overcome the negative impact of filamentation during PHB production, an essential cell division protein FtsZ, could be over-expressed in recombinant *E. coli*, allowing to reach a high PHB productivity [28].

Improvement of cell respiratory capacity can beneficial for the bacterial growth and PHA production. The expression of *Vitreoscilla* globin gene (*vgb*) in *E. coli* can induce, under low DO conditions during the fermentation process, the parent promotion effect on cell growth and PHB accumulation; *arcA* mutant, which confers high respiratory capacity of the host under microaerobic conditions, also gave rise to higher polymer accumulation [6].

Typically, for PHA production in non-native PHA-producing strains like *E. coli*, genes from native PHA producers are expressed to generate PHA precursors. It was showed that is necessary to use a stable plasmid, as also a high gene dosage in order to reach high PHA concentration values in *E.coli* [41, 42]. The instability of the plasmid can be controlled by using antibiotics in the culture to select for plasmid-bearing cells, but is not totally effective and normally means a significant cost increase for the final product [41, 42]. Others frequently used methods are, regulation of the expression of genes via a heterologous promoter or, alternatively, to regulate expression by controlling gene dosage in recombinant *E. coli* [40, 43].

One other cause that could have impact on the economy of the production process is related to species-specific variations in codon usage. The presence of rare codons, which are correlated with low levels of their cognate tRNAs species in the cell, can reduce the translation rate and induce translation errors, and therefore decrease yields and productivity of the process [43]. To surpass these obstacles, one could employ two different strategies. The first, assigns the most abundant codon of the host or a set of selected genes to all instances of a given amino acid in the target sequence ("one amino acid-one codon"). The second uses translation tables, based on the frequency distribution of the codons in an entire genome or a subset of highly expressed genes, to attach weights to each codon. In this case, codons are assigned randomly with a probability given by the weights ("codon randomization") [40, 44].

PHA production can be improved through utilization of complex media over a defined media, considering the primary metabolism of the bacteria. PHB synthesis and accumulation is related with the amount of acetyl-CoA available, as already explained. This metabolite is also, necessary for the synthesis of the major intermediary metabolites such as amino acids, vitamins, and fatty acids, and other competing pathways such as, synthesis of acetate and citrate [43, 45]. For a defined medium, the amount of acetyl-CoA available for PHB synthesis would be less than in a semi defined or complex medium, due to its use for synthesize these biosynthetic intermediates and to generate energy. However the use of such media composition is indicated as the major factor for PHA higher price, when compared with conventional plastic materials, limiting their use. For that reason, much research effort is currently devoted to improving productivity from cheaper carbon [24, 36].

Table 3 - Examples of PHA production in *Escherichia coli*.

Organism	Gene origin	Biopolymer	Substrate	Cell conc. ($g.L^{-1}$)	PHB conc.($g.L^{-1}$)	PHB content (%)	Specific production rate ($g.L^{-1}.h^{-1}$)	Reference
Escherichia coli HMS174	*Cupriavidus necator*	P(3HB)	Molasses	39.5	-	80	1	[12]
Escherichia coli GCSC4401	*Alcaligenes latus*	P(3HB)	Whey (lactose)	194	-	87	4.6	[12]
Escherichia coli XL1-Blue	*Alcaligenes latus*	P(3HB)-*co*-3HV	Glucose propanoic acid; oleic acid supplementation	203.1	158.8	78.2	2.88	[12]
Escherichia coli XL1-Blue	*Cupriavidus necator, Clostridium kluyveri*	P(4HB)	Glucose, 4-hydroxy-butyrate	12.6	4.4	36	0.07	[12]
Escherichia coli RS3097	*Pseudomonas aeruginosa*	mcl-PHA	Decanoic acid	2.6	-	38	0.06	[46]
Escherichia coli XL1-Blue	*Alcaligenes euthrophus*	P(3HB)	Glucose	153.7	101.3	65.9	2.8	[47]
Escherichia coli XL1-Blue	*Alcaligenes latus*	P(3HB)	Glucose	194.1	141.6	73	4.63	[12]
Escherichia coli XL1-Blue	*Alcaligenes euthrophus*	P(3HB)	Glucose	204.3	157.1	77	3.2	[47]

2.5 Substrates for PHA production

For PHA production, the carbon source utilized and how is metabolized within the cell (specifically PHA synthase presented in the host microorganism), have to be considered since monomer precursors used for PHA accumulation can contain unsaturated monomers and a wide variety of functionalized groups in the side chain (phenoxy, epoxy, hydroxyl, halogens and methylester groups) that will influence the PHAs monomer composition, PHAs physicochemical properties of biopolymers and therefore determine the type of applications and fields of application of a particular type of polymer [3, 48]. Several cheaper carbon sources have been studied for PHA production, most of them are produced in industrial branches that are closely related to agriculture, wood-processing, paper and are available in quantities that are appropriate for industrial process demands (Table 3) (Figure 4) [2, 3, 48].

