Final Master Thesis

Study of the effect of oxygen concentration on poly-3-hydroxybutyrate production by *Ralstonia eutropha* cultivation

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Abstract

Different oxygen saturation concentrations were tested for *Ralstonia eutropha* cultivation to study its effect on microbial growth, polymer production and polymer molecular weight. The cell in the culture medium was measured by spectrophotometry, and PHB concentration and molecular weight were measured by Gel Permeation Chromatography (GPC).

Ralstonia eutropha CECT 4635 strain was used for the batch process production of poly(3-hydroxybutyrate) (PHB) in a 1-L fermentor. Glucose was used as a carbon source and PHB accumulation was induced by nitrogen limitation, so NaOH solution was used for pH control.

Using an oxygen concentration of 65%, the 48 hours-batch cultivation resulted in a final cell concentration of 9,43 g/l, a PHB concentration of 9,45 g/l and a PHB productivity of 0,20 g/l·h. These values are 50 times higher than the ones obtained when using air at the same conditions. The molecular weight of the final polymer was 54.000 Da. Results suggested, though, that the nitrogen limitation had just started when terminating the fermentation, what justifies such low values.

Nevertheless, PHB content, cell yield from glucose and PHB yield from glucose when using 65% oxygen saturation were the highest ever reported to date in *R. eutropha* cultivation, with values of 99,8%, 0,47 g/g and 0,47 g/g respectively.

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Abbreviations

BM	Biomass
CDW	Cell dry weight
CECT	Colección Española de Cultivos Tipo
DO	Dissolved oxygen
GPC	Gel Permeation Chromatography
HFIP	Hexafluoro-2-propanol
Mn	Number average molecular weight
Mp	Molecular weight of the highest peak
Mw	Weight average molecular weight
OD	Optical Density
OTR	Oxygen transfer rate
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
RBM	Residual biomass

Although in this thesis the name "*Ralstonia eutropha*" is used to refer to the bacteria specie employed for the fermentation, this bacterium has had several names since its discovery such as *Alcaligenes eutrophus* and *Wautersia eutropha*, and it was finally renamed to *Cupriavidus necator*, which is the name officially used.

Introduction

Introduction

Growth in the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment. Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation [1]. For this reason, there is special interest in producing plastics from materials that can be readily eliminated from our biosphere in an "environmentally friendly" fashion.

The allure of bioplastic is also linked to diminishing petrochemical reserves. The industrialized world is currently highly dependent on fossil fuels as a source of energy for industrial processes and for the production of structural materials. Fossil fuels are, however, a finite resource. The world currently consumes approximately 140 million tons of plastics per annum. Processing of these plastics uses approximately 150 million tons of fossil fuels [1], which are difficult to substitute.

Polymers from renewable biomass are attracting much attention as a potential solution to these problems. Polyhydroxyalkanoates (PHAs) are polymers synthesized by a complete biological process, in which carbon sources are directly converted into PHAs by microbial fermentation, whereas most of the so-called biopolymers including polybutylene succinate (PBS), polytrimethylene terephthalate (PTT), and polylactic acid (PLA) are chemically synthesized using fermentation-derived monomers [2].

PHAs are the most versatile fully biodegradable polymers with properties similar to conventional plastics. Appreciable number of PHAs with more than 150 monomers has been identified with molecular masses ranging from 50,000 to 1,000,000 Da. As PHAs are biodegradable and immunologically inert, they have promising future applications, particularly in medical related fields, despite their expensive production. [3]

PHAs are biopolyesters that generally consist of 3-, 4-, 5-, and 6-hydroxycarboxylic acids. Many bacteria such as *Ralstonia eutropha* accumulate PHAs in their cytoplasm as carbon and energy storage materials when they encounter limited growth conditions in the presence of excess carbon sources [2].

However, commercial applications and wide use of PHA is hampered due to its price. The price of the product ultimately depends on the substrate cost, PHA yield on the substrate, and the efficiency of product formulation in the downstream processing [4]. This implies high levels of PHA as a percentage of cell dry weight and high productivity in terms of gram of product per unit volume and time [5]. The cost of PHA using the natural producer *R. eutropha* is US$16 per kg which is 18 times more expensive than polypropylene. With recombinant *E. coli* as producer of PHA, price can be reduced to US$4 per kg, which is close to other biodegradable plastic materials such as PLA and aliphatic polyesters [5]. The commercially viable price should come to US$3–5 per kg [4].

It is a prerequisite, then, to standardize and optimize all the fermentation conditions for the successful implementation of commercial PHA production systems.

In many previous processes producing PHB, dissolved oxygen in the medium was found to be the limiting nutrient [6]. In aerobic bioprocesses oxygen is a key substrate employed for growth, maintenance and product synthesis. Due to its low solubility in broths, which are usually aqueous solutions, oxygen must be continuously provided by a gas phase.

The transport of oxygen from air bubbles to the cells can be explained by the oxygen transfer rate (OTR) parameter. From its equation, various strategies can be employed to increase the oxygen supply. One of them, which has not yet been tested in *R. eutropha* cultivation, is increasing the concentration of oxygen in the gas phase. Despite its added costs to purchase purified oxygen, its positive impact on polymer production may more than offset its cost.

The overall objective of this report is to study the effect of oxygen saturation concentrations on microbial growth and PHB production in *Ralstonia eutropha* cultivation, as well as assess the batch process parameters and their influence in polymer formation.

The study also aims to set out guidelines for more productive bioprocesses and more effective analyses in further investigations.

Chapter I

Theoretical framework

Chapter 1
Theoretical framework

1.1. POLYHYDROXYALKANOATES: AN OVERVIEW

Polyhydroxyalkanoates (PHAs) are biopolyesters which are composed of 3-hydroxy fatty acid monomers, which form linear, head-to-tail polyester (Figure 1). PHA is typically produced as high molecular weight polymers in the range of 200,000 to 3,000,000 Da, depending on the microorganism and growth conditions [7], which accumulate as inclusions of 0.2–0.5 μm in diameter (Figure 2). These inclusions or granules are synthesized and stored by both gram-positive and gram-negative bacteria without hazardous effects to the hosts [1], and the number and size of the granules vary depending on the organism [8].

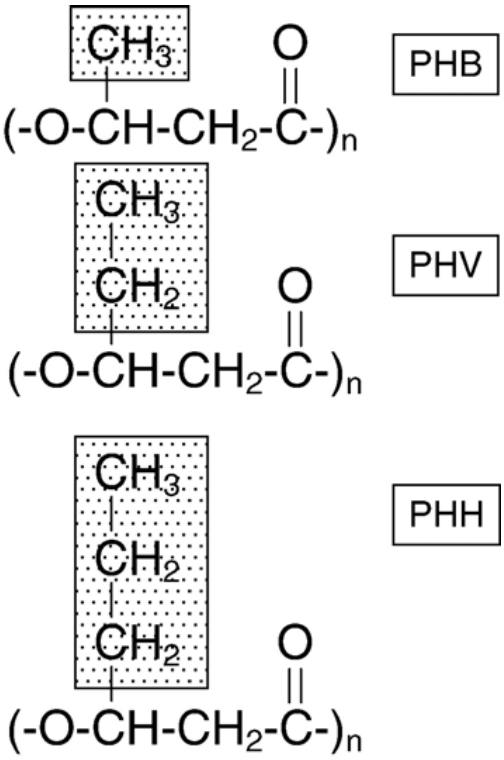


Figure 1. Chemical structure of PHAs. The pendant R groups (shaded boxes) vary in chain length from one carbon (C1) to over 14 carbons (C14). Structures shown here are PHB (R=methyl), PHV (R=ethyl), and PHH (R=propyl). [1]

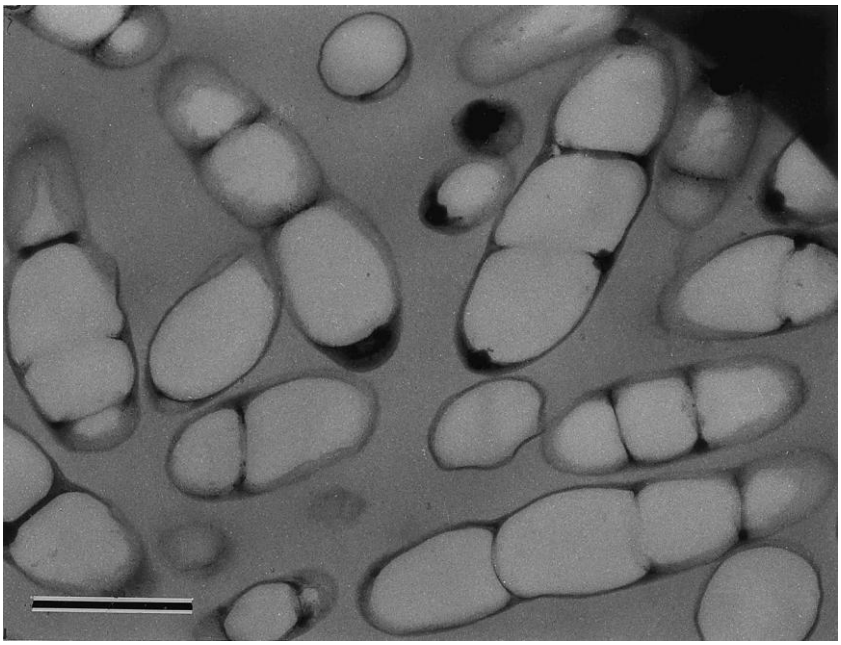


Figure 2. Transmission electron micrograph of thin sections of recombinant *R. eutropha* PHB24 cells containing large amounts (90% of the dry cell weight) of P(3HB-co-5 mol% 3HHx). Bar represents 0.5 mm. [7]

PHA accumulation occurs when the cells experience a nutrient imbalance such as excess carbon with limited nitrogen, phosphorus or oxygen, which are essential for growth [1][9]. The bacteria store the excess nutrients intracellularly by forming insoluble biopolymers from soluble molecules. The biopolymers become mobilized when conditions for normal growth return.

Of all the characterized PHAs, alkyl groups, which occupy the R configuration at the C-3, vary from one carbon (C1) to over 14 carbons (C14) in length. PHAs can be subdivided into three broad classes according to the size of comprising monomers. PHAs containing up to C5 monomers are classified as short chain length PHAs (scl-PHA). PHAs with C6–C14 and >C14 monomers are classified as medium chain length (mcl-PHA) and long chain length (lcl-PHA) PHAs, respectively [10].

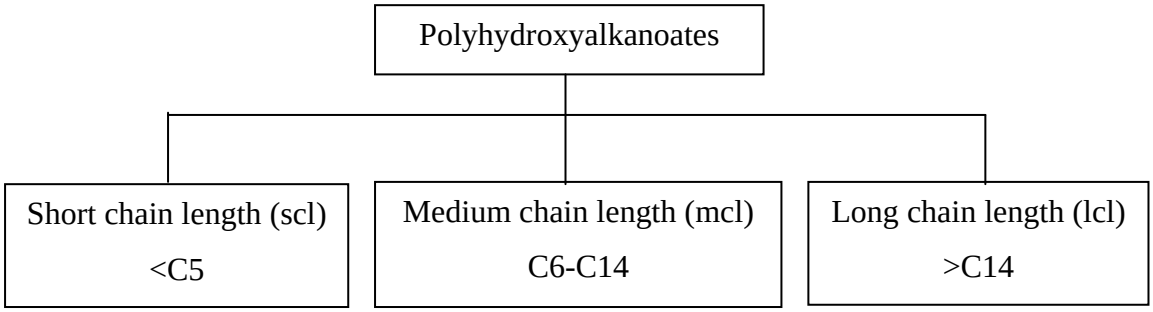


Figure 3. PHA classification according to their monomer composition.

Monomer content influence PHA physical and chemical characteristics, which is affected by many factors: type of microorganisms (e.g. Gram-negative or Gram-positive), media ingredients, fermentation conditions, modes of fermentation (batch, fed-batch, continuous) and recovery [3]. scl-PHAs normally have properties close to conventional plastics while the mcl-PHAs are regarded as elastomers and rubbers [1]. There are also reports on functional modification of the monomers to improve the properties of the resulting bioplastic, such as the introduction of unsaturated and halogenated branched chains. As well, heteropolymers can be formed by polymerization between more than one kind of monomer.

PHB is the most common type of scl-PHA and this homopolymer of 3-hydroxybutyric acid has been studied most extensively. Copolymers of PHA can be formed containing 3-hydroxybutyrate (HB), 3-hydroxyvalerate (HV), 3-hydroxyhexanoate (HH) or 4-hydroxybutyrate (4HB) monomers. Most of the microbes synthesize either scl-PHAs containing primarily 3HB units or mcl-PHAs containing 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD) as the major monomers [1].

Regarding PHA molecular weight, it depends on the production and recovery conditions. Extraction with organic solvents leads to polymers with higher molecular weight, compared with the extraction based on sodium hypochlorite or other chemicals.

The mechanisms affecting and determining the molecular weight of the PHA in bacterial cells are not yet fully understood, but is generally attributed mainly to the kind of microorganism and the carbon source used [11]. Because of this, there are PHAs with a variety of molecular weights, as seen in Table 1. Overall average P3HB produced by wild bacteria molecular weight is in the range of $1 \cdot 10^4$-$3 \cdot 10^6$ with a polydispersity in the range of 1.8 to 2.7.

Table 1. Molecular weight reported for PHA production from different bacteria. [11]

Polymer	Molecular weight (g/mol)	Polydispersity
P3HB from *R. eutropha*	939.000-1.400.000	1,9-2,25
PHA from *P. oleovorans*	178.000-330.000	1,8-2,4
PHA from *P. putida*	56.000-112.000	1,6-2,3

PHAs have a wide range of applications owing to their novel features. Initially, PHAs were used in packaging films mainly in bags, containers and paper coatings. Similar applications as conventional commodity plastics include the disposable items, such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, shampoo bottles and cups [8].

In addition to potential as a plastic material , PHA are also useful as stereoregular compounds which can serve as chiral precursors for the chemical synthesis of optically active compounds [8]. Such compounds are particularly used as biodegradable carriers for long term dosage of drugs, medicines, hormones, insecticides and herbicides. They are also used as osteosynthetic materials in the stimulation of bone growth owing to their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements.
However, the medical and pharmaceutical applications are limited due to the slow biodegradation and high hydraulic stability in sterile tissues.

1.2. BIOSYNTHETIC PATHWAY

Many species of bacteria synthesize PHAs. The list of such microorganisms is growing and currently contains more than 300 organisms [8]. The chemical diversity of PHAs is large; of which the most well-known and widely produced form is PHB, which is the one studied in the present thesis. The synthesis of PHB is considered the simplest biosynthetic pathway. The process involves three enzymes and their encoding genes (Figure 4).

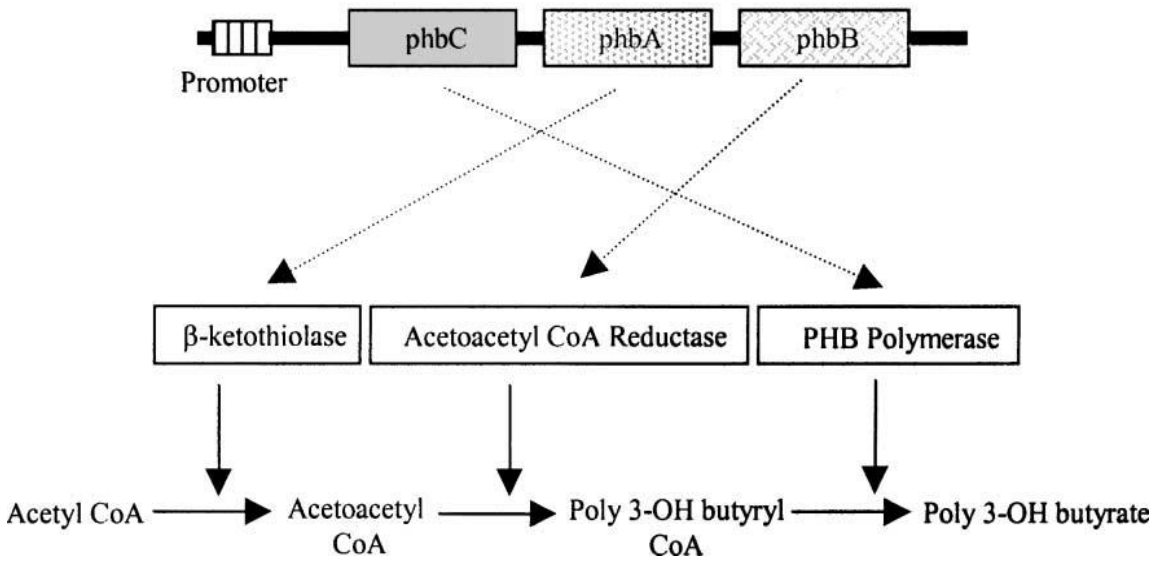


Figure 4. Biosynthetic pathway of poly(3-hydroxybutyrate). P(3HB) is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA), acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three-step pathway. The genes of the phbCAB operon encode the three enzymes. The promoter (P) upstream of phbC transcribes the complete operon (phbCAB) [5].

phaA gene encodes β-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. The next step is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA catalyzed by the acetoacetyl-CoA reductase [12]. The enzyme is encoded by the phaB gene and is NADPH-dependent. The last reaction is the polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase, which is encoded by the phaC gene. All three enzymes for PHB synthesis are located in the cytosol of the cell where PHB accumulation takes place [8].

PHA synthase in *Ralstonia eutropha*, formerly known as *Alcaligenes eutrophus*, reacts with a narrow range of substrates, with chain length of C3–C5 and prefers C4-substrates [12]. Therefore, PHAs obtained by this pathway contain short-chain-length monomers. Apart from PHB, bacteria also synthesize a wide range of other PHAs.

1.3. PHA PRODUCTION CONDITIONS

PHAs production is commonly carried out by fermentation using aqueous medium and different types of bacterial strains.

PHA synthesis, like all other biochemical processes, is affected by many factors: type of microorganisms (e.g. Gram-negative or Gram-positive), media ingredients, fermentation conditions and mode of fermentation (batch, fed-batch, continuous). For this reason, process conditions, including operating mode and control parameters, must be controlled to enhance suitable cell growth and metabolite production. These parameters are discussed below.

1.3.1. Operating mode

1.3.1.1. Batch culture

Batch fermentation refers to a partially closed system in which most of the materials required are loaded onto the fermentor, decontaminated before the process starts and then, removed at the end. The only material added and removed during the course of batch fermentation is the gas exchange and pH control solutions.

The principal disadvantage of batch processing is the high proportion of unproductive time (down-time) between batches, comprising the charge and discharge of the fermentor vessel, the cleaning, sterilization and re-start process. [13]

In PHA production, depending on the microorganism used, the substrate and the fermentation volume, the experiment can be performed in 24 to 48 hours. During this time, the microorganism goes through the main phases: lag, growth, stationary and finally death phase. The PHA produced in the fermentation can be consumed by the same microorganism; therefore this method rarely gives an indication of the maximum capacity of the cells to accumulate PHA [14].

1.3.1.2. Continuous culture

Continuous culture is a technique involving feeding the microorganism used for the fermentation with fresh nutrients and, at the same time, removing spent medium plus cells from the system. A unique feature of the continuous culture is that a time-independent steady-state can be attained which enables to determine the relations between microbial behavior (genetic and phenotypic expression) and the environmental conditions.

Continuous cultures are highly attractive for PHA production, but it has not been applied yet on a large scale [15].

1.3.1.3. Fed-batch processes

In fed-batch cultures, cells are grown under a batch regime for some time, usually until close to the end of the exponential growth phase. At this point, the reactor is fed with a solution of substrates, without the removal of culture fluid. This feed should be balanced enough to keep the growth of the microorganisms at a desired specific growth rate and reducing simultaneously the production of by-products that can lead to product inhibition or even to early cell death [16].

A fed-batch is useful in achieving high concentration of products as a result of high concentration of cells for a relative large span of time. Fed-batch fermentations can be also the best option for some systems in which the nutrients or any other substrates are only sparingly soluble or are too toxic to add the whole requirement for a batch process at the start.

Classically, PHA production is accomplished under fed-batch feeding conditions, where substrates are supplied to the fermentation broth when required [15]. However, in the present study, batch fermentation with nutrient limitation was used.

1.3.2. Microbial growth

To be able to live, reproduce and make products, a cell must obtain nutrients from its surroundings. A cell produces more cells, chemical products and heat from chemical substrates such as carbon as an energy source, nitrogen and phosphor.

In the present thesis, microorganisms are cultivated in a batch culture. Because no fresh medium is provided during incubation in this kind of processes, nutrient concentrations decline and concentrations of wastes increase, so certain phases of growth can be detected (Figure 5). The appearance and the length of each phase depend on the type of organisms and the environmental conditions [17][18].