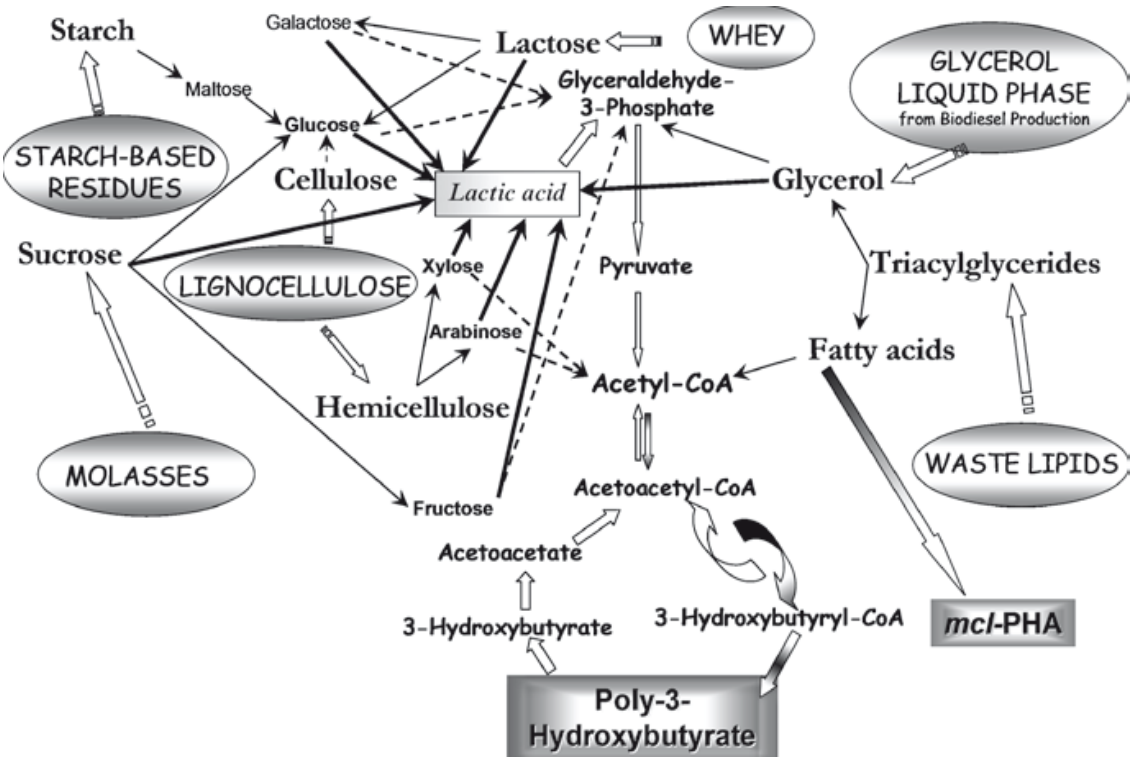

Figure 4 - The basic routes and the central metabolism for PHA production based on different substrates. (Koller, M et al, 2010)

Lignocelluloses (consisting of lignin, cellulosic and hemicellulosic fibres) and cellulosic materials are one of the most abundant waste materials to be provided as feedstock. The optimization of methods for digestion of lignocellulose and the development of effective biocatalysts for the breakdown of cellulose and hemicellulose into microbial convertible sugars (hexoses and pentoses), are the prerequisite for an efficient biotechnological conversion of these promising raw materials into desired end products [2, 5]. Other examples of cheap raw materials include molasses from the sugar production, whey and whey hydrolysates, fats, vegetables and waste cooking oils, glycerol and from wastewaters [12, 48]. However the efficiency of the fermentation of these raw materials are low and requires further developments in order to increase final PHAs concentration, productivity and lower costs.

2.6 Recovery

Recovery/purification costs correspond to a major part of the total production cost of PHA production. A few methods have been developed in order to surpass this problem, most of them fall in two categories: systems employing chemicals, or other additives, and systems employing the strains which are able to spontaneously liberate PHAs from cells [1, 21].

The extraction of PHAs with organic solvents and sodium hypochlorite were the first employed. This method is based on two strategies, PHAs solubilization and non-PHA cell mass (NPCM) dissolution. However, the application of these methods are unattractive not also because large quantities of solvent required, turning the procedure economically and environmentally feasible, but also could lead to degradation of PHB into a lower molecular weight. A non-pollution method that could be employed is the use of enzymes to digest NPCM, but the high cost of enzymes is a drawback in further development of this method [1, 21].

Spontaneous PHAs liberating systems have been developed for *E. coli* combining *phaCAB* genes and phage lysis genes to release PHB granules. The expression of lysis genes can be controlled by thermo sensitive expression systems or manipulating the initial inoculum size and the composition of the medium [28]. Several others techniques

such as, mechanical disruption, supercritical fluids, gamma radiation, flotation and aqueous two phase systems, have been studied as complement to the ones already in practice or as new methods in order to decrease costs [49].

2.7 Applications of PHAs

Some of the physical and material properties, exhibit by PHA, their biodegradability (Jendrossek et al., 1996) and the possibility to be processed using the equipment commonly used for the handling of polyolefin and other synthetic plastics, indicates that PHA can be used as replacement or partial substitute of petrochemical polymers and be considered for several applications, such as: in the packaging industry, medicine, pharmacy, agriculture and food industry, or as raw materials for the synthesis of enantiomerically pure chemicals and the production of paints [21, 27].

Applications focus in particular on packaging, containers and films but can also be employed in biodegradable personal hygiene articles into toners for printing applications and adhesives for coating applications; in electronic products, like mobile phones; agricultural applications (encapsulation of seeds, encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities). The use of PHAs as osteosynthetic materials, bone plates, surgical sutures, and other materials in medicine has also been proposed [9, 21]. However, because of the high level of specifications for plastics used in the human body, not every PHA can be used in medical applications. PHA used in contact with blood has to be free of bacterial endotoxins and consequently there are high requirements for the extraction and purification methods for medical PHAs [9, 21, 27].

After their utilization as plastic items, PHAs can not only be composted, but can also be easily depolymerized to a valuable source of optically pure *R*-(−)-configured bifunctional hydroxy acids which are of interest for the synthesis of chiral high-value chemicals such as vitamins, antibiotics, pheromones and aromatics. Some of these acids also exhibit important biological properties such as antimicrobial and antiviral activity [2].

3. Material and Methods

3.1 Bacterial strain and plasmid

The strain AF1000 originated from the *E. coli* K12 strain MC4100 was constructed by first transducing relA^{+} linked to *argA*::Tn10 into MC4100 [50]. This strain which lacks all constituents of the lactose operon, grows on minimal salt medium and is able to metabolize a vast range of substrates such as glucose, arabinose or xylose [30].

Cells were transformed with pCJY02 plasmid by heat shock treatment. The plasmid construct was provided by Xue Gao [51]. pCJY02 plasmid is a derivative of pBHR69, a high copy number derivative of pBluescriptSKM, harboring ampicillin resistance gene (Amp^{R}.), *phbA* and *phbB* genes from *Cupriavidus necator*. The *PhaC2* gene was cloned from *Pseudomonas stutzeri* 1317 and was added to form pCJY02 plasmid [52].

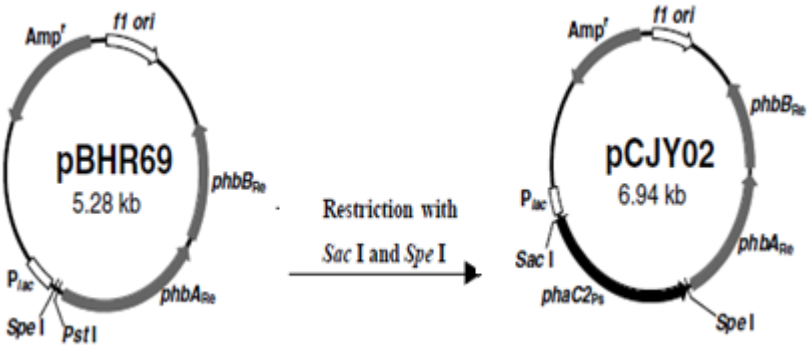

Figure 5 - Plasmid pCJY02 construct. (Chen et al, 2004)

In order to verify the transformation, after cells were grown in selective plates with ampicillin (100 µg.ml^{-1}), they were harvested, digested and the plasmid was purified using GeneJET ™ Plasmid Miniprep Kit by Fermentas. After purification the plasmid was digested with the enzymes *HindIII* and *SacI*.

3.2 Cultivation Medium

The cultivations were performed in a minimal medium consisted of 7 $g.L^{-1}(NH_4)_2SO_4$; 1.6 $g.L^{-1}$ KH_2PO_4; 6.6 $g.L^{-1}$ $Na_2HPO_4.2H_2O$; 0.5 $g.L^{-1}$ $(NH_4)2–H–$Citrate. The medium was autoclaved inside the bioreactor or shake flasks at 121^{o}C for 20min, and supplemented with 1 ml 1 M MgSO4 and 1 ml of trace element solution (0.5 g/L $CaCl_2$_$2H_2O$; 16.7 g/L $FeCl_3.6H_2O$; 0.18 $g.L^{-1}$ $ZnSO_4.7H_2O$; 0.16 $g.L^{-1}$ $CuSO_4$_$5H_2O$; 0.15 $g.L^{-1}$ $MnSO_4.4H_2O$; 0.18 $g.L^{-1}$ $CoCl_2.6H_2O$; 20.1 $g.L^{-1}$ Na-EDTA). These solutions were sterile-filtered (Sartorius 0.2 lm) into the bioreactors after sterilization. The production plasmid pCJY02 was induced with 200 µM of IPTG. An antifoam agent Breox and ampicillin (100 $\mu g.ml^{-1}$), was added when necessary. Glucose was autoclaved separately and added to a final concentration of 10 $g.L^{-1}$, in shake flask cultivations and batch bioreactor cultivations.

3.3 Cell Stock

After transformation a cell stock was prepared, by picking colonies from selective agar plates and inoculated into 100 ml of minimal salt medium with glucose (10 $g.L^{-1}$ as final concentration) and complemented with trace elements and $MgSO_4$, in 1000 ml shake flasks. Cells were harvested when the optical density at 600nm reached 1.4.Two parts of cell suspension and three parts of glycerol solution (43%) were collected in a sterile eppendorf (1,5ml), giving a final glycerol concentration of about 25%. The tubes were stored at -80^{o}C until further use.

3.4 Inoculum

One liter shake flasks with baffles were cultivated with a working volume of 100 ml, to improve oxygen transfer. The flasks were kept at 37 ^{o}C in an incubated shaker at 180 rpm (Infors Minitron). The medium was inoculated with 1.5 ml the frozen stock of AF1000 cells harboring the pCJY02 plasmid (OD = 0.7) and ampicillin (100 $\mu g.ml^{-1}$). These shake flask culture were used to inoculate the bioreactors, with 1 ml of the exponentially grown cell suspension with an optical density (OD) value of 1.6.

3.5 Bioreactors

The experiments were performed in a 10L bioreactor with a working volume of 7,5L (Belach Bioteknik AB) or a 15L bioreactor with a working volume of 10 L (Belach Bioteknik AB). Temperature was set to 37 ^{o}C and pH was kept at $\pm$7 in all cultivations, by addition of ammonia (25% w/w) for the cultivations without nitrogen limitation and by addition of NaOH solution, for the cultivation performed with nitrogen limitation.

The bioreactor was equipped with a polarographic electrode to monitor the dissolved oxygen tension (DOT). This parameter was controlled by manual changes of the stirring speed (200–1500 rpm) or the gas flow into the bioreactor (1–15 L.min^{-1}) and was kept above 10% in all cultivations.

3.6 Fed-batch cultivation

The fed-batch cultivation was performed and executed in two stages. The first stage was performed in order to reach the cell density till a value considered reasonable **(**OD value of 1 to 2**)** to start the second stage, the production.

The fed-batch was started with the addition of 10 g.L^{-1} of glucose, in order to avoid growth limitation due to excess of carbon source. The inoculum size was 1ml with OD of 2 in order to allow the cell grow over night till the desirable optical density (OD 1). After induction at OD 5, more glucose was added to the cultivation in order to follow the production stage.

3.7 Fed-batch cultivation (exponencial feed)

The fed-batch cultivation was started after a batch phase on glucose in which an initial glucose concentration of 1,6 g.L^{-1} was added in order to obtain a theoretical OD value of 2. After the initial glucose concentration was exhausted, the exponential feed was started. When glucose was consumed the increase in the oxygen signal was used as a signal to start of the exponential feed. This feed was set to give the theoretical growth

rate of 0.5 h^{-1}, which correspond to less than the maximum growth rates of AF1000 + pCJY02 cells (0.66 h^{-1}).

3.8 Nutrient limited cultivation

The nutrient limited cultivation was executed with nitrogen as the limiting nutrient. 1.85 $g.L^{-1}$ of $(NH4)_2SO_4$ was used in the medium preparation, giving a theoretical cell dry weight of 3,2 $g.L^{-1}$, using a glucose concentration of 10 $g.L^{-1}$. The inoculum size was 1ml with OD of 2 in order to allow the cell grow over night till the desirable optical density (OD 1). After this first stage the cells were induced and more glucose was also added to follow the production stage.

3.9 Analytical methods

3.9.1 Cellular dry weight

Cell growth was followed by measurement of light scattering (optical density/OD) at 600 nm in a Novaspec II Spectrophotometer (Amersham Pharmacia Biotech AB). Biomass concentration (CDW) was determined by centrifugation (Wifug Lab,10 min, 4,500 rpm) of three samples of 5 ml cell suspension, in pre-dried and pre-weighed test tubes after which the pellet was collected and dried overnight at 100 ^{o}C before weighing.