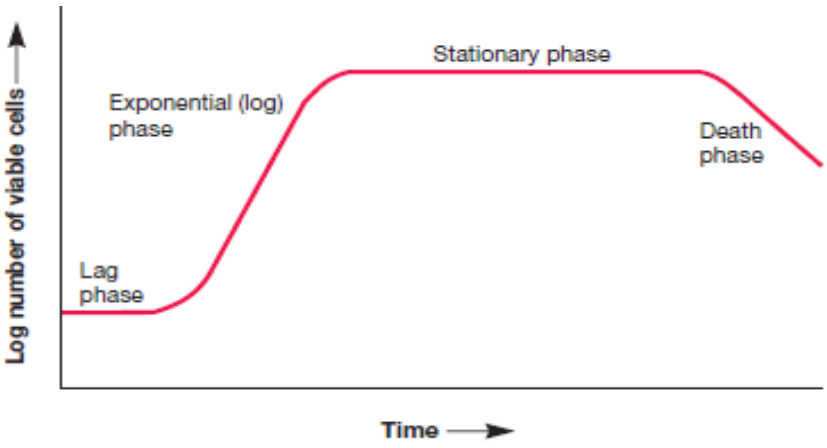


Figure 5. Microbial Growth Curve in a closed system. [17]

The first phase in the growth, where the growth rate stays almost constant, is the lag phase. The lag phase is caused for many reasons: the cells may be old and depleted of ATP, essential cofactors, and ribosomes, which must be synthesized before growth can begin; the medium may be different from the one the microorganism was growing in previously, so new enzymes would be needed to use different nutrients; possibly the microorganisms have been injured and require time to recover. Whatever the causes, eventually the cells begin to replicate their DNA, increase in mass, and finally divide.

During the exponential (log) phase, microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions. Their rate of growth is constant during the exponential phase; that is, they are completing the cell cycle and doubling in number at regular intervals [17].

In a closed system such as a batch culture, population growth eventually ceases. The growth rate slows down until it reaches zero and the stationary phase starts. In the stationary phase the number of the cells remains practically constant. Microbial populations enter the stationary phase for several reasons, for example, for nutrient or oxygen limitation, accumulation of toxic waste products or when a critical population level is reached [17]. When cells enter the stationary phase, PHA production starts due to nutrient limitation, as previously mentioned.

The last phase is called the death phase. During the death phase the cells begin to die and the growth rate decreases.

To get the highest production yields, many PHA fermentations are carried out in two stages. The aim is to produce a high cell density culture in the first stage (growth) and then to increase the concentration of PHAs during the second stage that is usually a nutrient limited fermentation [3]. During the second stage the biomass keeps on increasing even though the cells have stopped growing since they produce PHA intracellularly.

1.3.3. Control parameters

1.3.3.1. Medium

The choice of media is important not only to supply optimal conditions for production of a variety of PHAs in different bacteria but also to do so with high volumetric productivity to provide a final product that is economically competitive with the traditional plastics.

The choice of media, partly, depends on whether the microorganism is wild type or recombinant and whether it needs nutrient limiting conditions. Production of homopolymers or copolymers is another factor in the choice of media ingredients. A variety of homopolymers and copolymers with molecular weights between 50,000 and 1,000,000 Da and more than 100 different monomers, produced using different media, have been reported [3]. The carbon source used in this project was glucose, since it has been proved to obtain high PHB concentrations in previous investigations [19][20][21].

1.3.3.2. Aeration and foam control

Aerobic organisms, like *Ralstonia eutropha*, the one used in this study, are completely dependent on atmospheric O_2 for growth. For a suitable growth, it is necessary to provide extensive aeration. This is because the oxygen that is consumed by the organisms during growth is not replaced fast enough by diffusion from the air. Forced aeration of cultures is therefore frequently needed and can be achieved either by vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium through a fine glass tube or porous glass disc. Aerobes usually grow much better with forced aeration than when oxygen is provided by simple diffusion [17].

Oxygen supply is essential for aerobic cellular respiration, but high aeration rates could cause the microorganisms to die due to certain oxygen derivatives that are toxic to microorganisms [17]. Oxygen in its ground state is referred to as triplet oxygen ($^3O_2$). However, other electronic configurations of oxygen are possible. One major form of toxic oxygen is called singlet oxygen ($^1O_2$), a higher energy form of oxygen in which outer shell electrons surrounding the nucleus become highly reactive and are able to carry out a variety of spontaneous and undesirable oxidations within the cell. Singlet oxygen is produced both photochemically and biochemically, the latter through the action of various peroxidase enzymes. Other highly toxic forms of oxygen include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$), all of them produced as by-products of the reduction of O_2 to H_2O in respiration (Figure 6). Flavoproteins, quinones, thiols, and iron-sulfur proteins, found in virtually all cells, can also carry out the reduction of O_2 to O_2^- [17].

$O_2 + e^- \rightarrow O_2^-$	Superoxide
$O_2^- + e^- + 2H^+ \rightarrow H_2O_2$	Hydrogen peroxide
$H_2O_2 + e^- + H^+ \rightarrow H_2O + OH\cdot$	Hydroxyl radical
$OH\cdot + e^- + H^+ \rightarrow H_2O$	Water
$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	(Overall reaction)

Figure 6. Four-electron reduction of O_2 to H_2O by stepwise addition of electrons. All the intermediates formed are reactive and toxic to cells except for water, of course. Adaptation from [17].

High aeration rates can also cause excess of foam. In small vessels foaming is minimal, but in big fermentors the formation of foam is an issue that requires intervention. It can lead to reduced yields since bursting bubbles can damage proteins [22]. It can also result in a loss of sterility if the foam escapes, over-pressure if a foam-out blocks an exit filter, and a loss of culture suspension containing PHA and biomass.

1.3.3.3. pH and temperature

Temperature can affect living organisms in either of two opposing ways. As the temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates, and growth becomes faster. However, above a certain temperature, particular proteins may be irreversibly denatured, and cell functions fall sharply to zero. Moreover, below a certain temperature, enzymes cease to be catalytic. Therefore, for every organism there is an optimum temperature at which growth is most rapid [17].

Regarding pH effect, each organism has a definite pH growth range and pH growth optimum.

In PHA production, fermentation conditions depend on the demands of the microbe and often a temperature range of 30–37ºC is adopted, and pH is either left uncontrolled or is regulated linking to substrate (e.g. glucose) addition [3].

1.4. COMMERCIALIZED PHAs

The history of commercialized PHAs goes back to 1959.W. R. Grace and Company produced PHB in the U.S. for possible commercial applications. However, the company shut down the process due to low production efficiency and a lack of suitable purification methods [23].

In 1970, PHBV was commercialized by Imperial Chemical Industries Ltd. under the trade name of Biopol™ [23]. In 1996, the technology was sold to Monsanto and then to Metabolix, Inc. In 2008, Metabolix, Inc. announced the combined production of PHA Bio-based Polymers and Biomass Energy with a target to obtain PHA from switchgrass at a level of 20% of dry-cell weight, 75% of which could be recovered. Thus, if switchgrass yields are 10 to 15 tons per acre, then each acre will yield 1.5 to 2.25 tons of PHA bio-based polymers or derived chemicals, and 1 million acres will yield 3.3 to 5 billion pounds of PHAs [23].

Procter and Gamble, in partnership with Kaneka Corporation, Tsinga University in China, and the Riken Institute in Japan, has developed a wide range of applications for PHB and PHBH (Nodax™) as fibers, nonwoven materials, aqueous dispersions, and disposable products. However, Nodax technology was sold in 1993 [23]. Recently, Kaneka Corporation has announced its plan to launch the production of a plantderived soft polymer called Kaneka PHBH in 2010, with a production capacity of 1000 tons per year at Takasago City, Hyogo, Japan.

A German company, Biomer Inc. (Kraaling,Germany) produces PHB on a commercial scale for special applications. In 1993, Biomer acquired expertise and microbes for PHB products from the Austrian company Petrochemia Danubia and registered the trade name Biomer™ in 1995.

In Brazil, one of the largest sugar-exporting countries, PHB Industrial S.A. (Serrana) uses sugar cane to manufacture PHB (Biocycle™) in a joint venture started in 1992 between a sugar producer (Irmaoes Biagi) and an alcohol producer (the Balbo Group). The company has been running a pilot plant at 50 tons per year and plans to increase production capacity to 3000 tons per year [23].

In Canada, Biomatera Inc. specializes in the manufacture of PHA by fermentation of agricultural residues. The biopolymers are used in the manufacture of creams and gels that are used as slow-release agents in drug manufacturing and as cosmetic agents and tissue matrix regeneration [23].

In Japan, Mitsubishi Gas Chemical has made progress on the production of PHB from methanol fermentation (BioGreen™). Table 2 shows the potential of commercialized PHA to replace some petroleum-based plastics.

Table 2. The potential of commercialized PHA to replace the petroleum-based plastics [23].

Polymer	LDPE	PP	HDPE	PS	HI-PS	PVC	PET	PA	PBT
Mirel™	++	+	++		±	+	++	±	-
Biomer®	-	++	++	+	-	-	-	-	-
Nodax™	+	++	++	-	-	+	+	-	-
Biocycle®	-	++	++	+	-	-	-	-	-

(++) Means probable; (+) means possible; (±) means doubtful; (−) means unlikely.

1.5. A BIOPROCESS KEY PARAMETER: THE OXYGEN TRANSFER RATE

As previously said, in aerobic bioprocesses oxygen is a key substrate employed for growth, maintenance and in other metabolic routes, including product synthesis. Due to its low solubility in broths, which are usually aqueous solutions, oxygen must be continuously provided by a gas phase, and thus the knowledge of oxygen transfer rate (OTR) is needed for bioreactor design and scale-up.

During aerobic bioprocess, the oxygen is transferred from a rising gas bubble into a liquid phase and ultimately to the site of oxidative phosphorylation inside the cell, which can be considered as a solid particle. The transport of oxygen from air bubbles to the cells can be represented by a number of steps and resistances, as schematized in Figure 7; the liquid film resistances around bubbles usually control the overall transfer rate.

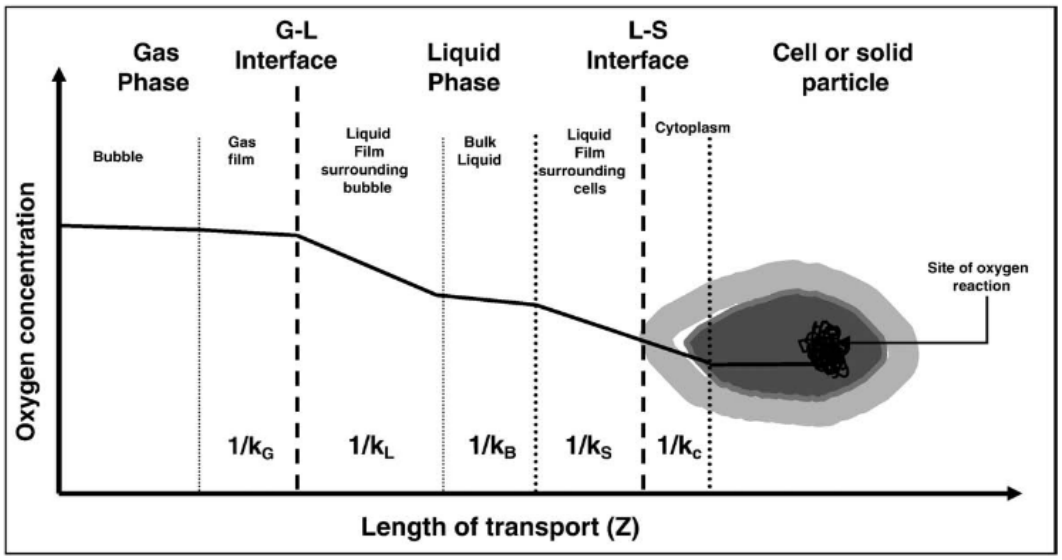


Figure 7. Steps and resistances for oxygen transfer from gas bubble to cell. (i) transfer from the interior of the bubble and gas film; (ii) movement across the gas–liquid interface; (iii) diffusion through the relatively stagnant liquid film surrounding the bubble; (iv) transport through the bulk liquid; (v) diffusion through the relatively stagnant liquid film surrounding the cells; (vi) movement across the liquid-cell interface; and (vii) transport through the cytoplasm to the site of biochemical reaction. [24]

The simplest theory on gas-liquid mass transfer is the two film model [25] and usually the gas–liquid mass transfer rate is modeled according to this theory (Figure 8). From the two film model, the equation for quantitatively determining the oxygen transfer rate (Eq. 1) is obtained, which is proportional to the volumetric mass transfer coefficient 'k_La'. The driving force is the gradient between the concentration of the oxygen at the interface and that in the bulk liquid (average concentration). Factors affecting this gradient include the solubility and metabolic activity. Gas (oxygen) solubility is mainly dependent on the temperature, the pressure, concentration and type of salts present and the chemical reactions.

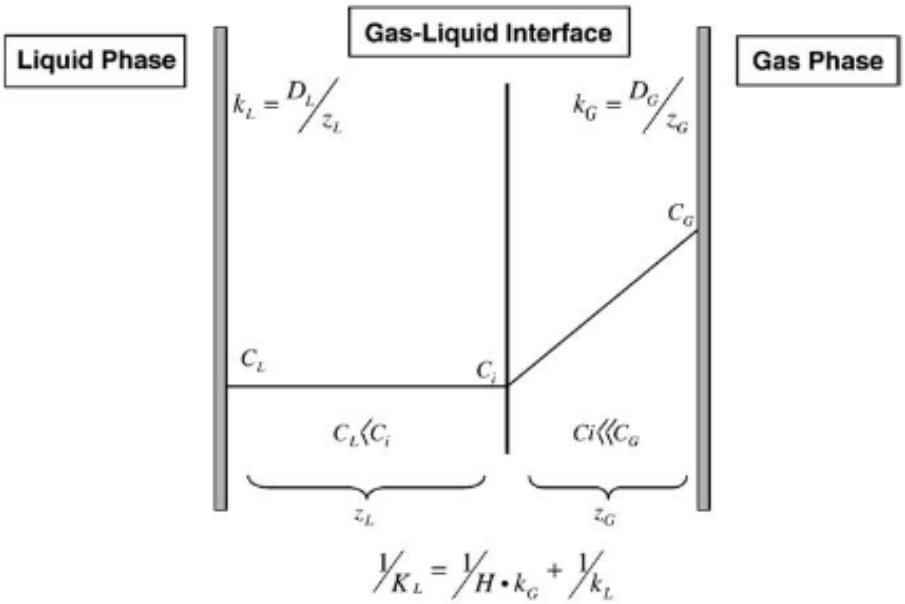


Figure 8. Schematic representation of the gas–liquid interface, concentrations and mass transfer coefficients K_L, k_L and k_G according to film theory. [24]

$$\text{Oxygen transfer rate (OTR)} = k_La\cdot(C^*-C_L) \qquad \text{Eq. 1}$$

Where C* is the saturation concentration of O_2 in the liquid; C_L is the actual liquid concentration (measured by Dissolved Oxygen probe); and k_La is the volumetric mass transfer coefficient.

Dissolved oxygen in the medium was found to be the limiting nutrient in all processes producing PHB [6]. To increase the oxygen supply, either k_La or C* can be increased. k_La can be enhanced, for example, by means of stirrer speed or optimizing the flow of gas into the bioreactor. C* is the saturation concentration of O_2 in the liquid phase, ant it can be enhanced by increasing the concentration of O_2 in the gas phase, feeding pure gas or a mix of air and pure oxygen.

In this project, different oxygen saturation concentrations will be tested for *Ralstonia eutropha* cultivation to study its effect on microbial growth and metabolite production.

Chapter II

Experiments and operation procedure

Chapter 2
Experiments and operation procedure

2.1. PROCESS DESCRIPTION

The batch fermentation was carried out in a 1L fermentor using strains of *Ralstonia eutropha* CECT 4635, following the steps discussed below.

2.1.1. Recovery of *Ralstonia eutropha* lyophilized culture

The freeze-dried *Ralstonia eutropha* culture was supplied in a glass ampoule containing a dried pellet with the microbe like the one shown in Figure 9. It also contained a cotton plug and a label on the outside.

Figure 9. Example of a freeze-dried culture vial.

For the culture recovery, the tip of the outer vial was heated in a flame and a few drops of sterile water were squirted on the hot tip using a sterile Pasteur to crack the vial glass. The broken glass was then stroke with ethanol-sterilized forceps to completely remove the vial tip. The insulation, the inner vial and the cotton plug were also gently removed with forceps. All the material and reagents used were previously sterilized, and the whole procedure was performed close to a flame to avoid contamination.

0,5 ml of liquid medium were aseptically added to the freeze-dried material with a sterile Pasteur pipette and mixed well to resuspend it. Part of the total mixture was transferred to a test tube containing 6 ml of the recommended broth medium, and the last few drops of this suspension were transferred to a slant tube containing solid medium. Both cultures were incubated under the appropriate conditions provided by the supplier (Table 3). As it was said, two different media were used (liquid and solid) in case one of them did not work, but since both of them grew well, only the liquid culture was used for further cultivations.

Table 3. Nutrient broth and incubation conditions for *Ralstonia eutropha* growth recommended by the Colección Española de Cultivos Tipo (CECT) organization.

Broth medium concentrations	
Beef extract	5 g/l
Peptone	10 g/l
NaCl	5 g/l
Agar powder [a]	15 g/l
pH	7,2
Temperature	30ºC

[a] Only for solid media.

After thousands of cells are formed in a culture medium it is possible to identify them with a microscope based on varied appearance since each species of bacteria exhibit characteristic morphology. However, bacteria are difficult to see with a microscope since they are small and, in order to see their shape, it is necessary to use a magnification of about 400x to 1000x. The available microscope was only 100x, so the cells could be seen (Figure 10) to prove the culture grew well but they could not be identified. Visual appearance identification was used instead, since *Ralstonia eutropha* broths have a characteristic white color.

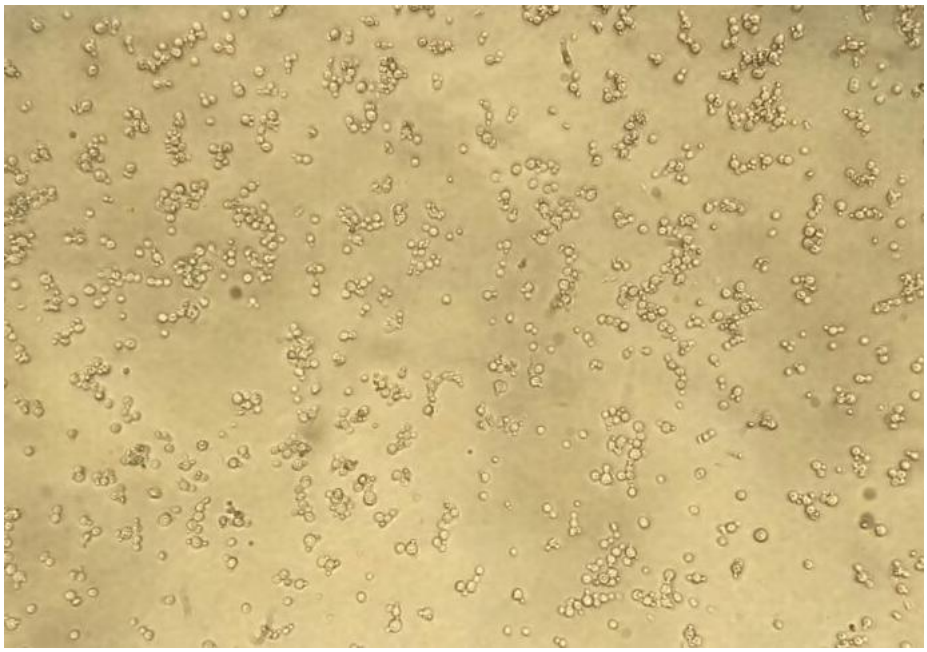


Figure 10. Cells in the recovered lyophilized culture using a 100x magnification microscope.

2.1.2. Strain preservation

Once the freeze-dried culture was recovered, part of the liquid culture obtained was re-cultivated in a 500 ml erlenmeyer using 100 ml of the same broth medium and the same conditions (Table 3). After 24h of incubation, several eppendorfs were filled with the culture medium and their conservation was performed using two different methods:

- Long-term preservation: the strain can be stored for a long period in case a focus of contamination appears if it is frozen. For that purpose, equal amounts of glycerol solution (30%) and the culture broth were mixed in eppendorfs and kept in the freezer.

- Short-term preservation: bacteria strains were kept for daily work in the liquid medium previously mentioned (Table 3) in 2 ml eppendorfs at 4ºC. These eppendorfs served as inoculums for each fermentation, and they were subcultivated every 15 days at 30ºC during 24h.

2.1.3. Seed culture preparation

For seed culture preparation, all the material used (Erlenmeyer, Büchner funnel and kitasato) was previously sterilized in the oven by dry heat sterilization.

For each fermentation, 100 ml of a growth medium (composition sown in Table 4) was sterilized by filtering using a 0,45 µm filter size and it was transferred into a 500 mL Erlenmeyer. The seed culture was inoculated with cells kept in the fridge (see section 2.1.2) and it was then incubated for 48h at 30°C and a pH value of 7,2 on a shaker at 700 rpm (Figure 11).

Table 4. Composition of the seed culture medium.

Component	**Concentration (g/l)**
Glucose	10
$(NH_4)_2SO_4$	1
$MgSO_4 \cdot 7H_2O$	0,2
KH_2PO_4	1,5
$Na_2HPO_4 \cdot 12H_2O$	9
Citric acid	-
Trace element solution	1ml/l

Table 5. Composition of the metal traces solution.

Component	**Concentration (g/l)**
$FeSO_4 \cdot 7H_2O$	10
$ZnSO_4 \cdot 7H_2O$	2,25
$CuSO_4 \cdot 5H_2O$	1
$MnSO_4 \cdot 5H_2O$	0,5
$CaCl_2 \cdot 2H_2O$	2
$Na_2B_4O_7 \cdot 7H_2O$	0,23
$(NH_4)_6Mo_7O_{24}$	0,1
HCl 35%	10 ml/l

Figure 11. Seed culture incubation in a 500mL Erlenmeyer on a shaker at 700 rpm.

For a correct bacterial growth it is necessary to add a certain concentration of the metal traces solution shown in Table 5 to the seed culture. This solution contains the metal ions required by *Ralstonia eutropha* to act as cofactors for essential enzymatic reactions in the cell [15].

During the cultivation, the Optical Density of the media at 546 nm (OD_{546}) was measured to control the correct growth of the bacteria. When the desired OD_{546} value is achieved, enough growth is obtained to transfer the inoculum to the next stage. According to literature [26], this OD value is 0,6 when diluted 10 times. Generally, this stage requires a period of time of 24 to 48h, but such high values were never obtained in this study after 48 hours. The concentrations obtained were half of those needed.

2.1.4. Fermentative process and operating conditions

A 6425-214 chemical reactor (AceGlass Co) was used for the PHB production process. The initial vessel, which allowed pressure conditions up to 35 psig, was replaced by a 1L vessel which can only work at standard pressure conditions. A picture of the bioreactor and the assembled equipment is shown in Figure 12, and the process flow diagram is shown in Appendix B.

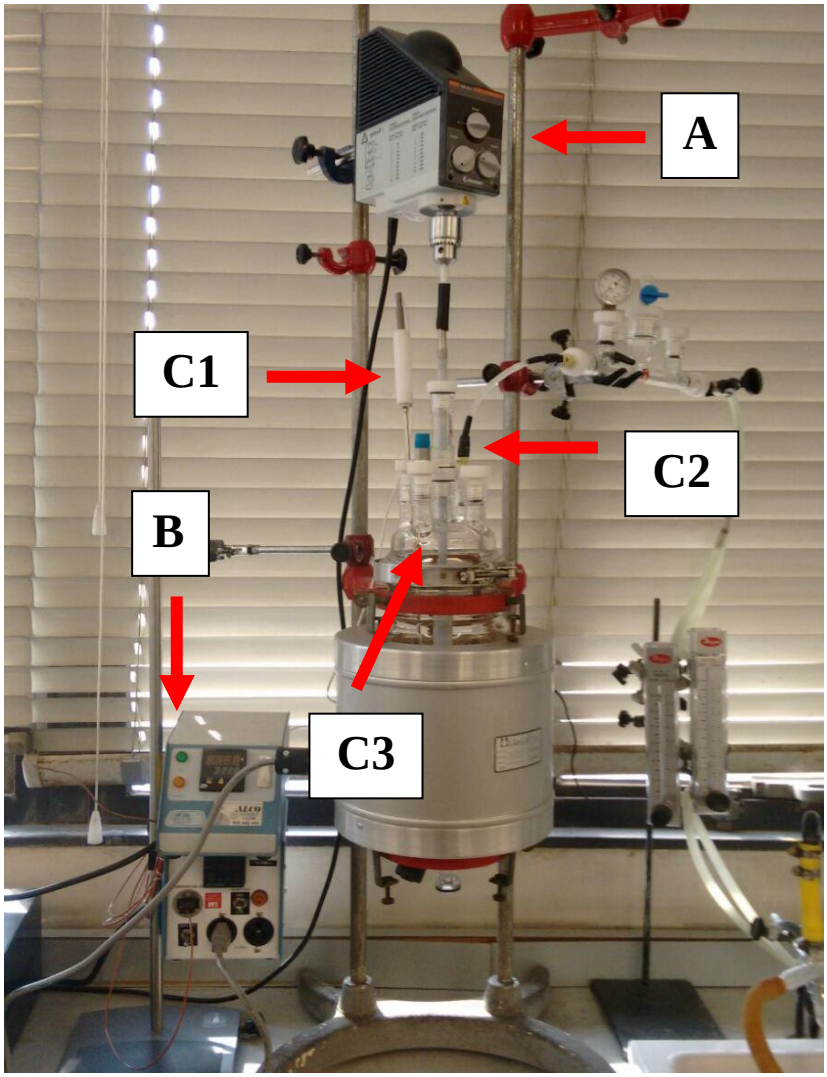


Figure 12. 6425-214 chemical reactor (AceGlass Co) with a 1L vessel.

The whole assembly is composed of a IKA RW20 stirring motor which achieves an agitation speed up to 2000 rpm (A); a temperature controller (B); a pure N_2 bottle and a pure O_2 bottle (not shown); and several ports: the temperature probe port (C1), the O_2 and N_2 connection (C2) and the sample-taking port (C3).

To start the fermentation, the reactor was first sterilized with boiling water for 2 hours, and it was left to cool down when the sterilization was finished. Afterwards, 600mL of the culture medium (Table 6) were sterilized by filtering using a 0,45 µm filter size, and it was transferred into the reactor. Once the culture was heated up to 30ºC using the temperature controller, the inoculation was carried out by transferring the seed culture (15 volume % of the total broth medium) to the bioreactor through a blind port with a sterile funnel previously cleaned with ethanol.

The pH was then adjusted to 6,8 with NaOH and HCl solutions, and the desired atmosphere was set allowing the oxygen and nitrogen to flow for some minutes through the reactor at the desired O_2 concentration. Finally, the reactor was closed tightly and the culture was incubated at 30 °C for 48 hours with a stirrer speed of 500 rpm. The stirrer speed was limited for structural reasons, since higher rates caused the reactor to vibrate.

Table 6. Composition of the culture medium.

Component	**Concentration (g/l)**
Glucose	20
$(NH_4)_2SO_4$	4
$MgSO_4 \cdot 7H_2O$	1,2
KH_2PO_4	13,3
$Na_2HPO_4 \cdot 12H_2O$	-
Citric acid	1,7
Trace element solution	10ml/l

Five different fermentations were conducted varying the oxygen concentration inside the reactor, which are summed up in Table 7. The last fermentation concentration was decided taking into account the results obtained in fermentations 1-4.

Table 7. Experiments carried out in this study.

Fermentation	1	2	3	4	5
O_2 concentration (%)	21 (air)	50	75	100	65

During all the fermentation, three samples were daily taken through a sample taking port with a Pasteur pipette, and they were kept in the fridge until their analysis. After harvesting, the reactor was completely cleaned with a diluted bleach solution and rinsed with distillate water.

2.2. QUANTITATIVE AND QUALITATIVE ANALYSIS

2.2.1. Biomass determination

Biomass determination was carried out by means of turbidity and cell dry weight.

2.2.1.1. Turbidity measurement

A rapid and quite useful method of estimating cell numbers is by turbidity measurements by spectrophotometry. A cell suspension looks cloudy (turbid) to the eye because cells scatter light passing through the suspension. The more cells that are present, the more light is scattered, and hence the more turbid the suspension.

Spectrophotometry depends on the fact that microbial cells scatter light that strikes them. Because microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. At high cell concentrations, however, light scattered away from the photocell by one cell can be rescattered back by another. To the photocell this makes it appear as if light had never been scattered in the first place. At such high cell densities, the correspondence between cell number and turbidity therefore drifts from linearity

The extent of light scattering can be measured by a spectrophotometer and is called the absorbance (optical density) of the media, which is almost linearly related to cell concentration (Figure 13).

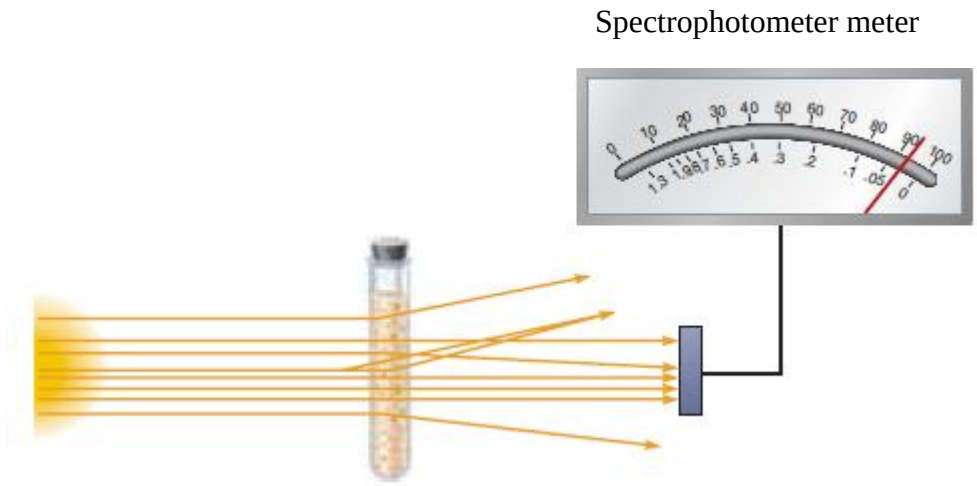


Figure 13. Determination of microbial mass by measurement of light absorption. As the population and turbidity increase, more light is scattered and the absorbance reading given by the spectrophotometer increases. [18]

2.2.1.2. Dry cell weight

Biomass concentration was also determined by dry cell weight. For that purpose, the supernatant present in a 1 mL sample of the culture medium was taken out from an eppendorf after centrifugation to separate it from the cells. The eppendorfs with the cells inside were then dried at 100ºC overnight. The next day, they were put in a dryer for 1 hour and the dried up samples were finally weighed. The analysis was carried out three times per sample.

The CDW can be calculated as follow:

$$\mathrm{CDW}\left(\frac{\mathrm{g}}{\mathrm{l}}\right) = \left(\mathrm{m}_{\mathrm{epp\ +pellet}} - \mathrm{m}_{\mathrm{epp}}\right) \mathrm{x}\ 1000 \qquad (\mathrm{Eq.}\ 2)$$

Where m_{epp} is the empty eppendorf weight and $m_{epp+pellet}$ is the dried samples mass.

2.2.2. PHB concentration and molecular weight determination

To follow up the PHB concentration along the fermentation processes, an analysis of the culture medium was carried for each sample. To that end, Gel Permeation Chromatography (GPC) was used. The PHB molecular weight was determined by GPC as well.

2.2.2.1. GPC operation mode

GPC is a type of liquid chromatography and so solid stationary and liquid mobile phases are used. However, the separation mechanism relies solely on the size of the polymer molecules in solution, rather than any chemical interactions between particles and the stationary phase.

A GPC instrument consists of a pump to push the solvent through the instrument, an injection port to introduce the test sample onto the column, a column to hold the stationary phase, one or more detectors to detect the components as they leave the column, and software to control the different parts of the instrument and calculate and display the results.

The polymer sample is first dissolved in a solvent. Once they have been dissolved, the molecules coil up on themselves to form a coil conformation. These coiled up polymer molecules are then introduced into the mobile phase and flow into the GPC column. The dissolved polymer molecules move past the beads as the mobile phase carries them down the column. Small polymer coils that can enter many pores in the beads take a long time to pass through the column and therefore exit the column slowly. Conversely, large polymer coils that cannot enter the pores take less time to leave the column, and polymer coils of intermediate size exit the column somewhere between these examples. This separating mechanism is shown in Figure 14.

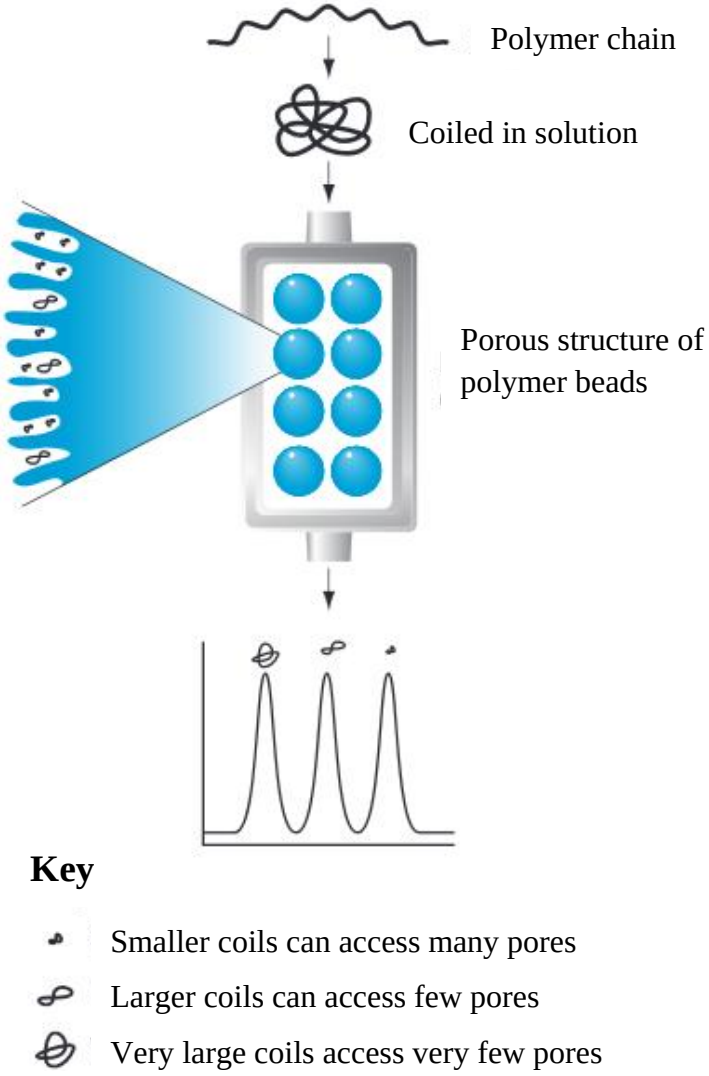


Figure 14. Scheme of how GPC separates molecules of different sizes. [27]

As the components exit the column they are detected in various ways, and the elution behavior of the sample is displayed in a chromatogram. The chromatogram shows how much material exited the column at any one time, with the higher molecular weight eluting first, followed by successively lower molecular weight (and therefore smaller) chains emerging later. The time it takes for a group of molecules of the same size (a fraction) to emerge from the column is called the retention time, because the molecules have been retained on the column

2.2.2.2. Calculations in GPC

For determining PHB concentration by GPC analysis, it is necessary to generate a calibration curve by injecting standards of several known concentrations. The peak for the component shown on the chromatogram is integrated and identified and the peak area is plotted against concentration to give a calibration curve. When an unknown sample is run, the peaks are integrated and identified based on their retention times, and the peak areas are related to a concentration from the calibration graph.

In addition to PHB concentration determination by using a calibration curve, GPC analysis also allows to determine the molecular weight of the polymer.

In polymers, molecular weight occurs not as a discrete value but as a distribution (Figure 15). There are several ways of describing molecular weight average:

- Number average molecular weight, abbreviated to Mn, marks the value at which there are equal numbers of molecules on each side, at higher and lower molecular weight. The value of Mn influences the thermodynamic properties of the molecule.

- Weight average molecular weight (Mw) is defined as the value at which there are equal masses of molecules on each side, at higher and lower molecular weight. Mw is large-molecule sensitive and influences the bulk properties and toughness of the polymer. Unsurprisingly, the Mw value is always greater than the Mn value unless the polymer is completely monodisperse. Mw affects many of the physical properties of polymers, and is the most often quoted molecular weight average.

- The ratio of Mw to Mn is used to calculate the polydispersity index (PDI) of a polymer, which provides an indication of the material's range of molecular mass. The broader the molecular weight distribution, the larger the PDI.

- Molecular weight of the highest peak (Mp) is the mode of the molecular weight distribution. Mp is quoted for very narrowly distributed polymers, such as polymer standards used in calibrations.

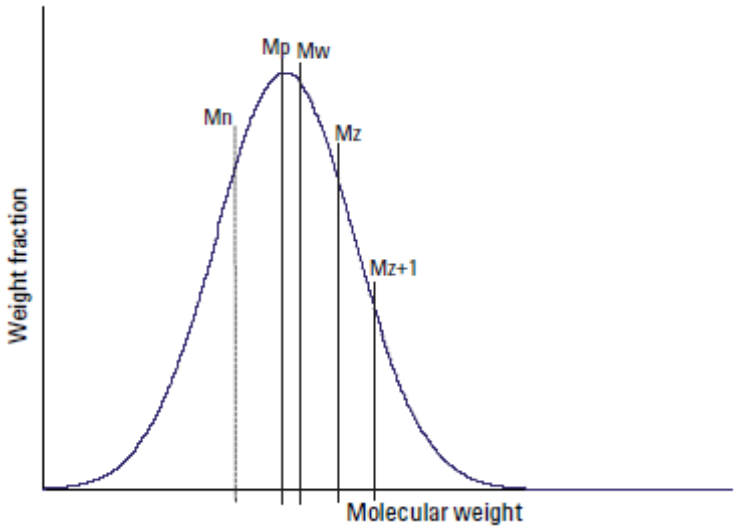


Figure 15. Average molecular weights of a mono-modal polymer. In this case, the distribution is nearly symmetrical. [27]

2.2.2.3. Sample preparation

Sample preparation is the key to getting the most out of GPC system. The aim is to obtain a crude extract free from the organic culture medium, because the chromatography becomes complicated with extraneous compounds.

The first step in preparing a sample for injection is to ensure it is completely dissolved and to remove particulate matter. Due to the organic character of the sample, it is needed to get rid of the water phase, which is done by centrifugation and supernatant removal. The pellet containing the cells and the polymer inside is then dissolved in hexafluoro-2-propanol (HFIP). Since the PHB concentration in some samples is too high for direct injection, the needed dilutions are performed before injection.

After dilution, using centrifugation to remove the residual biomass before injection protects the column filter from plugging and the system from pressure build-up.

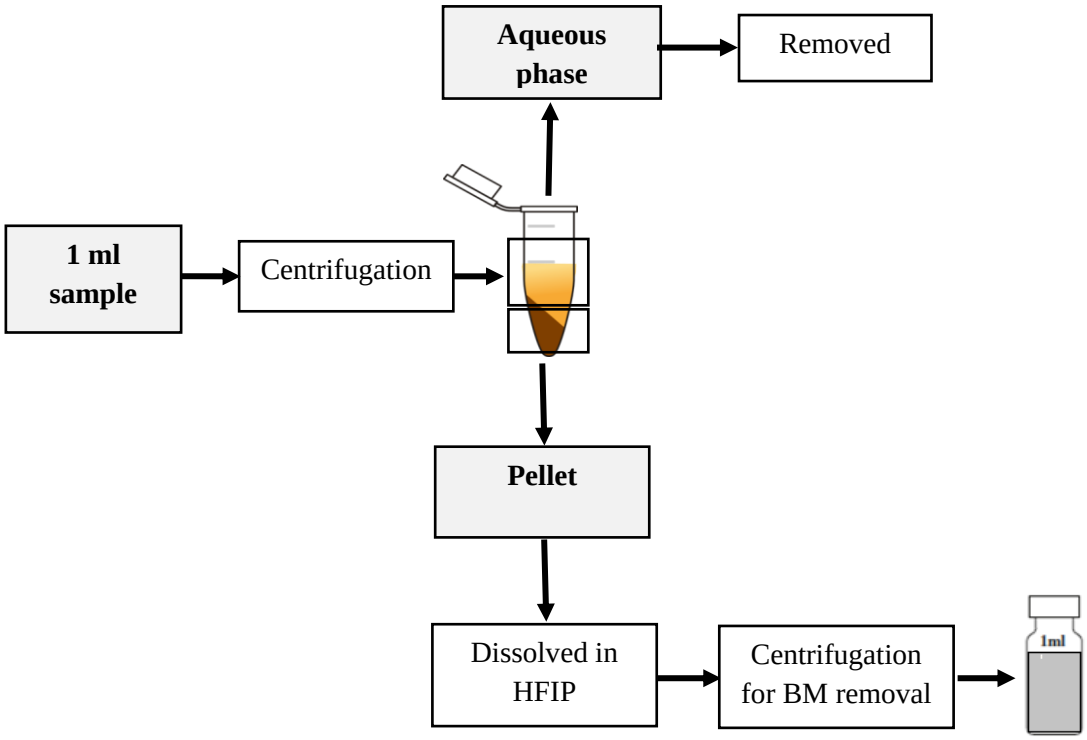


Figure 16. Scheme of the GPC sample preparation procedure.

2.2.2.4. Running the chromatograph

A 1260 Infinity chromatograph (Agilent Technologies) using a PL HFIPgel 300 mm X 7,5 mm column coupled to a UV detector was used for the analysis (Figure 17).

The mobile phase flow-rate was set at 1 mL/min, and temperature and pressure were room temperature and 35 bar respectively. The software used for the analysis is HPLC1260_GPC and its layout can be seen in Figure 18.

Figure 19 shows a typical PHB chromatogram, showing a broad peak at a retention time range of 4-8 min approximately.