3.9.2 Acetic acid quantification

The supernatant from the biomass determination was sterile filtered (Sartorius, 0.2 lm) and used for acetate analysis. The acetate concentration was analyzed by a commercial enzymatic kit Boehringer-Mannheim n^{o}. 148261. Each sample was analyzed three times from which the mean value was calculated

3.9.3 Glucose quantification

For glucose determination the samples were rapidly inactivated by sampling (under 0.1 s) into test tubes containing cold (+8 ^{o}C) perchloric acid (0.132M) and centrifuged at

4,500 rpm for 10 minutes. 3.5ml of the sample where transferred to another tube containing 0.075ml of concentrated potassium carbonate, placed on ice for 15min, centrifuged at 4500rpm for 5min and stored in eppendorf tubes at -20^{o}C. The analyses were performed using a commercial enzymatic kit protocol Boehringer-Mannheim. Each sample was analyzed three times and a mean value was calculated.

3.9.4 PHB extraction

The produced PHB was extracted by sodium hypochlorite digestion, a commonly used extraction method developed by Law and Slepecky [53]. The cultivation sample was centrifuged in order to remove the cultivation medium. The formed pellet was incubated in sodium hypochlorite for one hour at 37 ^{o}C, with an objective to digest the biomass and release the intracellular PHB. Through successive centrifugations (14000×g for 25min.) the cells debris were removed and the crude PHB was washed with distilled water (10ml), acetone (3 portions) and methanol.

3.9.5 PHB Quantification and PHB Standard Curve

After the successive washes the extracted PHB is dissolved in 10 ml of chloroform. One ml of this solution is transferred to a fresh glass tube and allowed to evaporate. Once the tube is dry, 10ml of concentrated H_2SO_4 is added and the tube is sealed. Then the tube is transferred to a boiling water bath (94^{o}C-96^{o}C) for 20 minutes to complete the conversion into crotonic acid. After cooling and vortex the sample is transferred to a silica cuvette for UV absorbance measurement at 235nm, in a spectrophotometer.

The concentration of PHB in the sample is determined through standard curve (Figure 3), constructed using known concentration (100 µg/ml) of commercial PHB (Sigma Aldrich) dissolved in chloroform and heated in a water bath (65^{o}C -70^{o}C). The solution is divided in different aliquots where 1 ml corresponds to 100 µg PHB, 900 µl corresponds to 90 µg and so on. After chloroform evaporation, 10 ml con.H_2SO_4, is added to the tubes and capped with glass stoppers. The tubes are heated in boiling water bath (94°C-96°C) for 20 minutes. After cooling and vortex the sample is transferred to a silica cuvette for UV absorbance measurement at 235nm, in a spectrophotometer.

3.10 Calculations

3.10.1 Feed profile (fed-batch)

The feed profile of the fed-batch cultivations was calculated from mass balances for exponential feed and of the limiting substrate, glucose, according to:

$$F(t) = F_0 \times e^{\mu t}$$

$$F_0 = (\mu \times V \times X)/(S_i \times Y_{X/S})$$

where F ($L.h^{-1}$) is the feed rate at a determinate time event t (h), F_0 is the initial feed rate at fed-batch start ($L.h^{-1}$), μ is the specific growth rate (h^{-1}), V is the cultivation volume (L), X the cell dry weight ($g.L^{-1}$) and $Y_{X/S}$ the theoretical yield coefficient of cell mass production resulting from the substrate uptake rate (approximated to 0.5 $g.g^{-1}$).

3.10.2 PHB content

The PHB content of the cells, expressed as PHB/CDW (%) (where PHB is the amount of product detected and CDW is the cellular dry weight), works as measurement of the cell's ability to accumulate PHB and how much can be potentially accumulated. High values, above 60%-70% are required in order to achieve an efficient and less costly extraction, also the cell mass should not be too low, since it will influence the results [32, 42].

3.10.3 Glucose

The initial glucose concentration to be used in the cultivations was obtained using the following equation:

$$S \times Y_{x/s} = X$$

where S is the substrate concentration ($g.L^{-1}$), $Y_{X/S}$ the theoretical yield coefficient of cell mass production resulting from the substrate uptake rate (approximated to 0.5 $g.g^{-1}$) and X is the cell dry weight ($g.L^{-1}$) . OD value was calculated with a conversion factor of 2.5 times the cellular dry weight.

Other formula was used to determine the amount of glucose present in the samples collected during the cultivations. The formula was obtained from the Boehringer-Mannheim protocol for D-Glucose quantification.

$$\Delta A = (A2 - A1)sample - (A2 - A1)blank$$

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ (g/L)$$

Where ΔA is the variation in the absorbance; A1 is for the absorbance of the solution not containing the enzymatic solution; A2 is for the absorbance of the solution containing the enzymatic solution; C is the glucose concentration ($g.L^{-1}$); V final volume (mL); MW molecular weight of the substance to be assayed (186.16 $g.mol^{-1}$); d light path (cm); ε extinction coefficient of NADPH (340 nm = 6.3 (l × mmol-1 × cm-1)); v sample volume (ml).

3.10.4 Acetic acid

The formula used for the acetic acid calculation was based in the Boehringer-Mannheim protocol Acetic acid quantification.

$$\Delta A = \left[(A2 - A0)sample - \frac{(A1 - A0)^2 sample}{(A2 - A0)^2 sample}\right] - \left[(A2 - A0)blank - \frac{(A1 - A0)^2 blank}{(A2 - A0)^2 blank}\right]$$

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ (g/L)$$

Where ΔA is the variation in the absorbance; A0 is for the absorbance of the solution not containing the enzymatic solution; A1 is for the absorbance of the solution containing only the first enzymatic solution; A2 is for the absorbance of the solution containing all the enzymatic solutions; C is the glucose concentration ($g.L^{-1}$); V final volume (mL); MW molecular weight of the substance to be assayed (60.05 $g.mol^{-1}$); d light path (cm); ε extinction coefficient of NADPH (340 nm = 6.3 (l × mmol-1 × cm-1)); v sample volume (ml).