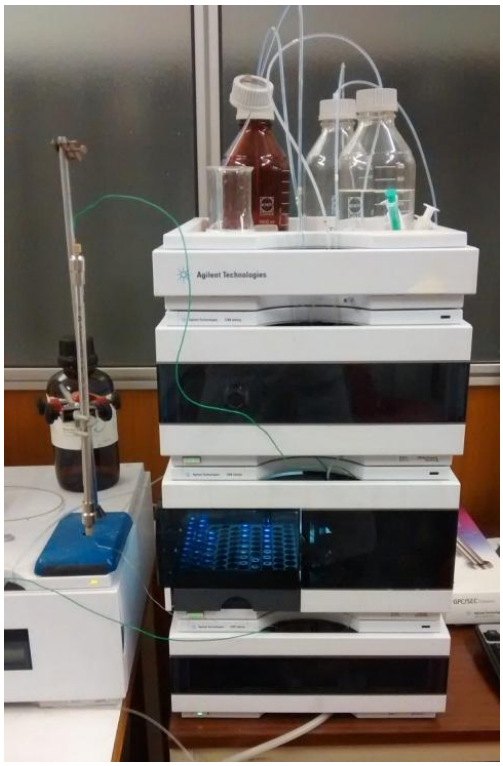


Figure 17. 1260 Infinity chromatograph GPC device.

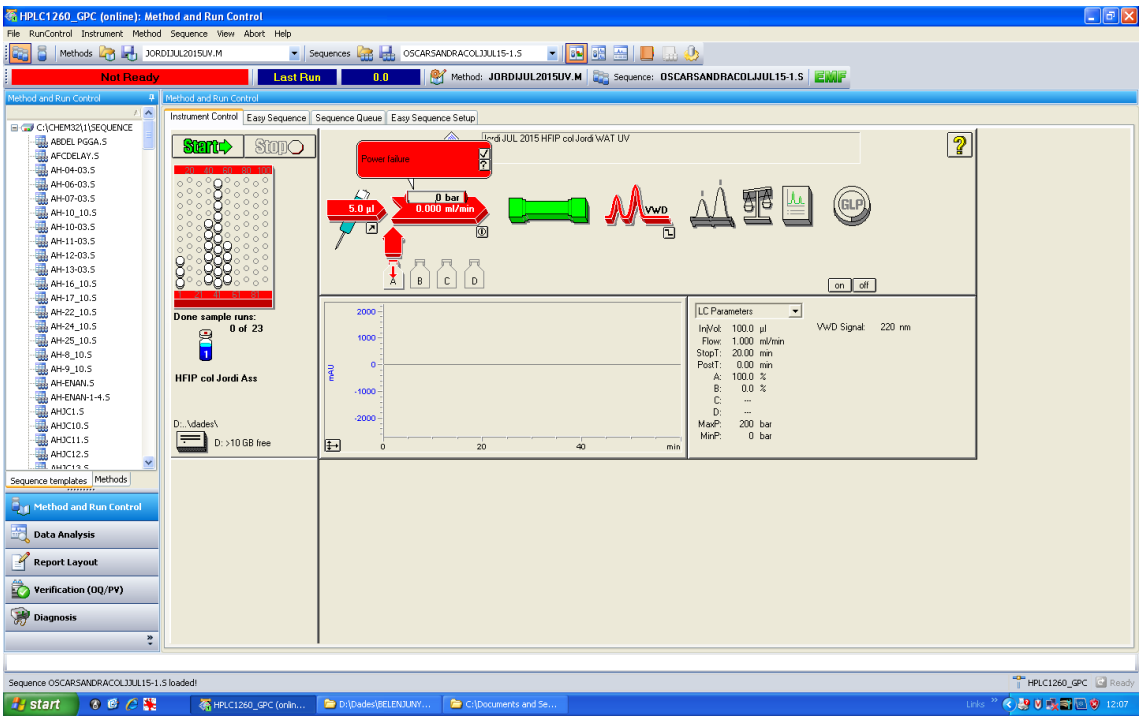


Figure 18. Layout of the HPLC1260_GPC software.

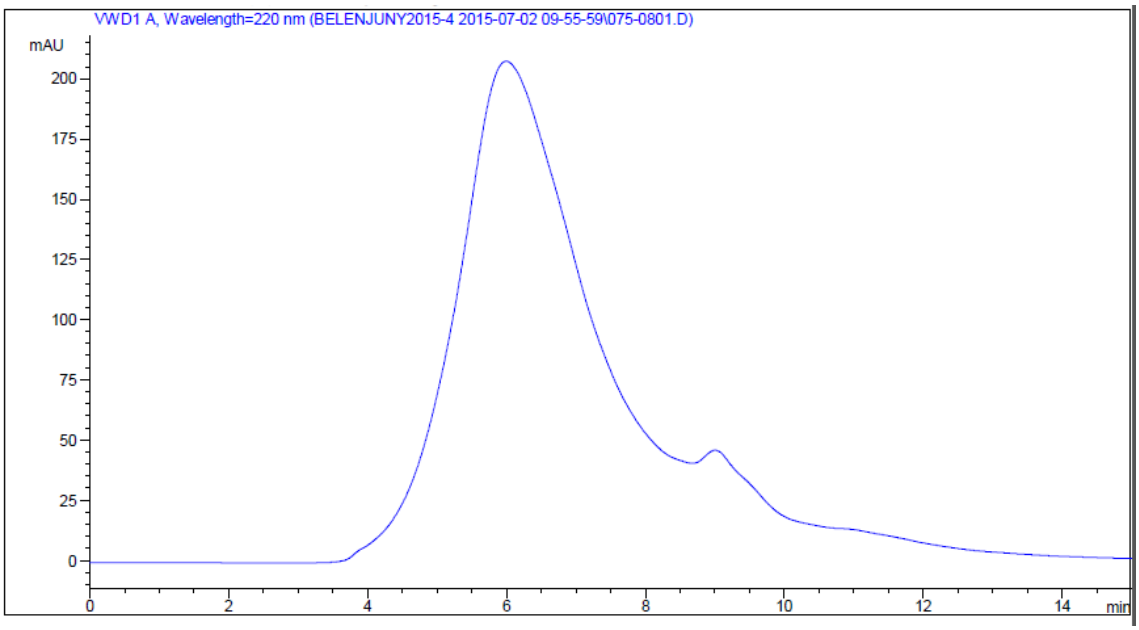


Figure 19. Chromatogram showing the analysis of a PHB solution by GPC analysis with UV detector. Column: PL HFIPgel 300 mm X 7,5 mm; room temperture; mobile phase: 1 mL/min HFIP.

2.2.3. PHB extraction

PHAs are intracellular granules surrounded by lipid molecules. For that reason, it is necessary to disrupt the cells to extract the product. The PHB was recovered from the fermentation broth using an adaptation of the method proposed by previous studies [28] with chloroform as solvent.

For that purpose, the final fermentation broth was concentrated by centrifugation, washed twice with water and dried. The biomass was then mixed with 50 volumes of chloroform for 48 hour at 25ºC to dissolve the polymer. The non-PHB biomass which did not get dissolved in chloroform was removed by filtration, and the resulting solution was left under the extractor hood so the chloroform got evaporated. The PHB extracted from 200 mL of the broth medium is shown in Figure 20.

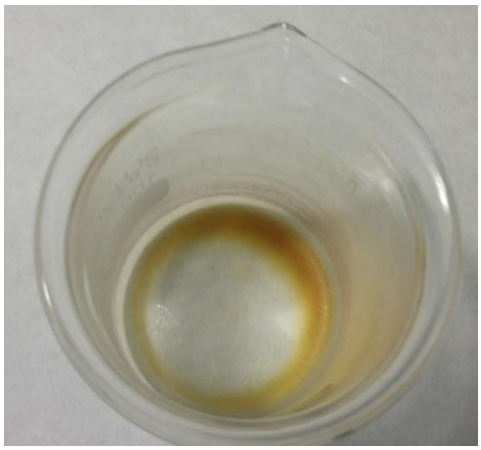


Figure 20. PHB extracted from 200 mL of the fermentation 5 broth medium.

2.2.4. Biopolymer characterization

The characterization of the biopolymer produced was done by FTIR analysis. A typical PHB FTIR-spectrum can be seen in Figure 21, which shows the characteristic peaks produced by the polymer.

In IR spectroscopy, an organic molecule is exposed to infrared radiation. When the radiant energy matches the energy of a specific molecular vibration, absorption occurs. The wavenumber, plotted on the X-axis, is proportional to energy; therefore, the highest energy vibrations are on the left. The percent transmittance (%T) or the absorbance (A) is plotted on the Y-axis. An absorption of radiant energy is therefore represented by a peak in the curve: zero transmittance corresponds to 100% absorption of light at that wavelength [29].

The IR spectrum of the PHB from is characterized by two intense absorption peaks; an absorption band at about 1730 cm^{-1} which is characteristic of carbonyl group, and a band at about 1280-1053 cm^{-1} which characterizes the valence vibration of the carboxyl group [30]. It is also important to consider another peak that characterizes PHB, which appears at 3440 cm^{-1} and is characteristic of the hydroxyl group at the end of the chain [15].

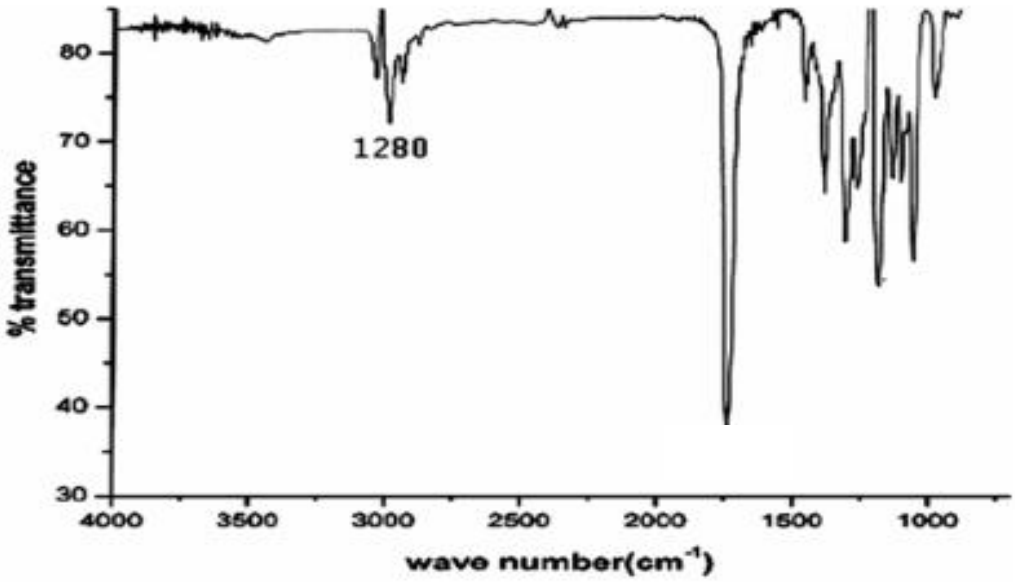


Figure 21. FTIR spectra of polymer purified from *B. thuringiensis* R1. [30]

For FTIR analyses, the samples were first dissolved in chloroform and the added to NaCl pellets. After complete solvent evaporation, FTIR spectra were recorded using a Fourier Transform Infrared Perkin Elmer spectrometer.

2.3. OVERALL OPERATION PROCEDURE

A scheme of the overall operation procedure is presented in Figure 22.

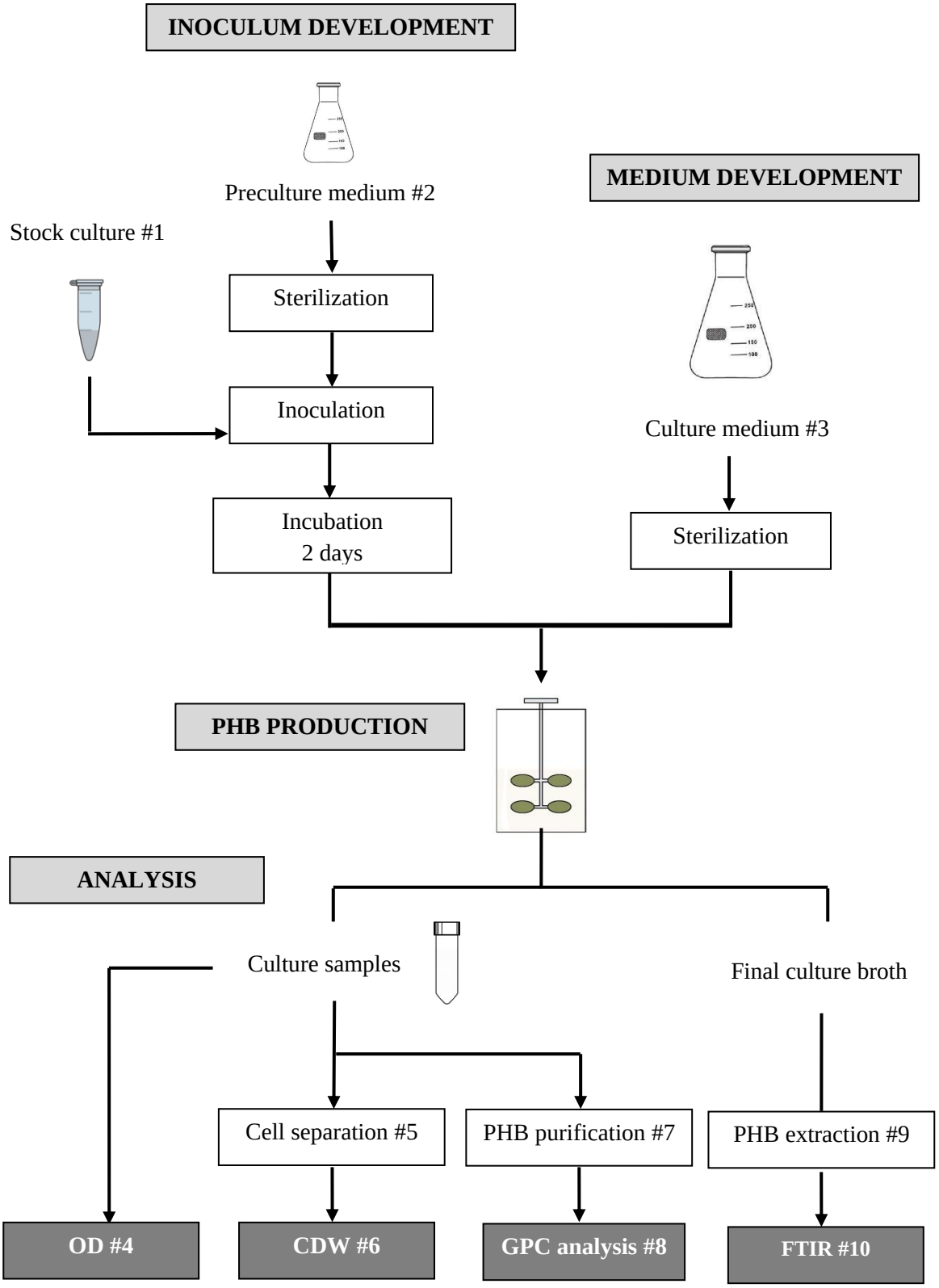


Figure 22. Generalized schematic representation of PHB production and separation process from *R. eutropha* bacteria. Number of experiment defined as #X.

Chapter III

Results and discussion

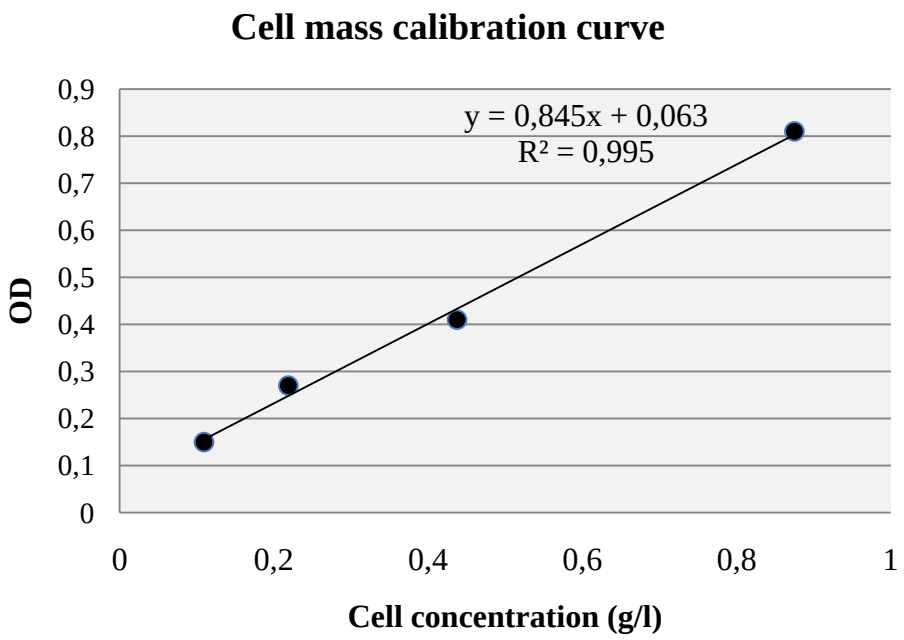
Chapter 3
Results and discussion

3.1. CALIBRATION CURVES

3.1.1. Cell mass calibration curve in spectrophotometer

To estimate the cell mass by spectrophotometry, a standard curve was first prepared to relate the cell concentration to the indirect measurement obtained by turbidity.

For that purpose, six solutions of different known cell concentrations were prepared with the biomass extracted from a seed culture of *Ralstonia eutropha*. Their optical density was measured and plotted against concentration (Graph 1).



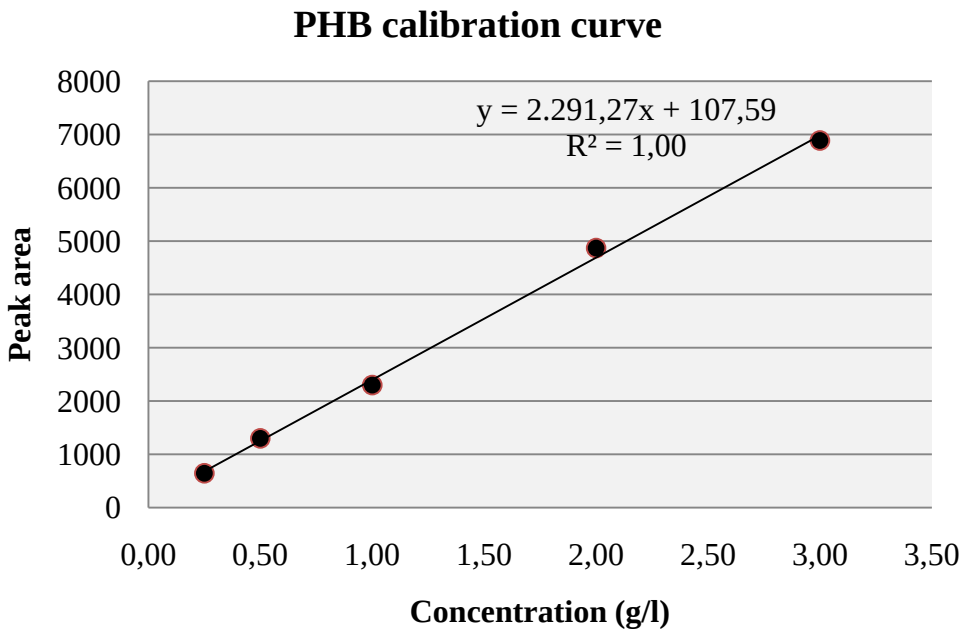
Graph 1. Turbidity calibration curve. Optical Density measured at 546 nm.

Solutions with higher cell concentrations were not in the range of linearity, so all further measures were performed within these limits.

3.1.2. PHB calibration curve in GPC

To generate the calibration curve, a constant volume of a pure PHB solution was injected at five different concentrations, individually, in the GPC.

Graph 2 represents the standard curve of PHB amount versus the average peak area obtained. The calibration curve is characterized by a high correlation coefficient, which leads to a low margin of error.



Graph 2. PHB calibration curve. Analysis performed by GPC. For detailed description of the measurement see “2.2.2.5. Running the chromatograph”.

Solutions with higher PHB concentrations were not in the range of linearity, so all further measures were performed within these limits.