3.10.5 Specific production rate

The formula for the specific production rate determination is the following one:

$$q_P = \frac{dP_i}{dt} + \mu P_i$$

Where dP_i/dt is the slope obtained from the plot of the product over time; μ is the growth rate during cultivation (h-1); P_i is for an intracellular product with intracellular concentration P_i (g gcells-1).

3.10.6 PHB quantification

Formula for the PHB quantification was obtained from standard curve (Figure 6)

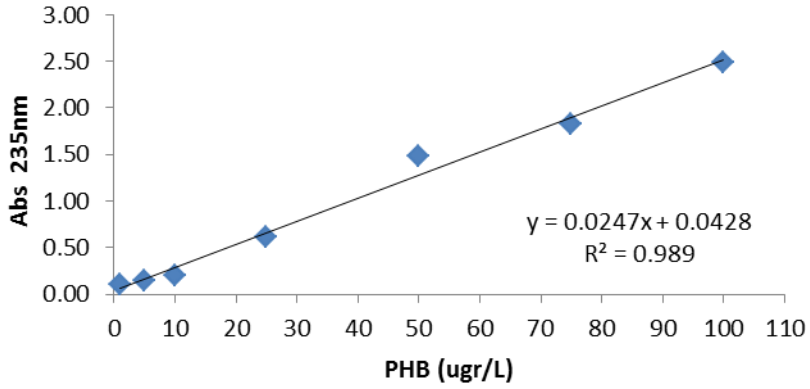

Figure 6 - PHB standard curve.

3.10.7 Real biomass

The real biomass value is obtained by subtracting the PHB value to the total biomass (cellular dry weight), which comprises the PHB value plus the biomass values.

$$real\ biomass\ (g/L) = total\ biomass\ (g/L) - PHB\ concentration\ (g/L)$$

3.10.8 Biomass Yield

$Y_{(X/S)}$ (g.g-1) represents the biomass yield coefficient that describes how much biomass is produced per consumed substrate. It was obtained according to:

$$Y_{(X/S)} = \frac{Biomass\ (g/L)}{Substrate\ (g/L)}$$

3.10.9 Product Yield

$Y_{(P/S)}$ (g.g-1) is a yield coefficient that describes how much of the consumed substrate that is converted to the product. It was calculated for PHB and acetic acid according to:

$$Y_{(P/S)} = \frac{Prodcut\ (g/L)}{Substrate\ (g/L)}$$

4. Results and Discussion

4.1 Production Strain construct

Escherichia coli is not a natural PHB-producing bacteria and requires the construction of the metabolic pathway by transferring the genes responsible for PHB production from a natural producer. Moreover it has many advantages over the natural PHB-producing organisms, namely it does not require a nutrient starvation step in order to accumulate the polymer; it has proven to be suitable as a heterologous expression host for foreign genes; high-cell-density cultivation strategies are well established; also does not have any PHA regulatory systems in place or enzymes that degrade PHAs [6, 20]. In this work, the Plasmid pCJY02, provided by Xue Gao and co-workers [51], harboring *phbA* and *phbB* genes from *Cupriavidus necator*, p*haC* gene from *Pseudomonas stutzeri* 1317, was used to transform *E. coli* AF1000.

Agarose gel electrophoresis showed that resultant fragments obtained after purification and enzymatic digestion had sizes in the ranges 2500 -3000bp and 4000-5000bp as predicted from the vector map (Figure 6)

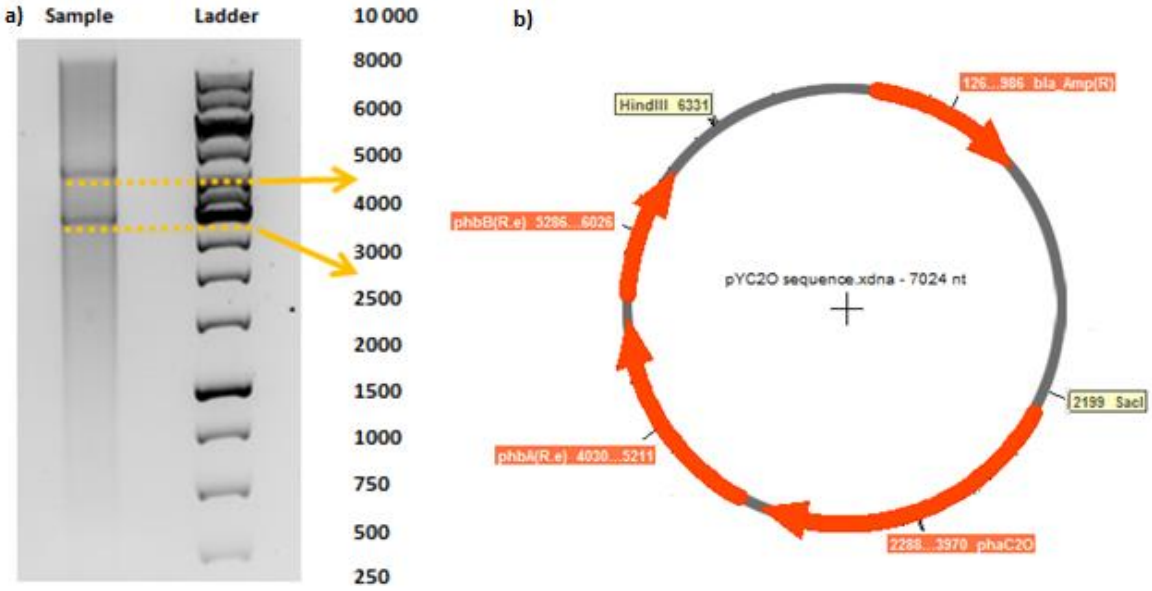

Figure 7 - a) Restriction analyses results; b) enzymes cutting sites (yellow)

4.2 Cultivations results

After the strain transformation, several cultivations were performed in order to test its ability to produce PHB. All cultivation were performed and executed in two stages. The first stage was performed in order to reach the cell density till a value considered reasonable (OD value of 1 to 2) to start the second stage, the production, with IPTG induction. Table 4 shows the results obtained from all three cultivations and the Figures 7, 8 and 9 showed the evolution of the cultivations throughout the time.

During the first cultivation (Figure 8) *E. coli* produced a maximum PHB amount of 0.769 $g.L^{-1}$, corresponding to a PHB content of 8%, maximum specific production rate of 0.320 $g.g^{-1}.h^{-1}$ and $Y_{(P/S)}$ yield of 0.296 $g.g^{-1}$. The active biomass obtained was 9.6 $g.L^{-1}$ with a yield $Y_{(X/S)}$ of 2.86 $g.g^{-1}$ and the amount of acetic acid produced was 3.0 $g.L^{-1}$ with correspondent $Y_{(Hac/S)}$ yield of 0.808 $g.g^{-1}$.

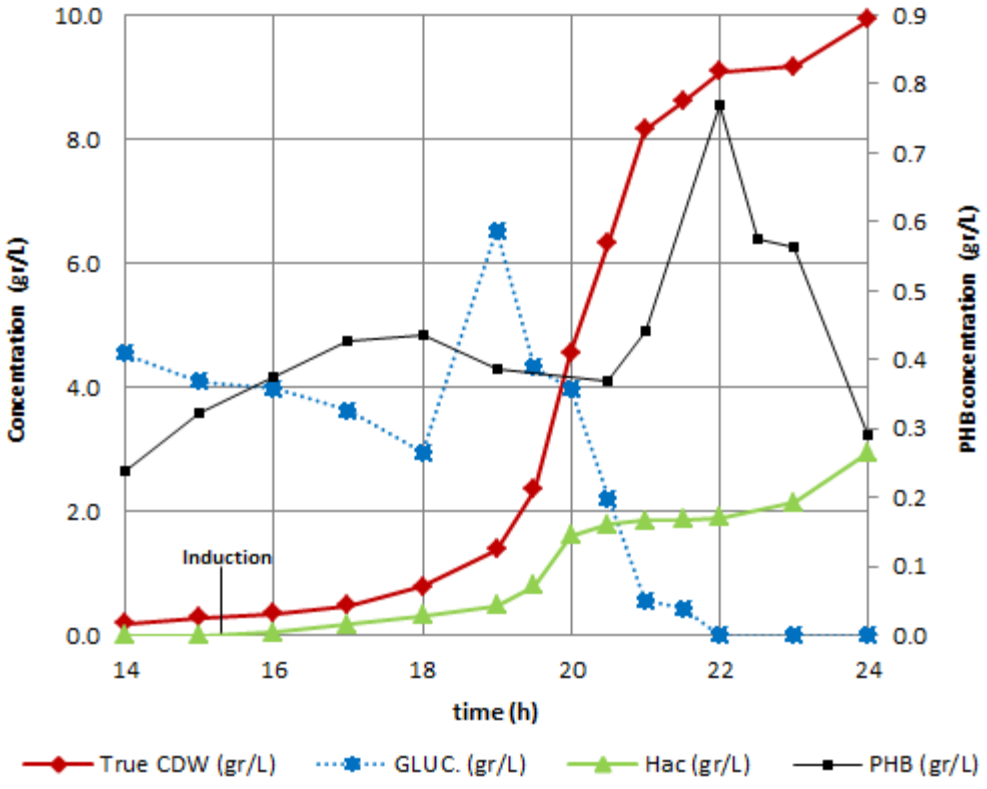

Figure 8 – Fed-batch cultivation results.

With the fed-batch cultivation with exponencial feed (Figure 9) it was possible to increase the amount of PHB in 26% (0.971 gr.L^{-1}), the specific production rate to 0.489 g.g^{-1}.h^{-1} and $Y_{(P/S)}$ yield of 0.399 g.g^{-1} As expected the amount of acetic acid also increased to 4.3 g.L^{-1} with correspondent $Y_{(Hac/S)}$ yield of 1.0 g.g^{-1}. However the PHB content decreased to 3.65 %, influenced by the amount of biomass obtained (23.0 g.L^{-1}; $Y_{(X/S)}$ of 3.71 g.g^{-1}) much higher than the previous cultivation, since the substrate was continuously added.

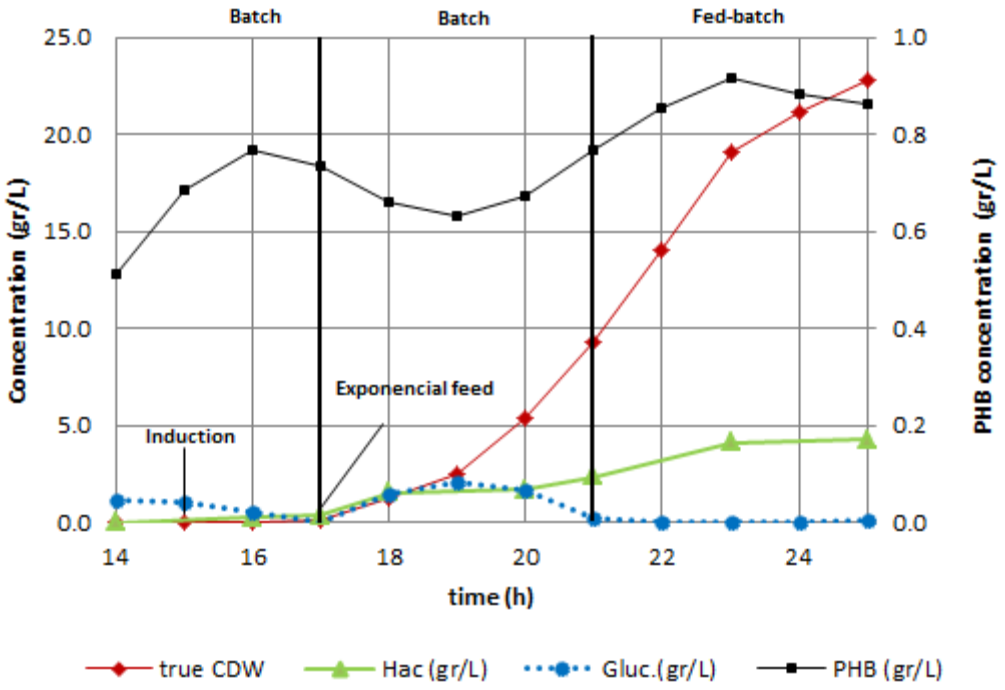

Figure 9 – Fed-batch cultivation with exponential feed results.