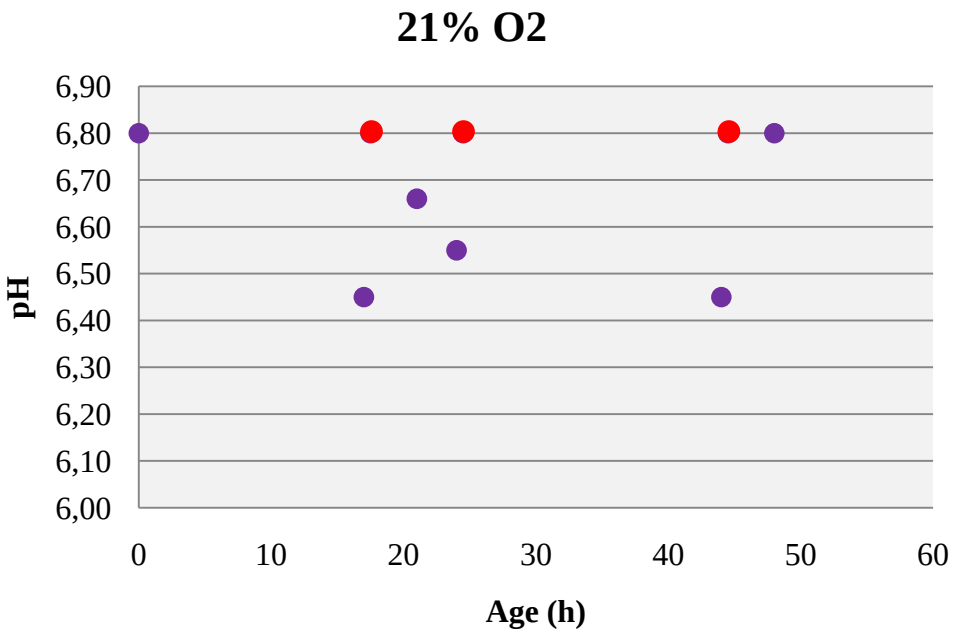
3.2. BIOPROCESS PARAMETERS

3.2.1. pH evolution

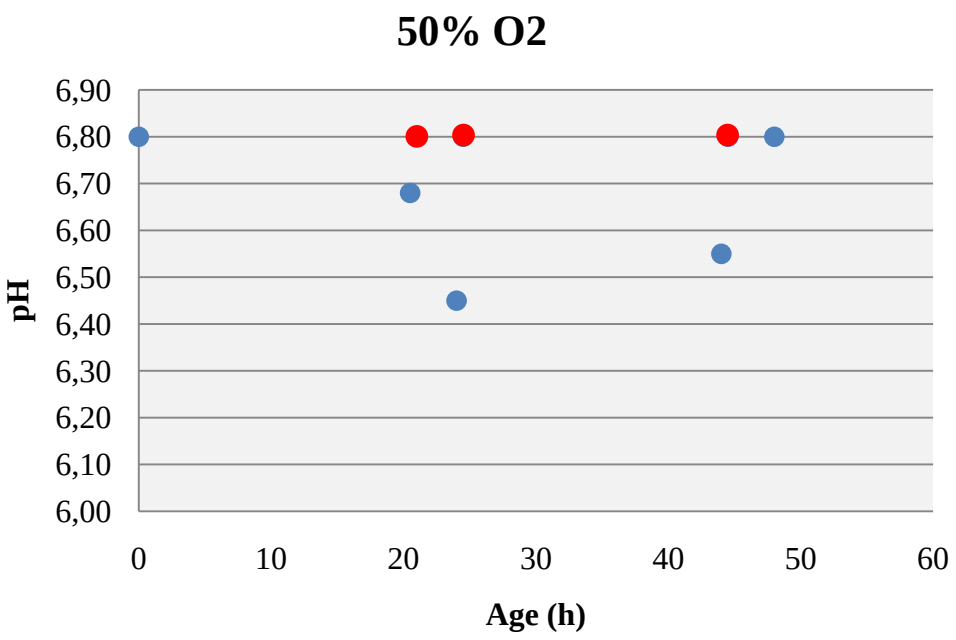
As previously said in Section 2.1.4, during all the fermentations carried out in the present thesis pH and temperature were maintained at 6,8 and 30ºC respectively.

However, pH was not regulated automatically but manually after sample-taking. Since this optimal value was not kept constant during the whole fermentation, the process behavior and results could be affected by these changes. A plot of the fermentation pH changes is shown in Graph 3, Graph 4, Graph 5, Graph 6 and Graph 7.

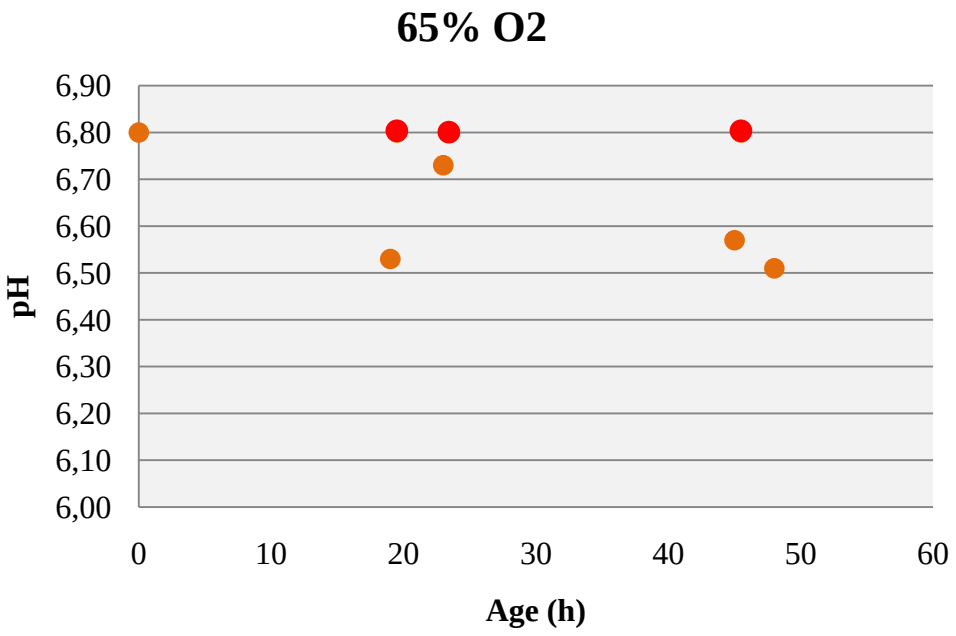
Variations in the pH of the culture medium may be indicative of metabolic activity. Bacteria extracellulary produce some agents influencing pH, usually acidic metabolitees when oxidizing sugars, which decrease pH value. Ammonia consumption as a nitrogen source also results in decrease of pH, since it causes the release of a proton.



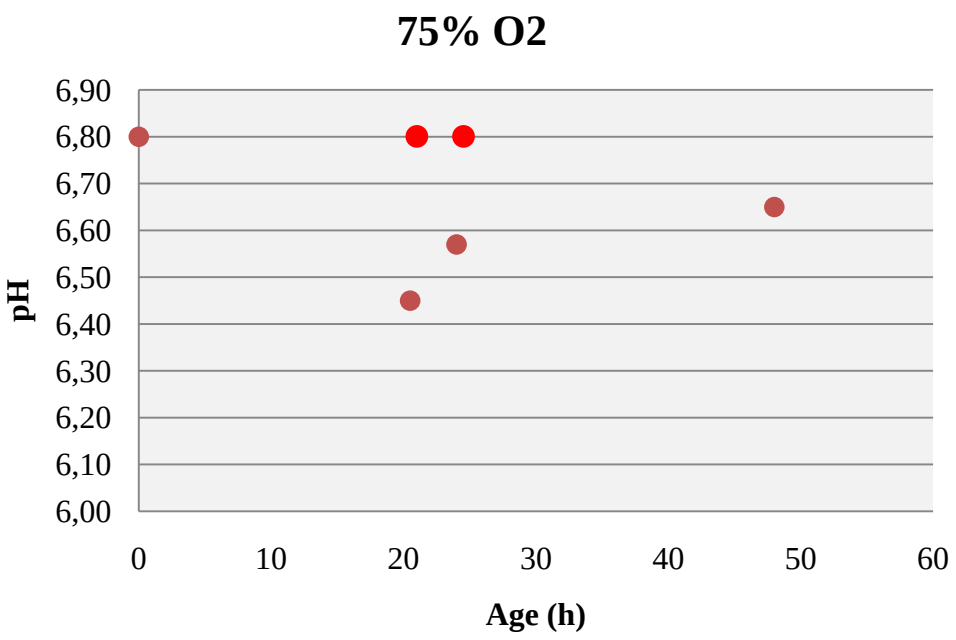
Graph 3. pH evolution of PHB production process from *R. eutropha* CECT 4635 in a 1L bioreactor with 21% air saturation. pH adjustment point is indicated by a red circle.



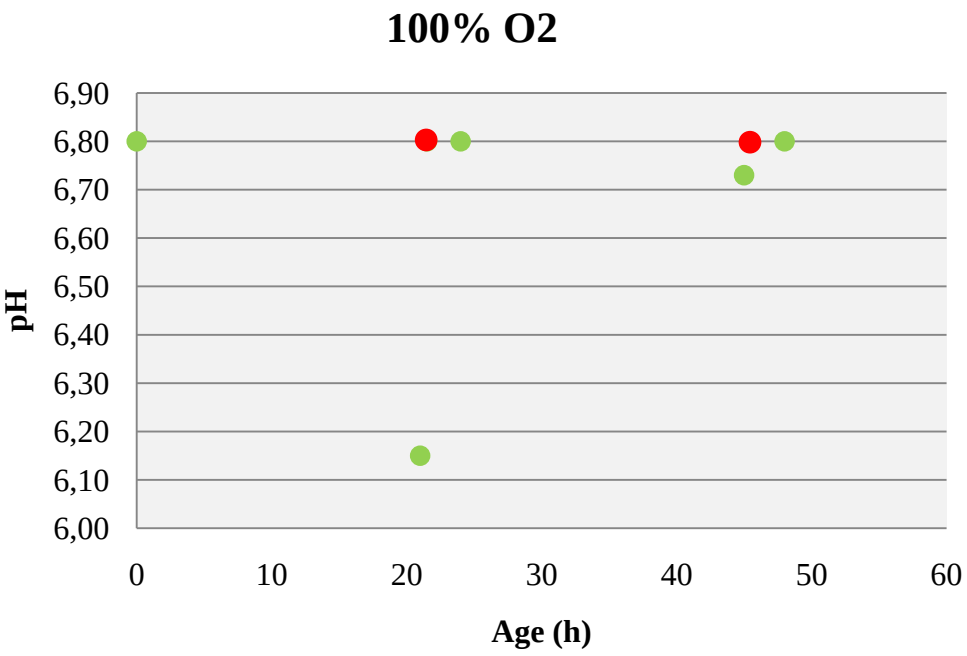
Graph 4. pH evolution of PHB production process from *R. eutropha* CECT 4635 in a 1L bioreactor with 50% air saturation. pH adjustment point is indicated by a red circle.



Graph 5. pH evolution of PHB production process from *R. eutropha* CECT 4635 in a 1L bioreactor with 65% air saturation. pH adjustment point is indicated by a red circle.



Graph 6. pH evolution of PHB production process from *R. eutropha* CECT 4635 in a 1L bioreactor with 75% air saturation. pH adjustment point is indicated by a red circle.



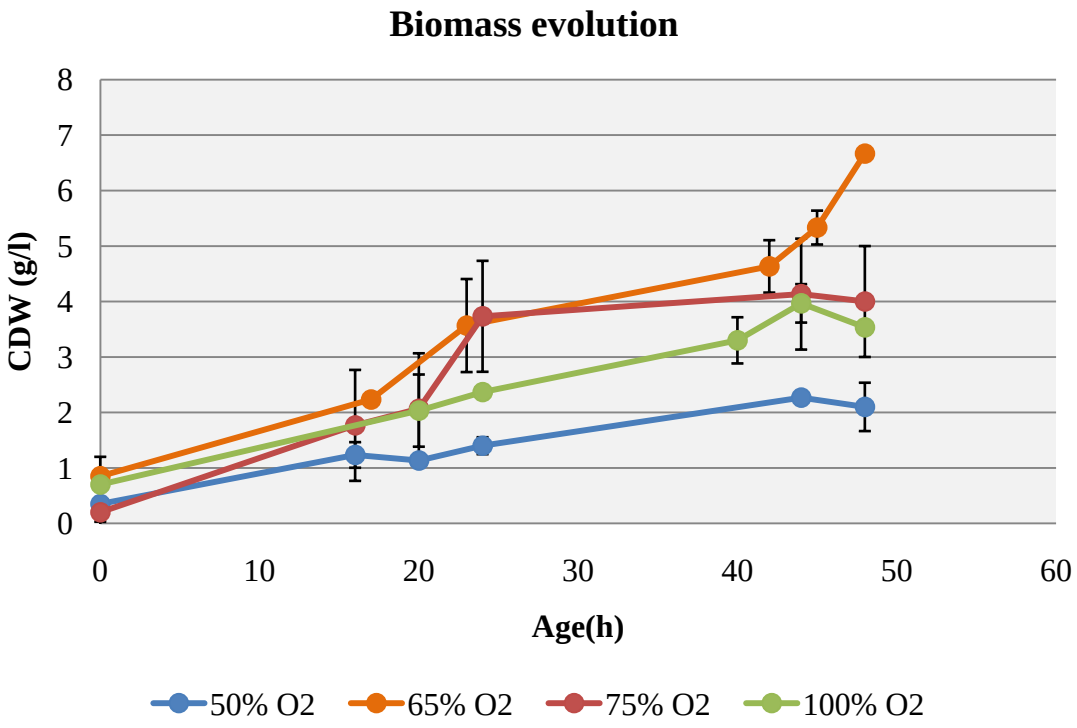
Graph 7. pH evolution of PHB production process from *R. eutropha* CECT 4635 in a 1L bioreactor with 100% air saturation. pH adjustment point is indicated by a red circle.

pH change may be related to sugar and ammonia consumption, as previously said, and it can be seen that it stops decreasing at high fermentation ages in all fermentations except the one with 65% air saturation. Nevertheless, pH changes might be indicative of many other metabolic processes.

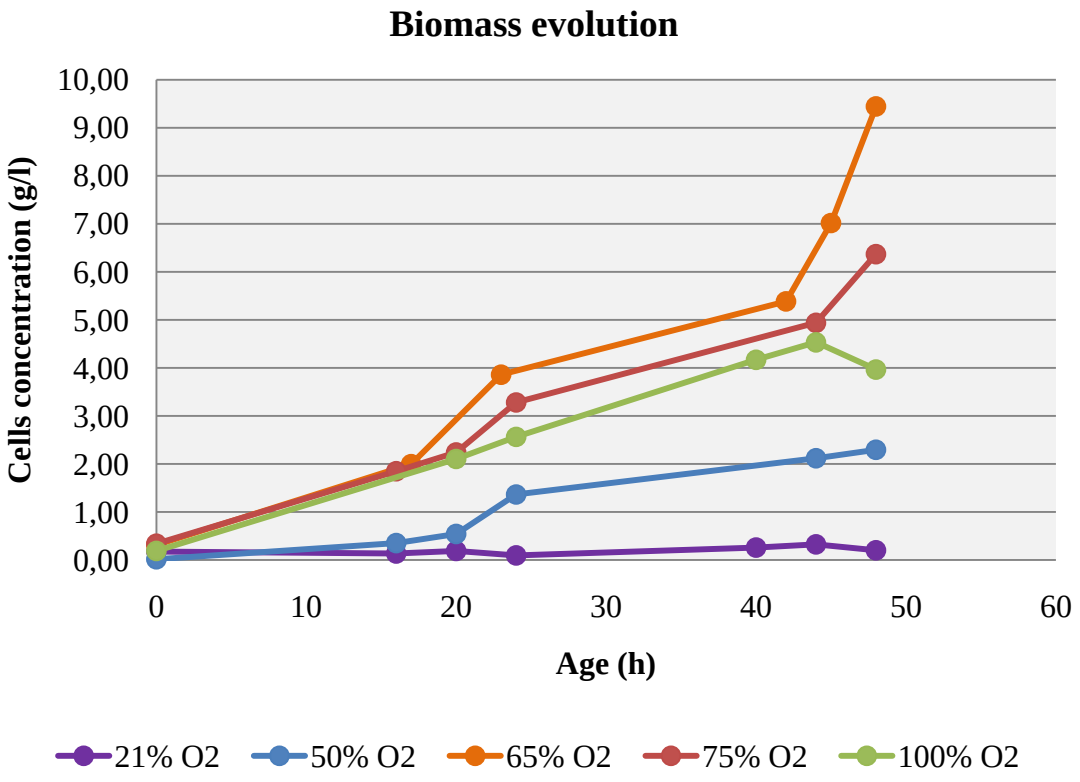
3.2.2. Cell concentration

Cells concentration was measured for each sample from all fermentations as well, using two different methods: OD measurement and CDW determination. The cell concentration evolution along the processes obtained by both methods is shown in Graph 8 and Graph 9.

Previous investigations [17] determined that, actually, the cell concentration remains constant after cells run out of an essential nutrient source, so that the CDW increase obtained in the results is due to the accumulation of material inside the cells. In previous investigations [31][19][20][21][32] nutrient limitation was obtained around 20 hours of fermentation.



Graph 8. CDW evolution of PHB production process from *R. eutropha* CECT 4635 with different oxygen concentrations. Standard deviation shown for 3 analyses per sample.



Graph 9. Cell concentration evolution measured by spectrophotometry of PHB production process from *R. eutropha* CECT 4635 with different oxygen concentrations.

CDW analysis for 21% air saturation fermentation was not carried out since the balance sensibility was not enough for weighting such low cell amounts.

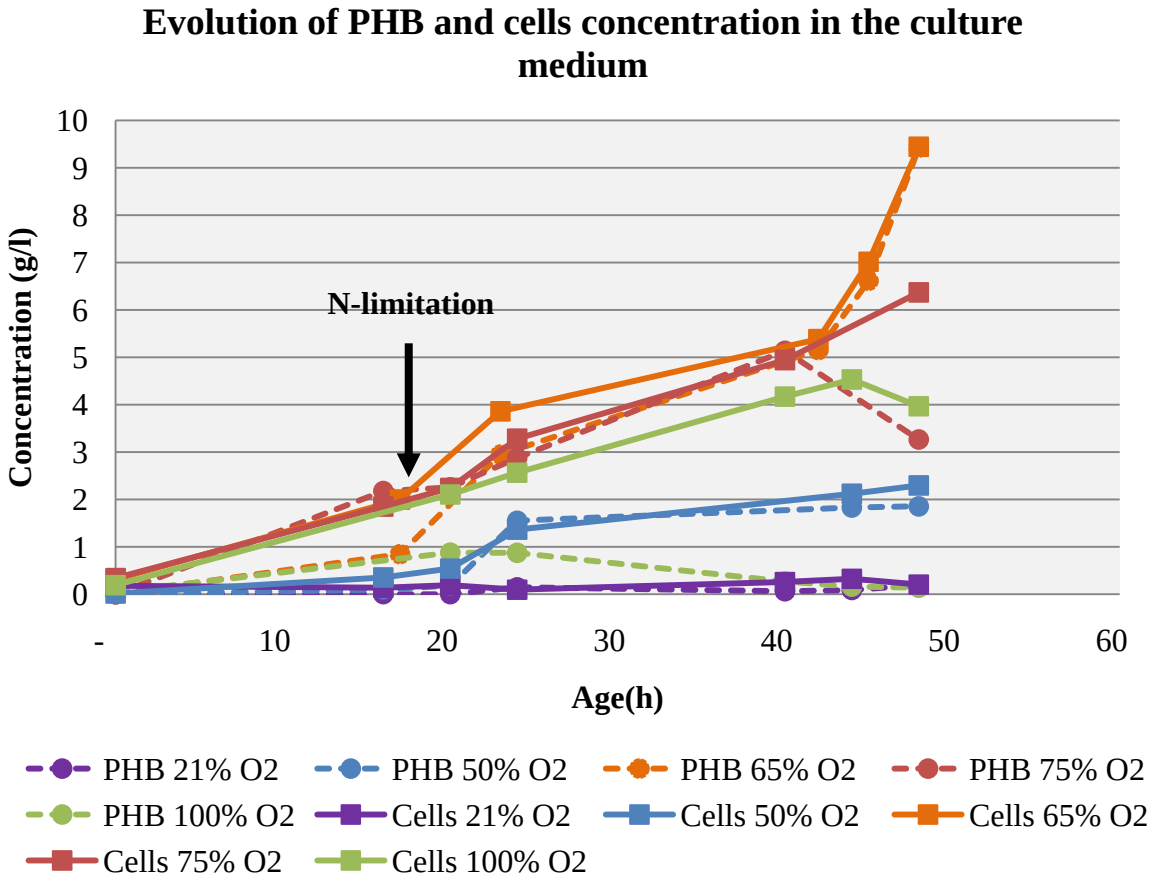
Both methods show the same trend in cells concentration, thought CDW analysis implies some biomass lost during the process. Moreover, it highly depends on the balance accuracy since very little cell amounts were measured. For this reason, lower values are obtained.

3.3. PHB RESULTS

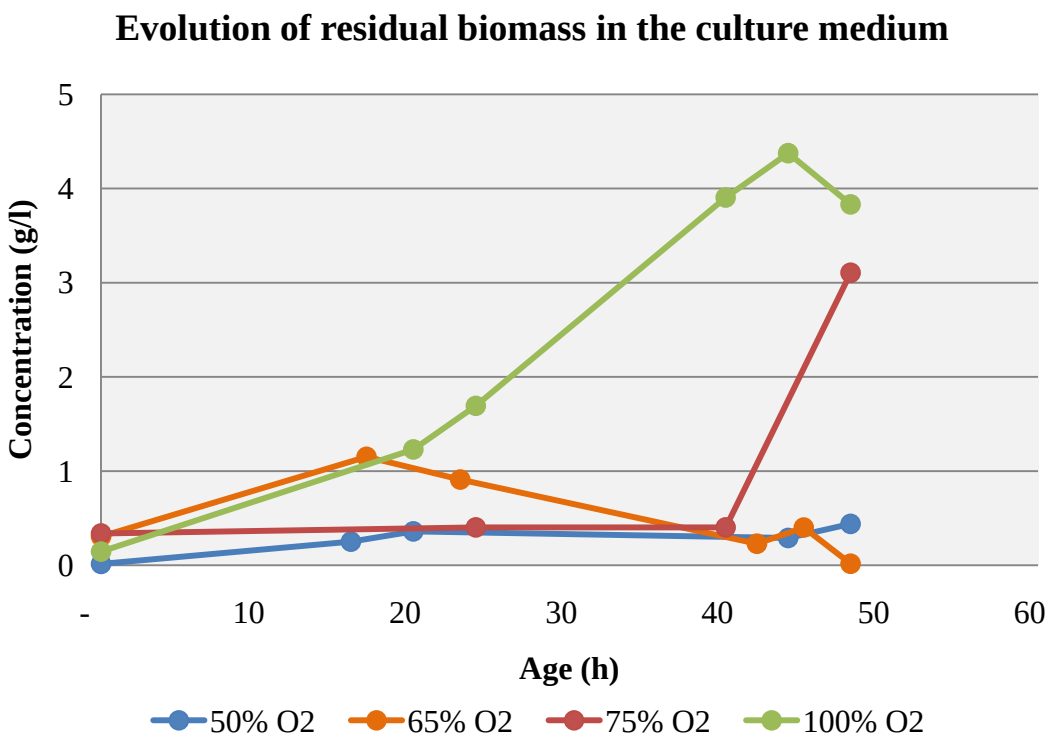
3.3.1. Evolution of the PHB concentration

After analyzing all the extracts by GPC, which were prepared as explained in Section 2.2.2.3, the broad PHB peak of the chromatograms (Appendix B) was identified by its retention time and integrated, obtaining the total peak area. The exact composition was then calculated using the equation of the calibration curve and applying the correspondent dilution factor.

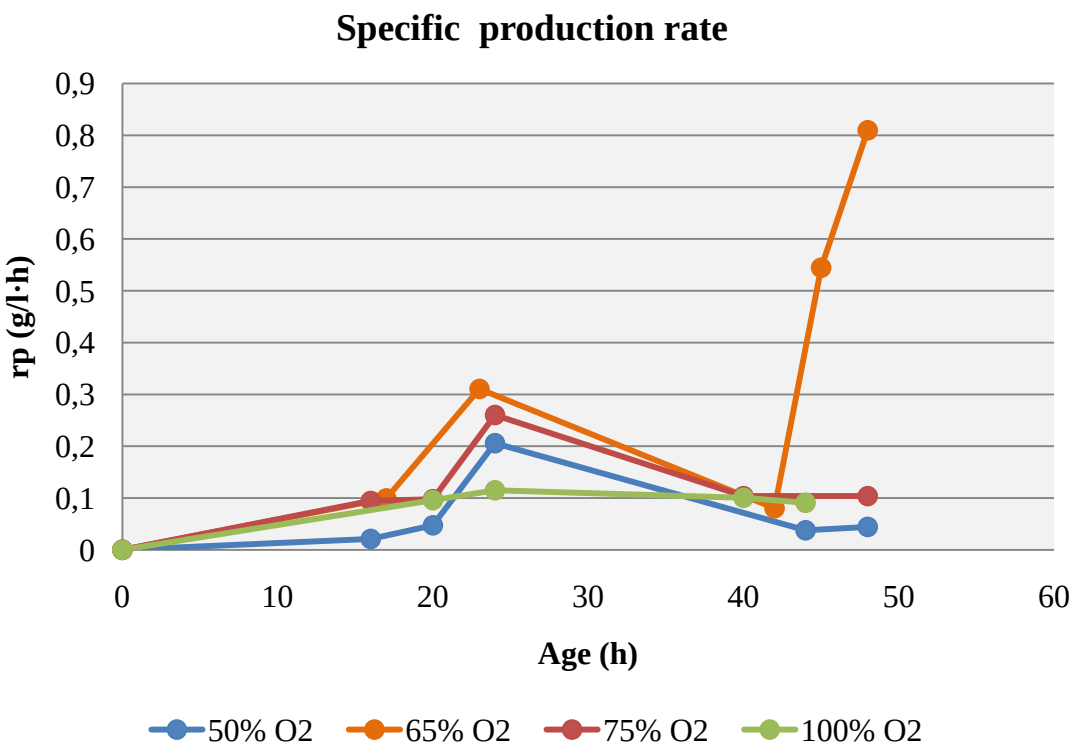
Graph 10 shows the PHB evolution for each fermentation as well as the cell concentration. Graph 11 shows the residual biomass evolution (RBM), calculated as the difference between the biomass concentration and the PHB concentration, and Graph 12 shows the specific production rate evolution (r_p).



Graph 10. Effect of oxygen in cell and PHB concentration evolution in *R. eutropha* CECT 4635 cultivation. N-limitation is supposed to start around 20h of fermentation. The process was terminated after 48 hours.



Graph 11. Effect of oxygen in residual biomass evolution in *R. eutropha* CECT 4635 cultivation. The process was terminated after 48.



Graph 12. Effect of oxygen in specific production rate evolution in *R. eutropha* CECT 4635 cultivation. The process was terminated after 48.

The production rate was calculated using Eq. 3, where P is the product concentration in g/L and t is the time in hours.

$$r_p = \frac{P_i - P_{i-1}}{t_i - t_{i-1}} \qquad (\mathrm{Eq.}\,3)$$

The specific growth rate was not determined since the cell growth was associated to PHB production when reaching the nitrogen limitation.

From the results, it can be said that the increase in cell growth was directly connected to an increase in PHB production in fermentations with 21%, 50%, 65% and 75% of oxygen concentration. In these cases, the residual biomass remains almost constant or even decreases, which means that the cell is accumulating a higher percentage of PHB inside.

However, with 100% of oxygen concentration cells do not seem to use glucose for PHB production but for their own growth, which can be seen in residual biomass increase. In this fermentation, moreover, high foam levels were obtained, which can cause a loss of culture suspension and biomass that may be the reason of cell concentration drop at 48 hours of fermentation. Besides, high oxygen concentrations can be toxic for cells after long exposure, as it was explained in Section 1.3.3.2.

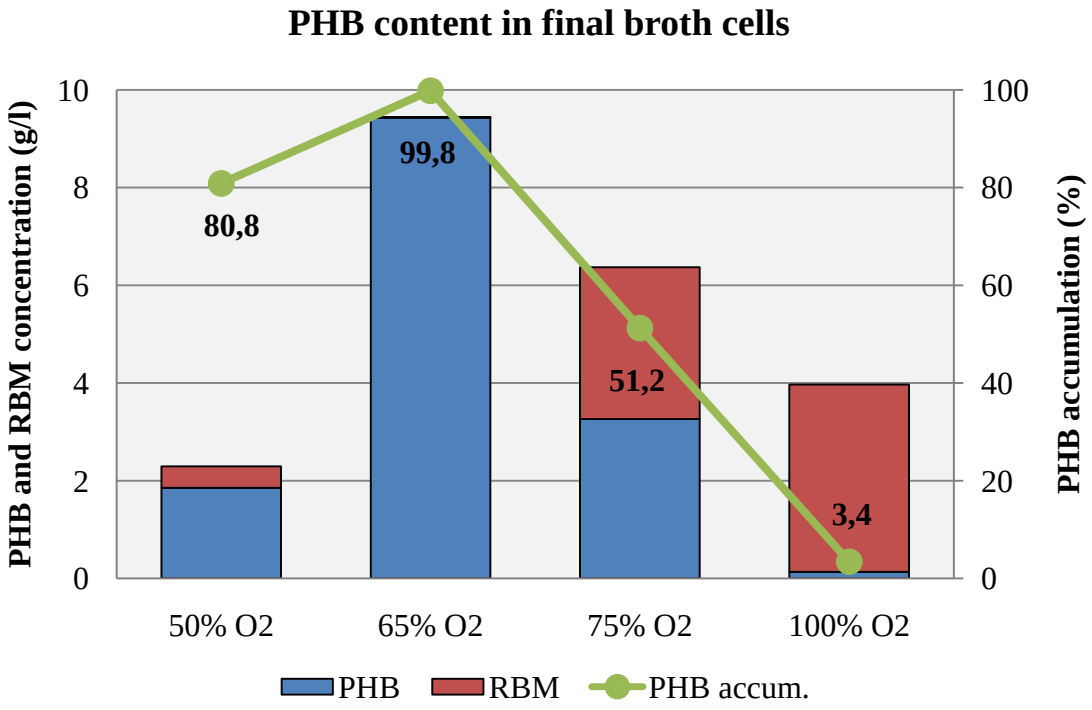
When using 65% of oxygen concentration it is seen that the polymer production starts around 20 hours, when the nitrogen limitation is supposed to start, but it keeps increasing even at the end of the fermentation. Probably the exponential production phase started at 40 hours instead of 20 hours, and this is not seen in the other fermentations because they need longer time to reach this phase. This could be tested, in further investigations, by increasing the total fermentation time and by monitoring dissolved oxygen (DO), carbon source and nitrogen source, which would give a lot of information about the bioprocess evolution.

The PHB decrease seen at the latest samples in 75% and 100% oxygen fermentation can be due to the cells taking up the intracellular PHB as a carbon source due to the lack of glucose, which is probably totally consumed at this point of the fermentation. In previous studies [31] it was shown that the glucose was totally consumed around 35 hours after starting the fermentation when operating with similar cell densities, which agrees with the moment in which this phenomenon is seen.

In previous investigations it was proved that cells of *P. aeruginosa* degraded the accumulated PHA at a rate comparable to that for the accumulation as soon as gluconate was depleted from the medium [33], and octanegrown cells of *P. ofeovorans* mobilized the accumulated PHA at a rather high rate [34]. In 1998 it was concluded that P3HB was a carbon and energy storage that slowed autolysis and cell death, since its degradation occurred rapidly in the absence of an exogenous carbon source and energy [35].

In other studies, fed-batch fermentation with glucose feeding was used to avoid carbon source depletion [20][21] and much higher cell densities and PHB values were obtained.

Taking into account the previous data, the final broth parameters can be obtained (Graph 13).

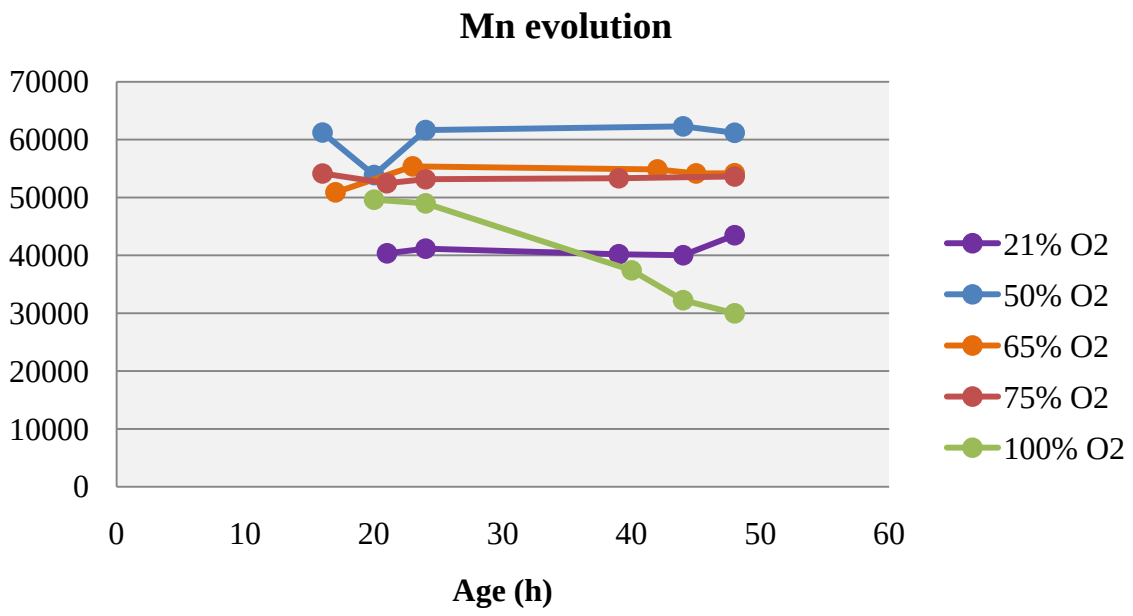


Graph 13. Effect of oxygen concentration on growth and accumulation of PHB in *R. eutropha* CECT 463 cultivation at the end of the fermentation (48 hours).

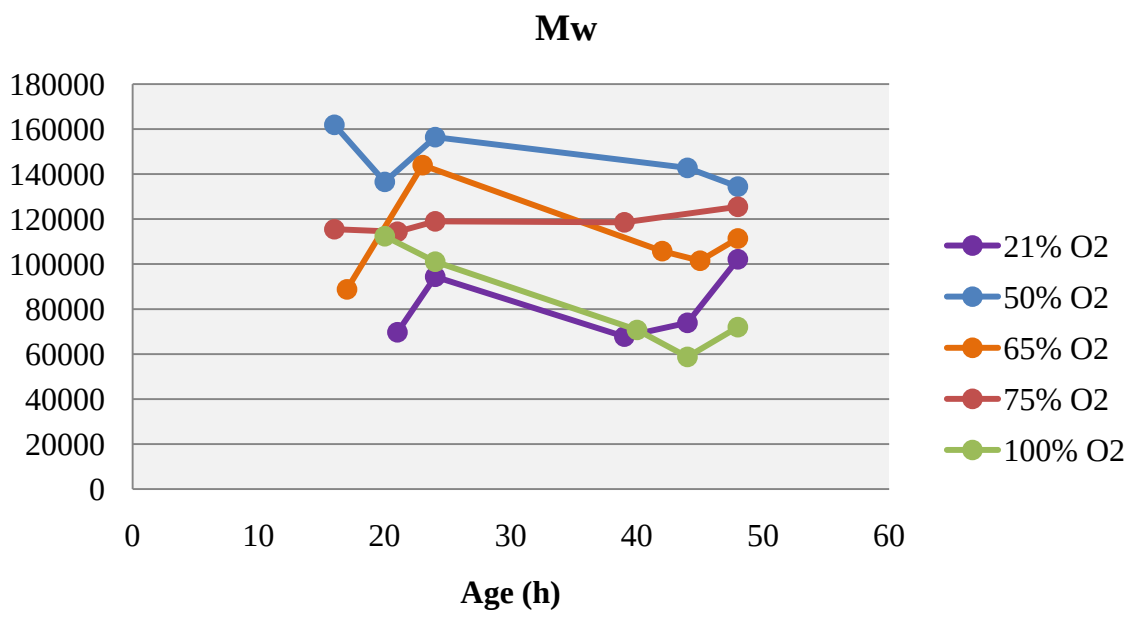
From Graph 13 it can be said that 65% oxygen concentration gave the best results for both PHB production in terms of total concentration and PHB accumulation in terms of percentage of total biomass, which had a high final value of 99,8% .

3.3.2. PHB molecular weight

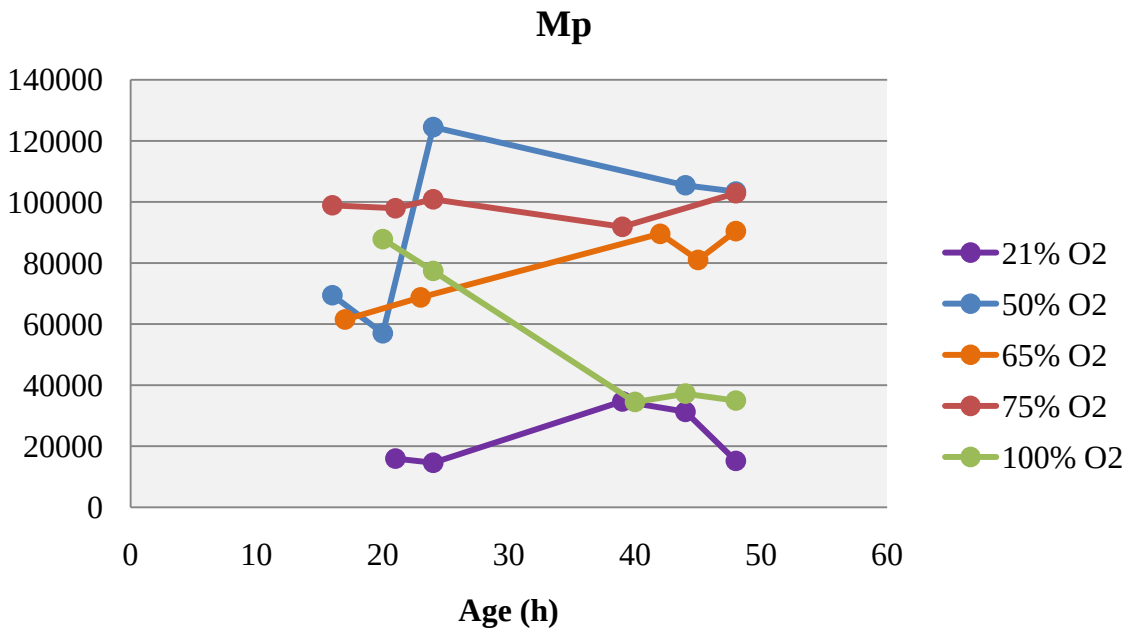
The evolution of the molecular weight along all the fermentations was also determined by GPC. Three parameters were calculated: the number average molecular weight (Mn), the weight average molecular weight (Mw) and the molecular weight of the highest peak (Mp) shown in Graph 14, Graph 15 and Graph 16.



Graph 14. Effect of oxygen concentration on PHB number average molecular weight.



Graph 15. Effect of oxygen concentration on PHB weight average molecular weight.



Graph 16. Effect of oxygen concentration on PHB average molecular weight of the highest peak.

The molecular weight decrease in fermentation with 100% of oxygen concentration may be due to the degradation of the polymer by the bacteria. This fact is in accordance with the initial hypothesis of the bacteria consuming the polymer produced due to an exhaustion of the carbon source.

However, the molecular weight values obtained for the rest of the fermentations, taking into account the experimental error made in preparing and analyzing the samples, are in the same order of magnitude, and they remain practically constant along the fermentation. For this reason, the molecular weight seems not to be significantly affected by the oxygen concentration.

The molecular weight values determined from the final broth polymer of all fermentations are shown in Table 8, which are also compared with data obtained in previous investigations.

Table 8. Comparison of PHB molecular weight produced by *R. eutropha* microorganism and different culture conditions.

Oxygen concentration (%)	Carbon source	Mn	Mw	Mw/Mn	Reference
21% O2	Glucose	43.400	102.100	2,3	This work
50% O2	Glucose	61.200	134.400	2,2	This work
65% O2	Glucose	54.200	111.350	2,1	This work
75% O2	Glucose	53.600	125.500	2,3	This work
100% O2	Glucose	30.000	71.900	2,4	This work
Air	DTDP[a]	76.000	251.000	3,3	36
Air	Soybean oil	330.000	-	3,3	37
Air	Soybean oil	400.000	-	4	37

[a] Thymidine diphosphate glucose

PHB accumulation data from Graph 13 and molecular weight data from Table 8, taken together, suggest a possible correlation between the amount of polymer accumulated and its Mn, as the values were slightly higher in cultures accumulating more PHB, opening the possibility of further increasing the Mn of the polymer obtained when optimizing PHB yields. This behavior was also observed in other investigations [38].

A low molecular weight is undesirable for industrial processing of the polymer, since the polymer properties highly depend on its molecular weight. The values obtained in this study are too low compared to those obtained in other investigations in *R. eutropha* cultures, but good polydispersity values were obtained, quite lower than those obtained in other references. Generally, polymers with polydispersity index close to one have better properties than those having an index much greater than one. On the one hand, low molecular weight species can act as plasticizers softening the material and not contributing at all to the polymer strength. On the other hand, high molecular weight species raise the viscosity of the melt polymer, increasing the difficulties in the forming process.

3.3.3. Polymer characterization

The FTIR spectrum of the final PHB for all fermentations are shown in Figure 23, Figure 24, Figure 25, Figure 26 and Figure 27, which highlight the main peaks that characterizes PHB.