Implementation of specific nutrient limitation for PHB production, it might stimulate the PHB synthesis through limitation of the cell growth, acetic acid production or other competitive pathways [54]. Since the concentration of acetic acid obtained in the previous cultivations, could be partially responsible for the low accumulation of PHB and PHB content, a nutrient limited cultivation was performed (Figure 10), in which nitrogen was limited. As expected less acetic acid was produced (1.3 g.L^{-1}; $Y_{(Hac/S)}$ 0.255 g.g^{-1}) and active biomass (2.3 g.L^{-1}; $Y_{(X/S)}$ of 0.468 g.g^{-1}).The PHB content increased 20% consequence of the low cell mass, however the results for PHB quantification were

insufficient in order to make conclusions about the real effect of the nutrient limitation due to step variation of the PHB amount during limitation period, more accentuated from 18th hour to 20th hour and the last two hour of cultivation.

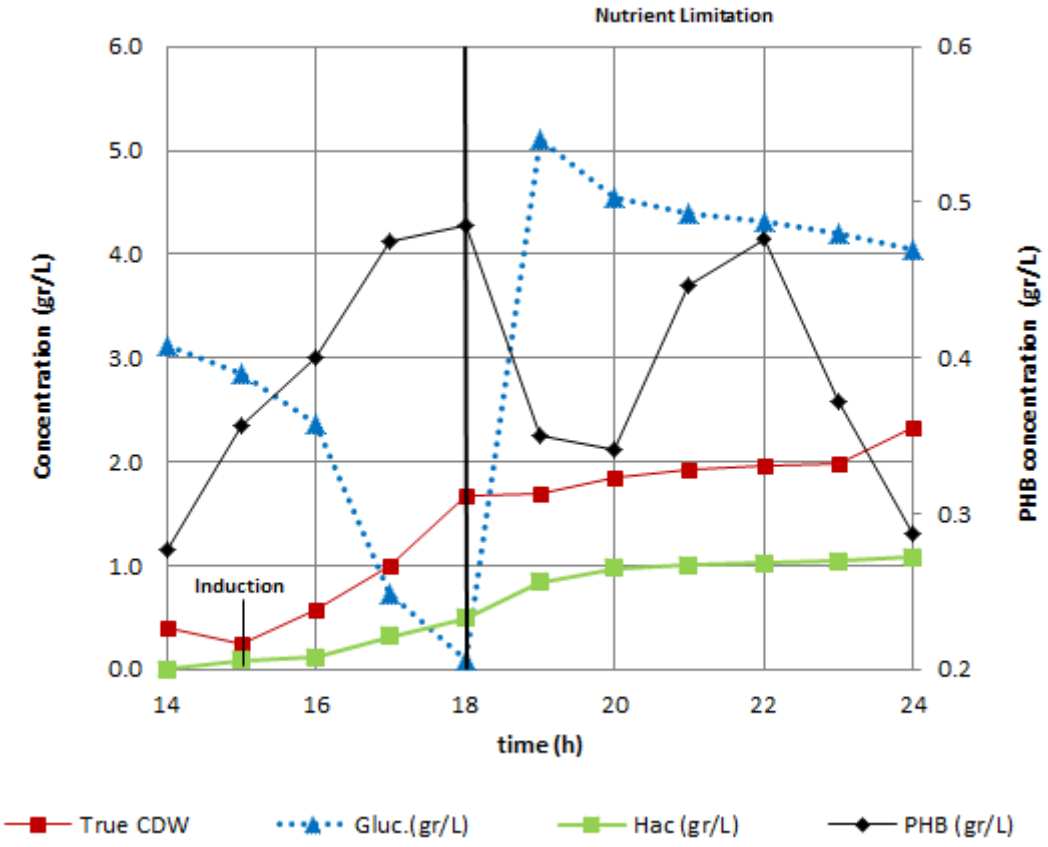

Figure 10 - Results from the cultivation with nutrient limitation.

This is not an isolated occurrence, since in all cultivations it was possible to observe a decrease in PHB concentration more extensive in the final hours of each test. In this case, the expected pattern for PHB accumulation during the nutrient limitation period is that the amount of PHB remained constant or presenting a small increase during remaining time. Nevertheless, the values that were possible to obtain were 0.477 gr.L^{-1} PHB, PHB content 20%, 2.3 gr.L^{-1} active biomass, specific production rate of 0.540 g.g^{-1}.h^{-1} and the yields $Y_{(X/S)}$,$Y_{(P/S)}$, $Y_{(Hac/S)}$ were 0.468 g.g^{-1}, 0.147 g.g^{-1} and 0.255 g.g^{-1}, respectively. However, according to Lee et al. (1994), high improvements of the PHB amounts, should not be expected since only minor enhancement was observed when

E. coli was grown in excessive carbon source and nutrient depletion, when compared with natural producers [54].

In the fed-batch cultivation it was also possible to observe a decrease at 17th hour of cultivation when the exponential feed was started. During the first two hours of this period a decrease in the PHB amount was observed, probably due to cell adaptation to the new conditions, since the feed was calculated for a μmax of 0.5 h $^{-1}$; or due to dilution from the cell mass increase.

All tests could have been influenced by cell lysis during cultivation or storage of the samples. The insertion of the *rel*A gene in *E. coli* AF1000 could turn the cell less prone to lysis, even if there is a higher chance to occur in the *rel*A mutants according to Shokri et al (2006) [50]. To confirm this hypothesis PHB quantification could be performed in the supernatant after cell mass removal by centrifugation, as suggested Wang et al, (2009)[55]. However since the free PHB present in the medium was not quantified in any of the cultures, this hypothesis was not possible to confirm. Other possibilities for the PHB variation could be related with the method used for quantification or loss of the genetic modification. The successive washes, centrifugations and dilutions required for the quantification, will always lead to some product loss. Therefore alternative and more reliable method such as gas chromatography must be implemented [56].

During all cultivations PHB was also detected before induction with IPTG. Small leakage from the plasmid is not uncommon to occur. However, comparing the values of specific production rate for each cultivation (Table 4), before induction (fed-batch – 304 gr.L^{-1}; fed-batch with exponential feed - 430 gr.L^{-1}; nutrient limited cultivation – 306 gr.L^{-1}) and after the induction (fed-batch - 320 gr.L^{-1}, fed-batch with exponential feed - 489 gr.L^{-1}; nutrient limited cultivation - 540 gr.L^{-1}), the addition of IPTG (200 μM) had small effect, achieving values near the maximum specific production detected when induced. The small effect of IPTG addition could be related with the fact that the used *E. coli* strain lacks all constituents of the *lac* promoter [30]**.** This will lead to a constant expression of the plasmid and PHB production, even without the induction since the cell does not possesses the required regulatory mechanisms of the *lac* promoter used in the plasmid.