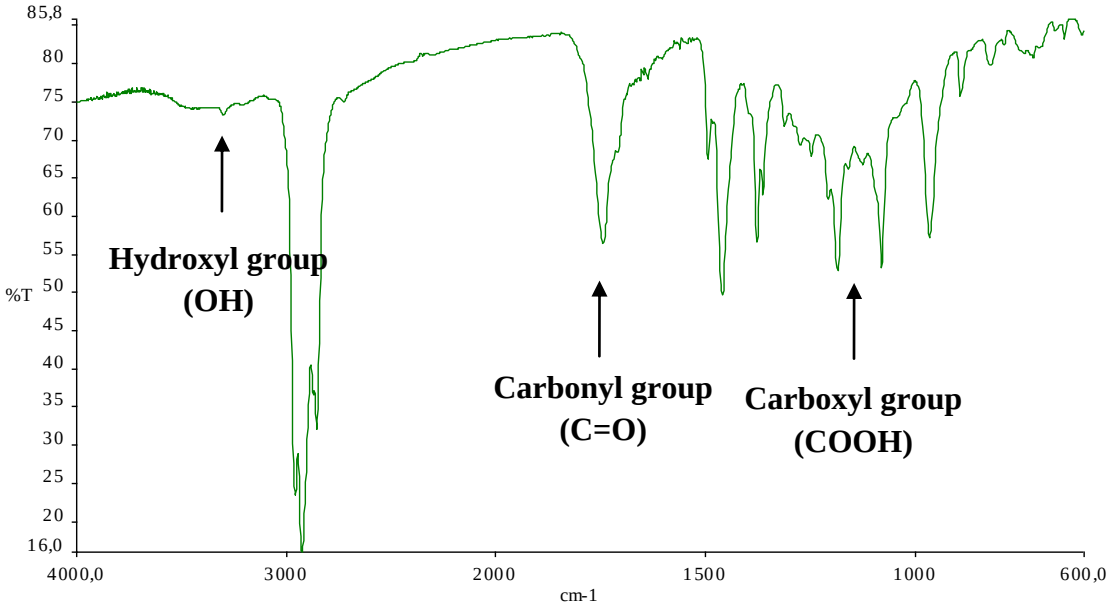


Figure 23. FTIR spectrum of the polymer extracted with chloroform from *R. eutropha* cultivation with 21% oxygen concentration.

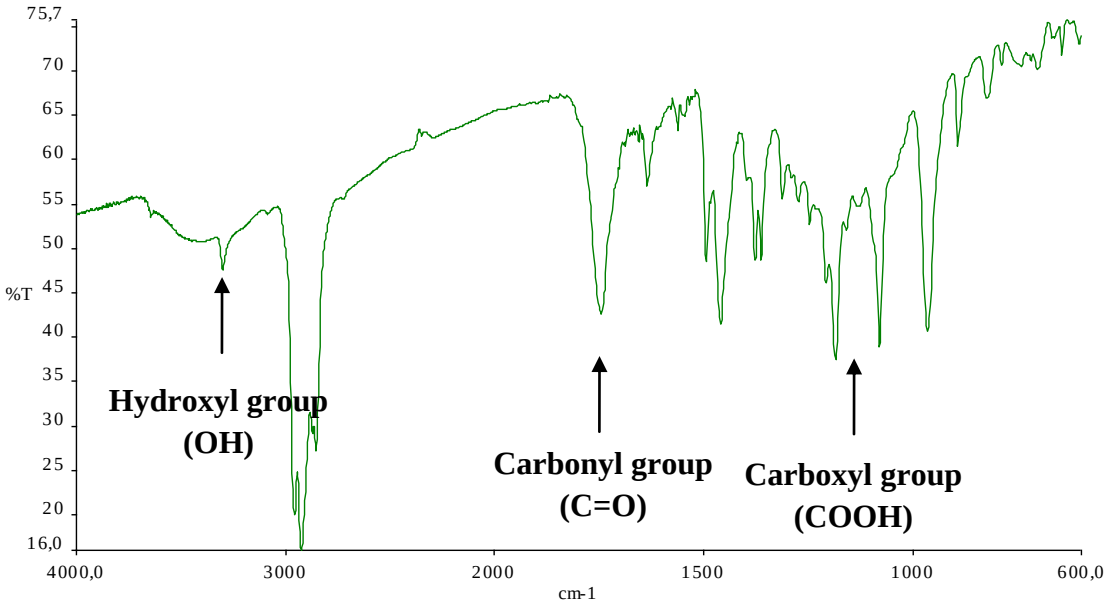


Figure 24. FTIR spectrum of the polymer extracted with chloroform from *R. eutropha* cultivation with 50% oxygen concentration.

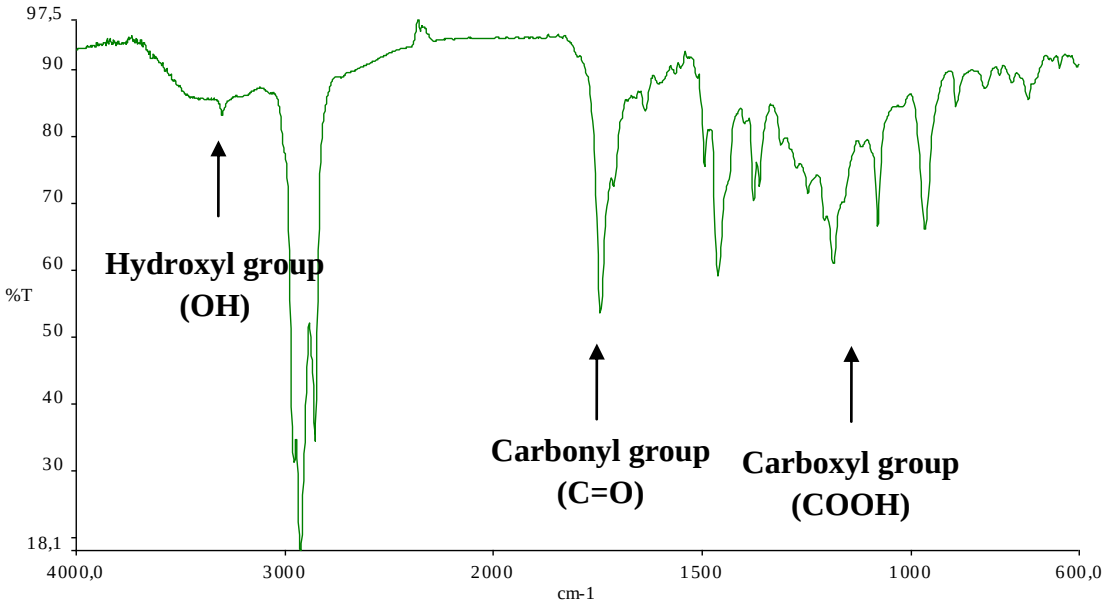


Figure 25. FTIR spectrum of the polymer extracted with chloroform from *R. eutropha* cultivation with 65% oxygen concentration.

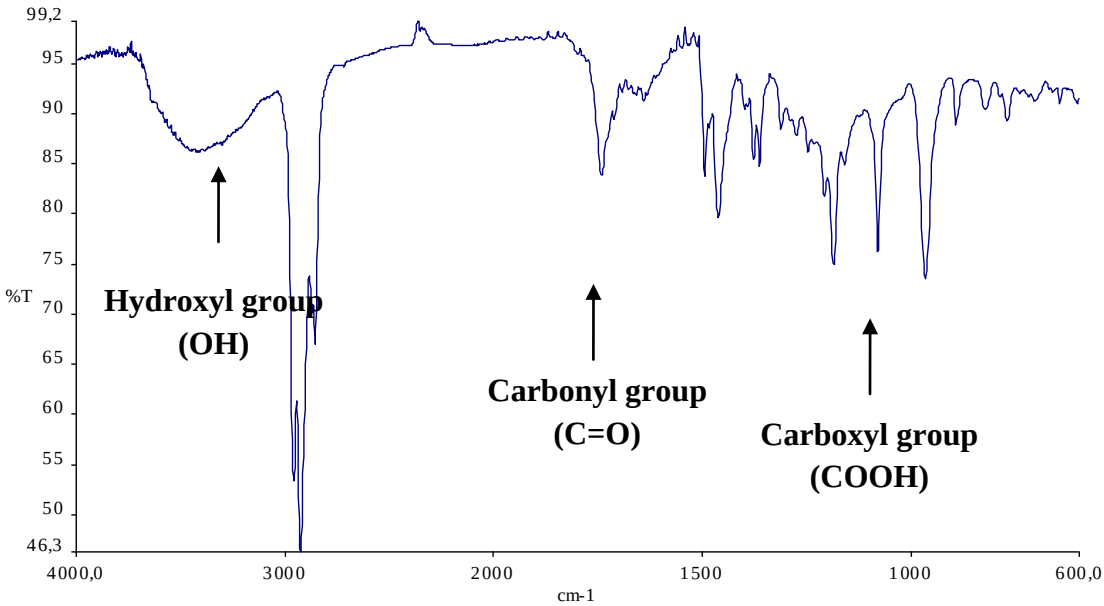


Figure 26. FTIR spectrum of the polymer extracted with chloroform from *R. eutropha* cultivation with 75% oxygen concentration.

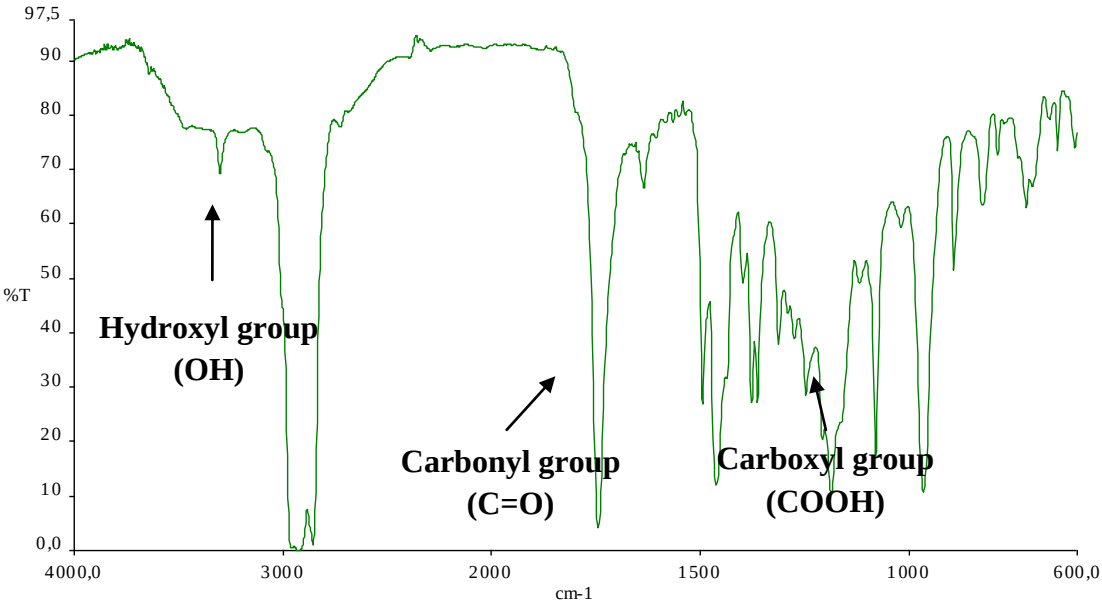


Figure 27. FTIR spectrum of the polymer extracted with chloroform from *R. eutropha* cultivation with 100% oxygen concentration.

A signal at about 3000 cm^{-1} which is characteristic of chloroform can be seen in al chromatograms, since the extraction of the polymer from the culture broth was done with this component.

The FTIR spectrums of the products obtained for each fermentation show no remarkable differences. For that reason, it can be concluded that the same type of polymer, the homopolymer PHB, was produced in all fermentations.

3.4. CULTIVATION YIELD PARAMETERS

Taking into the account all the previous the data, the final comparative parameters can be calculated (Table 9) which are also compared with data obtained in previous investigations.

Table 9. Comparison of PHB production by *R. eutropha* microorganism and different culture conditions.

Oxygen concentration (%)	Limitation source	Reactor volume (L)	Mode	Cell mass (g PHB/l)	PHB (g/l)	PHB content (%)	Productivity (g PHB/l·h)	$Y_{X/S}$	$Y_{PHB/S}$	Process time (h)	Reference
21% O_2	N (4 g/l)[a]	1	B	0,20	0,19	-	0,004	0,01	0,01	48	This work
50% O_2	N (4 g/l)[a]	1	B	2,29	1,86	80,8	0,039	0,11	0,09	48	This work
65% O_2	N (4 g/l)[a]	1	B	9,45	9,43	99,8	0,196	0,47	0,47	48	This work
75% O_2	N (4 g/l)[a]	1	B	6,37	3,26	51,2	0,068	0,32	0,16	48	This work
100% O_2	N (4 g/l)[a]	1	B	3,97	0,14	3,4	0,003	0,20	0,01	48	This work
Air	N (55 g/l)[b]	2,5	FB	124	92	74	1,87	0,41	0,30	49	39
Air	N (70 g/l)[b]	2,5	FB	164	121	76	2,42	-	-	50	39
Air	P (4,3 g/l)[a]	60	FB	221	180	81	3,75	0,45	0,37	48	20

[a] Initial nutrient concentration.

[b] Cell concentration at the onset of N limitation..

B= Batch; FB= Fed-Batch

An absolute concentration of 9,43 g PHB/l culture medium was obtained from the best fermentation carried out in this study. This value is far away from the ones obtained in other studies [39][20] using the same bacteria, medium, pH and temperature, but those processes were carried out in a fed-batch reactor.

To obtain higher PHB concentrations, several issues could be improved in further investigations, such as the bacteria concentrations achieved in the seed culture for the culture inoculation, which were half of those recommended in other articles [26]. Moreover, as it was said previously, the results obtained suggest that the exponential production phase associated with the nitrogen limitation was reached just before finishing the fermentation when using 65% oxygen concentration and it was not reached at all in the other fermentations.

Besides, other studies [39] concluded that *R. eutropha* produced much more polymer when operating with glucose concentrations of 10-20 g/l along all the fermentation, which can be achieved by carrying out fed-batch cultures with a nutrient feeding control by monitoring dissolved oxygen (DO), pH or carbon source. In this study, the carbon source was depleted when using 100% of O_2, and in the other fermentations the glucose concentration should be very low at the end of the process.

Nevertheless, it was proved that, when using a 65% of oxygen, bacteria produced a PHB amount 50 times higher than when using air at the same conditions. Besides, PHB content, cell yield from glucose and PHB yield from glucose were the highest reported so far in *R. eutropha* cultivation.

This increase favorably impacts on the raw material cost, which was concluded that accounts for 30–40% of the total cost of PHB [23]. Compared with the greatest success reported in terms of product yield using glucose as substrate (0,37 kg PHB/kg glucose) [20], the value obtained in this study (0,47 kg PHB/kg glucose) represents savings of 21% on the total carbon source cost.

Considering a mean value of 0,41 $/kg glucose (USDA Economic Research Service) a total cost of 0,87 $ carbon source/kg PHB is obtained. This value is more expensive than other cheaper substrates such as methanol (0,58$ C-source/kg PHB) or cane molasses (0,23$ C-source/kg PHB) [23]. However, this inexpensive carbon sources may incur additional costs due to pre-treatment steps, extended cultivation times, and purification.

Chapter IV

Conclusions

Chapter 4
Conclusions

Ralstonia eutropha CECT 4635 strain was used for the batch production process of 3-polyhydroxybutyrate (PHB) in a 1L fermentor with different oxygen concentrations. After approximately one day of cultivation, the cells seemed to reach the nitrogen limitation phase and started to produce PHB.

Chromatography results showed that, after that moment, the increase in cell growth was directly connected to an increase in PHB production and the residual biomass remained almost constant. However, when using 100% of air saturation, PHB production inside cells was very low since cells used glucose for their own growth.

Moreover, when dealing with high residual biomass concentrations, mainly obtained in fermentation with 100% of oxygen concentration, a PHB concentration drop at the end of the fermentation was seen likely due to carbon source depletion. This hypothesis was proved by the mean molecular weight decrease seen in this fermentation. As it was concluded in previous investigations, degradation of the polymer occurred rapidly in the absence of an exogenous carbon source and energy [35].

To prevent this to happen, fed-batch fermentation with glucose feeding should be used, which allows to maintain a certain glucose amount inside the bioreactor, and much higher cell densities and PHB values can be obtained. Other studies [39] concluded that *R. eutropha* produced much more polymer when operating with glucose concentrations of 10-20 g/l along all the fermentation.

Anyway, 65% of oxygen concentration was proved to be the best condition for both PHB production in terms of total concentration and PHB accumulation in terms of percentage of total biomass, which had a final value of 9,43 g/l and 99,8% respectively. The absolute PHB concentration in the medium was 50 times higher when using 65% oxygen concentration than when using air at the same conditions.

In terms of yield and productivity, which are essential for profitable production processes, several issues could be improved to obtain higher values, such as the bacteria concentrations achieved in the seed culture for the culture inoculation, which were half of those recommended in other articles [26], and, as previously said, the low glucose concentration maintained during the fermentations.

Nevertheless, PHB content, cell yield from glucose and PHB yield from glucose were the highest reported so far in *R. eutropha* cultivation, with values of 99,8%, 0,47 g/g and 0,47 g/g respectively. The high product yield obtained in this study represents savings of 21% on the total carbon source cost compared with the greatest success reported so far. However, to be economically profitable, higher productivity values should be obtained to compensate the additional cost of pure oxygen, since it would make the process uneconomic at industrial scale.

Regarding polymer molecular weight, PHB accumulation and molecular weight data suggested a possible correlation between the amount of polymer accumulated and its molecular weight, as the values were slightly higher in cultures accumulating more PHB. However, it could be also linked to the oxygen concentration, since the molecular weight decreased when increasing the air saturation.

The higher molecular weights were obtained in fermentations with 50% oxygen concentration (Mn=61.200 Da, Mw=134.400 Da, PDI=2,2) and 65% (Mn=54.200 Da, Mw=111.350 Da, PDI=2,1), giving a good polydispersity index but low molecular weight values compared with those reported by other studies (Mn= 300.000-400.000 Da) [36][37].

Finally, the FTIR spectrums of the products obtained show no significant differences, so it could be concluded that the same type of polymer, the homopolymer PHB, was produced in all fermentations.

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Appendices

APPENDIX A: TABLE OF REAGENTS

Table A-1. Table of reagents.

Compound name	Chemical formula	#[a]	Pictogram	H and P Phrases	M (g/mol)
Agar powder	-	1	None	This substance is not classified as dangerous according to European Union legislation.	-
Ammonium molybdate	$(NH_4)_6Mo_7O_{24}$	2,3		H302 Harmful if swallowed. H412 Harmful to aquatic life with long lasting effects. P264 Wash … thoroughly after handling. P270 Do not eat, drink or smoke when using this product. P273 Avoid release to the environment P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell. P330 Rinse mouth.	1163,9
Ammonium sulfate	$(NH_4)_2SO_4$	2,3		H319 Causes serious eye irritation. H315 Causes skin irritation. H335 May cause respiratory irritation. P280 Wear protective gloves/protective clothing/eye protection/face protection. P261 Avoid breathing dust/fume/gas/mist/vapours/spray.	132,1

				P302+P313 IF ON SKIN: Get medical advice/attention. P332+P313 If skin irritation occurs: Get medical advice/attention. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing. P501 Dispose of contents/container to local/regional/national/ international regulations.	
Beef extract powder	-	1		H319 Causes serious eye irritation. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	-
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	2,3		H319 Causes serious eye irritation. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	147,0
Citric acid	$C_6H_8O_7$	3		H315 Causes skin irritation. H318 Causes serious eye damage. P280 Wear protective gloves/protective clothing/eye protection/ face protection. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	192,1

Chloroform	$CHCl_3$	9,10	 	H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation. H331 Toxic if inhaled. H351 Suspected of causing cancer. H361 Suspected of damaging fertility or the unborn child. H372 Causes damage to organs through prolonged or repeated exposure cause the hazard. P302+ P352 IF ON SKIN: Wash with plenty of soap and water. P304 + P340 IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing. P308 + P310 IF exposed or concerned: Immediately call a POISON CENTER or doctor/physician.	119,4
Copper (II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	2,3	 	H302 Harmful if swallowed. H318 Causes serious eye damage. H400 Very toxic to aquatic life. P264 Wash … thoroughly after handling. P270 Do not eat, drink or smoke when using this product. P273 Avoid release to the environment.	249.7

				P280 Wear protective gloves/protective clothing/eye protection/ face protection. P312 Call a POISON CENTER or doctor/physician if you feel unwell. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	
Disodium phosphate dodecahydrate	$Na_2HPO_4 \cdot 12H_2O$	2	None	This substance is not classified as dangerous according to European Union legislation.	358,1
Disodium tetraborate heptahydrate	$Na_2B_4O_7 \cdot 7H_2O$	2,3		H360 – May damage fertility or the unborn child. P201 – Obtain special instructions before use. P202 – Do not handle until all safety precautions have been read and understood. P281 Use personal protective equipment as required. P307 + P313 IF exposed: Get medical advice/attention.	327,2
Glucose	$C_6H_{12}O_6$	2,3	None	This substance is not classified as dangerous according to European Union legislation.	80,2
Glycerol	$C_3H_8O_3$	1	None	This substance is not classified as dangerous according to European Union legislation.	92,1

Hexafluoro-2-propanol	$(CF_3)_2CHOH$	7,8		P260 Do not breathe dust/fume/gas/mist/vapours/spray. P271 Use only outdoors or in a well-ventilated area. P280 Wear protective gloves/protective clothing/eye protection/ face protection. H302+H312+H352 Harmful if swallowed, in contact with skin or if inhaled. H314 Causes severe skin burns and eye damage.	168,1
Hydrochloric acid 37%	HCl	2,3, F		H290 May be corrosive to metals. H315 Causes skin irritation. H319 Causes serious eye irritation. H335 May cause respiratory irritation. P302+ P352 IF ON SKIN: Wash with plenty of soap and water. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	36,5
Iron(II) sulfate heptahydrate,	$FeSO_4 \cdot 7H_2O$	2,3		H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation. P302+ P352 IF ON SKIN: Wash with plenty of soap and water. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	277,9

Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	2,3	None	This substance is not classified as dangerous according to European Union legislation.	246,5
Manganese(II) sulfate pentahydrate	$MnSO_4 \cdot 5H_2O$	2,3	 	H373 May cause damage to organs through prolonged or repeated exposure cause the hazard. H411 Toxic to aquatic life with long lasting effects. P273 Avoid release to the environment.	241,0
Nitrogen	N_2	F		H280 Contains gas under pressure; may explode if heated. P403 Store in a well-ventilated place.	
Oxygen	O_2	F	 	H270 May cause or intensify fire; oxidizer. H280 Contains gas under pressure; may explode if heated. P220 Keep/Store away from clothing/…/combustible materials. P240 Ground/bond container and receiving equipment. P370+P376 In case of fire: Stop leak if safe to do so.	
Peptone	-	1	None	This substance is not classified as dangerous according to European Union legislation.	-

Potassium dihydrogen phosphate	KH_2PO_4	2,3	None	This substance is not classified as dangerous according to European Union legislation.	136,1
Sodium chloride	NaCl	1		H319 Causes serious eye irritation. P264 Wash … thoroughly after handling. P280 Wear protective gloves/protective clothing/eye protection/ face protection. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	58,4
Sodium hydroxide	NaOH	F		H314 Causes severe skin burns and eye damage. H315 Causes skin irritation. H318 Causes serious eye damage. H319 Causes serious eye irritation. P280 Wear protective gloves/protective clothing/eye protection/ face protection. P260 Do not breathe dust/fume/gas/mist/vapours/spray. P301 + P330 + P331 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. P332+P313 If skin irritation occurs: Get medical advice/attention. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.	40,0

Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H2O$	2,3		H302 Harmful if swallowed. H318 Causes serious eye damage. H373 May cause damage to organs through prolonged or repeated exposure cause the hazard. H410 Very toxic to aquatic life with long lasting effects. P273 Avoid release to the environment. P280 Wear protective gloves/protective clothing/eye protection/ face protection. P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell. P305+P351+P338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing. P391 Collect spillage.	287,6

[a] Experiment number shown in Section 2.3. F means reagent added during the fermentation.