Table 4 - Resume of the results obtained in all tests; (*) after nutrient limitation; (**) before nutrient limitation;

Cultivation	Cell concentration ($g.L^{-1}$)	Maximum PHB concentration ($g.L^{-1}$)	PHB content (%)	Specific production rate ($g.g^{-1}.h^{-1}$)	Acetic acid ($g.L^{-1}$)	$Y_{X/S}$ ($g.g^{-1}$)	$Y_{P/S}$ ($g.g^{-1}$)	$Y_{Hac/S}$ ($g.g^{-1}$)
Values detected before induction								
Fedbatch	-	0.242	-	0.304	-	-	-	-
Fedbatch (exponencial feed)	-	0.490	-	0.430	-	-	-	-
Nutrient Limited	-	0.277	-	0.306	-	-	-	-
Values detected at the end of cultivaton								
Fedbatch	9.6	0.769	8.0	0.320	3.0	2.86	0.296	0.808
Fedbatch (exponencial feed)	23.0	0.917	3.65	0.489	4.3	3.71	0.399	1.00
Nutrient Limited	2.3* 1.6**	0.477* 0.486**	20.0* 20.0**	0.540* 0.486**	1.3* 0.5**	0.468	0.147	0.255

Several other reasons could be behind the low efficiency in PHB production. The first could be related to the medium used [47, 57]. Several authors report production of high amounts of PHA (Table 3), as high as 158.8 $g.L^{-1}$ or 101.3 $g.L^{-1}$, obtained with recombinant *E.coli* [12, 47]. Most of these results were obtained using complex, semi-complex or minimal mediums supplemented with complex nitrogen sources such as of tryptone, yeast extract, soybean hydrolysate among others. Cells were grown making available to them intermediaries metabolites (vitamins, aminoacids, etc.). This will diminish competition by different pathways, for the principal precursors used in PHB production [23, 24]. The major competitors pathways are acetate synthesis; TCA and fatty acids, all require acetyl-CoA and NADPH [19, 45]. However even with the use of a defined medium, Lee et al (1994), reported PHB production values of 16$g.L^{-1}$, supplemented with glucose, still high above those obtained in this work [32, 42].

The metabolic influence is not only promoted with the use of different mediums or carbon sources. Different PHB concentrations were obtained for different *E.coli* strains using the same plasmid. Lee et al. (1994) suggested that these variances could be due to different expression levels of the native enzymes involved in the pathways that will influence conversion rates of the precursors, concentration of the available metabolites or the expression levels of the cloned gene [32]. Also among the strains investigated, lowest PHB concentration was observed in *E.coli* K12 probably due to high production of acetate [32]. Since this strain is considered to be the wild type of *E. coli* AF1000 which has been used in this present work, it could explain the low PHB formation as high concentration of acetate was also obtained.

Other major influence in the obtained results regards to the plasmid and the used genes. The stabilization of the plasmid was indicated as one improvement that could be employed to increase PHB production [41, 42, 54]. The plasmid used in this work allowed for stable inheritance of the plasmid using the antibiotic ampicillin. However the use of antibiotic besides not being practical on industrial scale it may not guaranty that cells without the plasmid will not grow and normally means a significant cost increase for the final product [41, 42, 54]. Several techniques could be employed in order to increase the plasmid stability, Lee et al (1994) [54] used the *parB* locus of plasmid R1, which mediated stabilization via post segregational killing of plasmid-free cells, and

improve the PHB production. Gao and co-workers (2012) suggested the addition of different secondary structures within 5' untranslated regions (UTRs) of prokaryotic mRNA. These hairpin structures could improve translation and prevent fast degradation by RNases, consequently improving the amounts of PHB produced [51].

Regarding to the used genes, PhaC2$_{Ps}$ cloned from *Pseudomonas stutzeri 1317* is a type II synthase, besides being capable of synthesize scl-PHA it favored the formation of mcL-PHA [58]. Gao and co-workers (2012) addressed some attention to the reasons behind the poor performance of the PHA synthase PhaC2Ps cloned from *Pseudomonas stutzeri* 1317. It was reported that through codon optimization of PhaC2Ps gene using "one amino acid-one codon" strategy, the PHB production was improved. It is known that species-specific variations in codon usage deeply affects heterologus protein expressions, therefore codon analysis of the PhaC2Ps gene were executed, revealing that almost 60% codons were not preferred in *E.coli* [51]. Wen Shen *et al* (2011) also reported improvements in PHA production with the PhaC2Ps cloned from *Pseudomonas stutzeri* 1317, resultant from site-specific mutation of the enzyme. The target residues Ser326 and Gln482, have proven to be important in the PhaC2Ps activity but also in the determination of the substrate chain-length specificity [59].

5. Conclusions

The goal of this work was to study the production of polyhydroxybutyrate by *Escherichia coli* AF1000 strain. Since *E.coli* is not a natural producer, it was necessary to create the metabolic pathway to make possible PHB accumulation. This was performed by transforming *E.coli* AF1000 with plasmid pCJY02 harboring, *phbA*, *phbB* genes from *Cupriavidus necator* and *phaC* gene from *Pseudomonas stutzeri* 1317.

With the results obtained in this work, it was possible to conclude that besides the ability to synthesize and accumulate PHB, the present strain and plasmid construct were not suitable for an industrial process.

Further work regarding genetic modifications in the strain as in the plasmid construct must be considered in order to improve PHB production. More attention could be also addressed into find the right balance between *Escherichia coli* AF1000 growth and the PHB produced, leading to a more efficient process. The medium used in this work can also be supplemented with co-factors or more complex substrates, in order to fulfill the metabolic needs of the host microorganism. Since molecular weight is also an important criterion on PHB applications, analysis regarding this parameter should also be performed in order to follow possible alterations caused by the medium composition and extraction process.

An alternative method for PHB quantification such as gas chromatography must be employed, since the method employed in this work is laborious and the successive washes lead to product lost.

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