APPENDIX B: PROCESS FLOW DIAGRAM

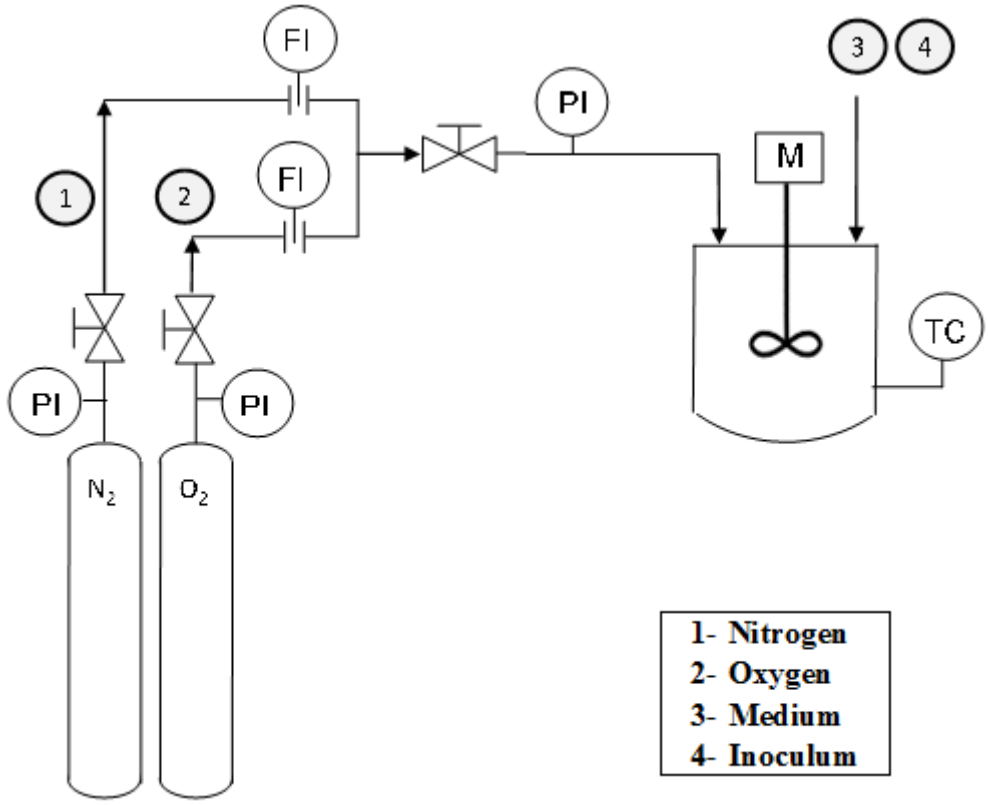
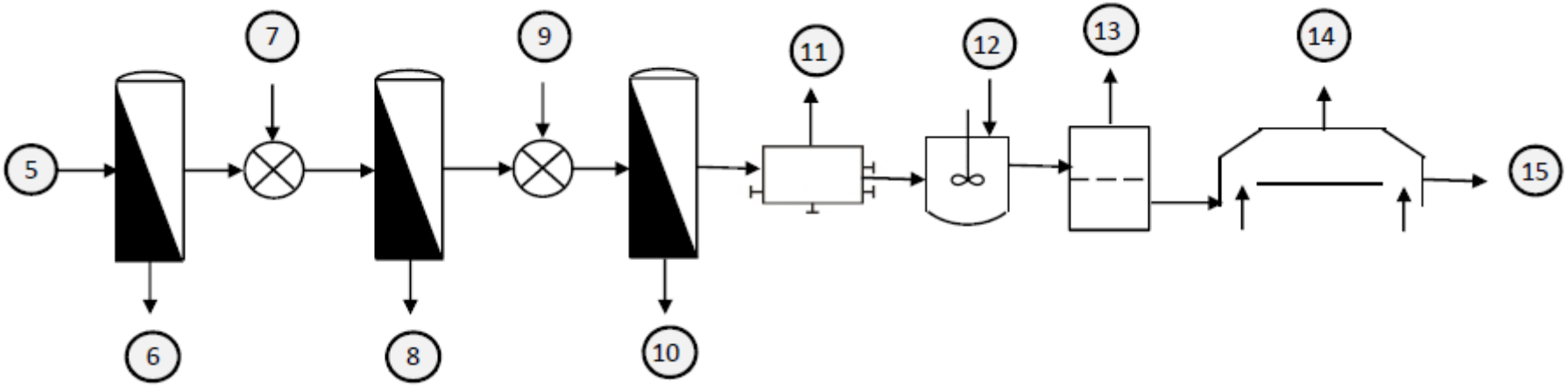


Figure B-1. Upstream Process Flow Diagram of the PHB production process.



5- Final broth	8- Supernatant	11- Water vapour	14- Chloroform
6- Undesirable medium	9- Water	12- Chloroform	15- PHB
7- Water	10- Supernatant	13- Biomass	

Figure B-2. Downstream Process Flow Diagram of the PHB production process.

APPENDIX C: CHROMATOGRAMS

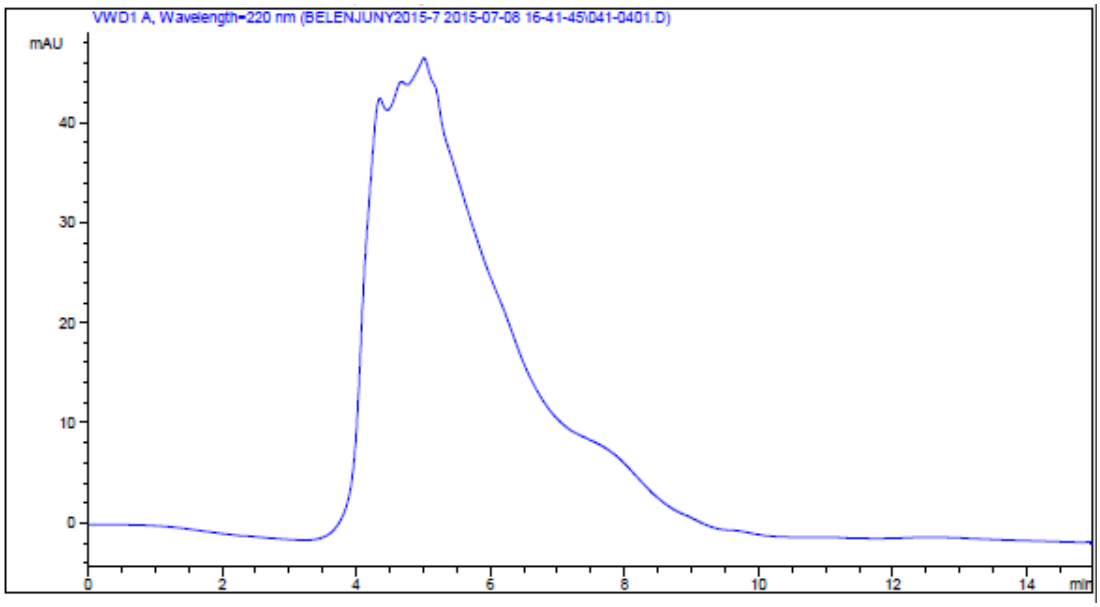


Figure C-1. Chromatogram showing the analysis of a 3mg/ml PHB solution for the calibration curve. Column details as previously explained.

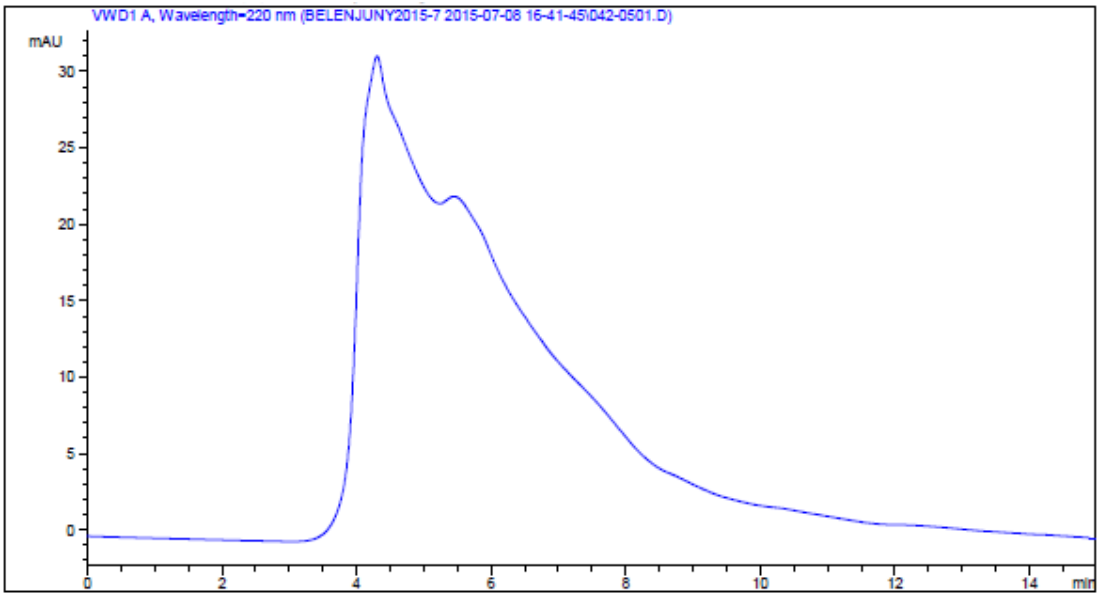


Figure C-2. Chromatogram showing the analysis of a 2mg/ml PHB solution for the calibration curve. Column details as previously explained.

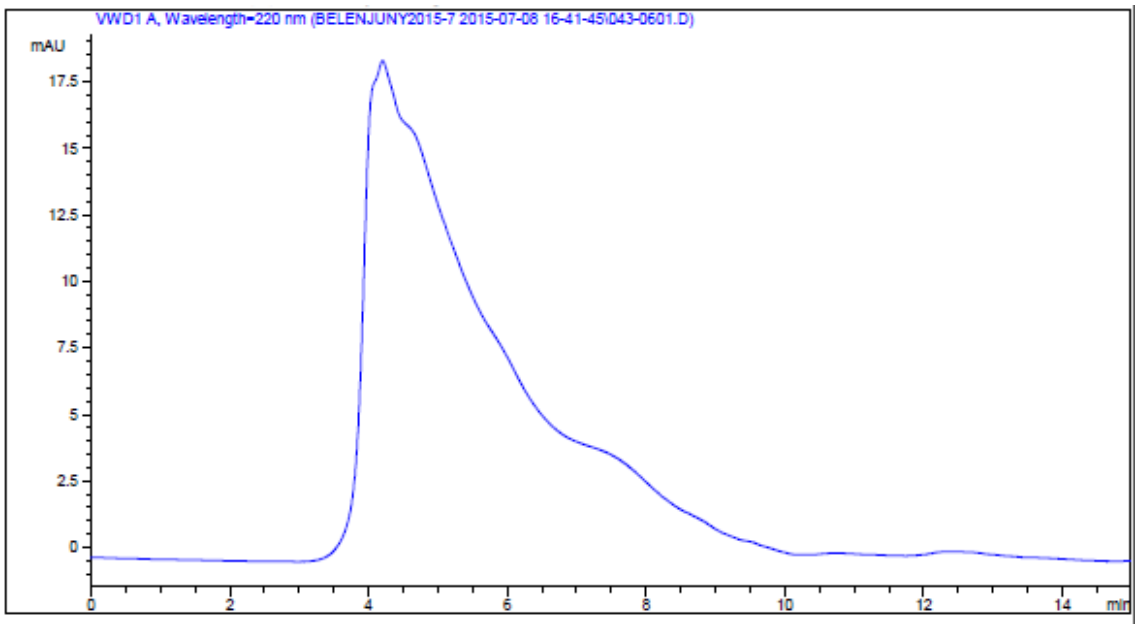


Figure C-3. Chromatogram showing the analysis of a 1mg/ml PHB solution for the calibration curve. Column details as previously explained.

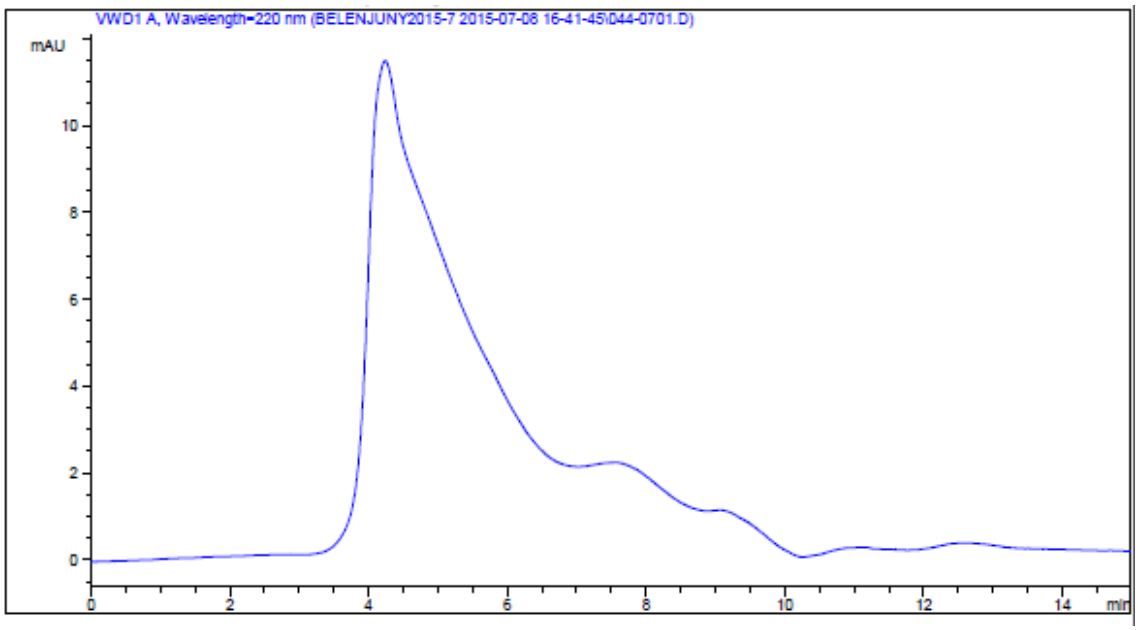


Figure C-4. Chromatogram showing the analysis of a 0,5mg/ml PHB solution for the calibration curve. Column details as previously explained.

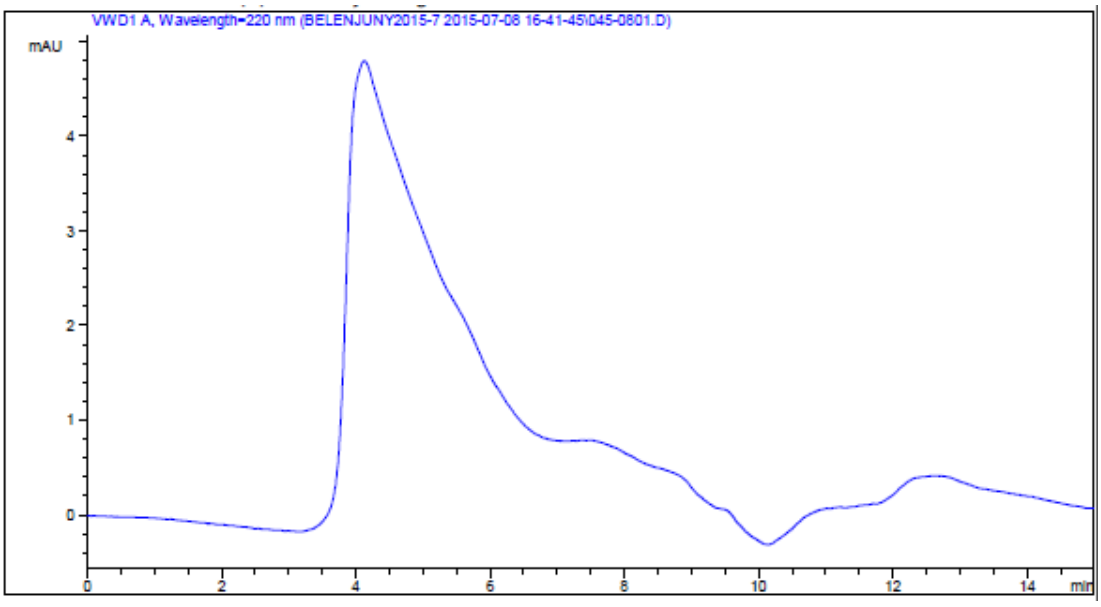


Figure C-5. Chromatogram showing the analysis of a 0,25mg/ml PHB solution for the calibration curve. Column details as previously explained.

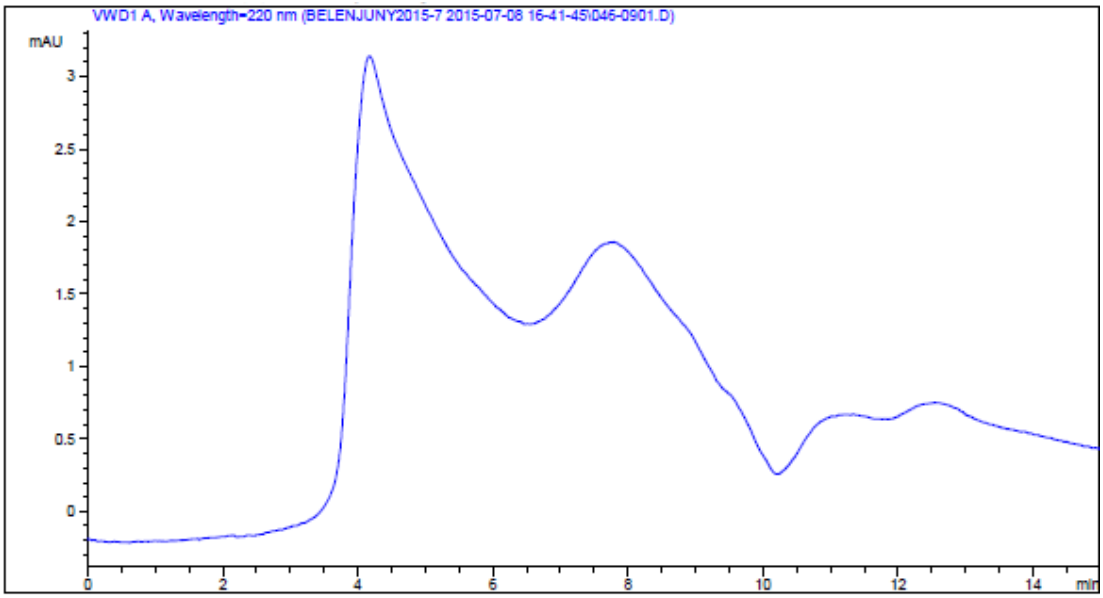


Figure C-6. Chromatogram showing the analysis of a 0,125mg/ml PHB solution for the calibration curve. Column details as previously explained.

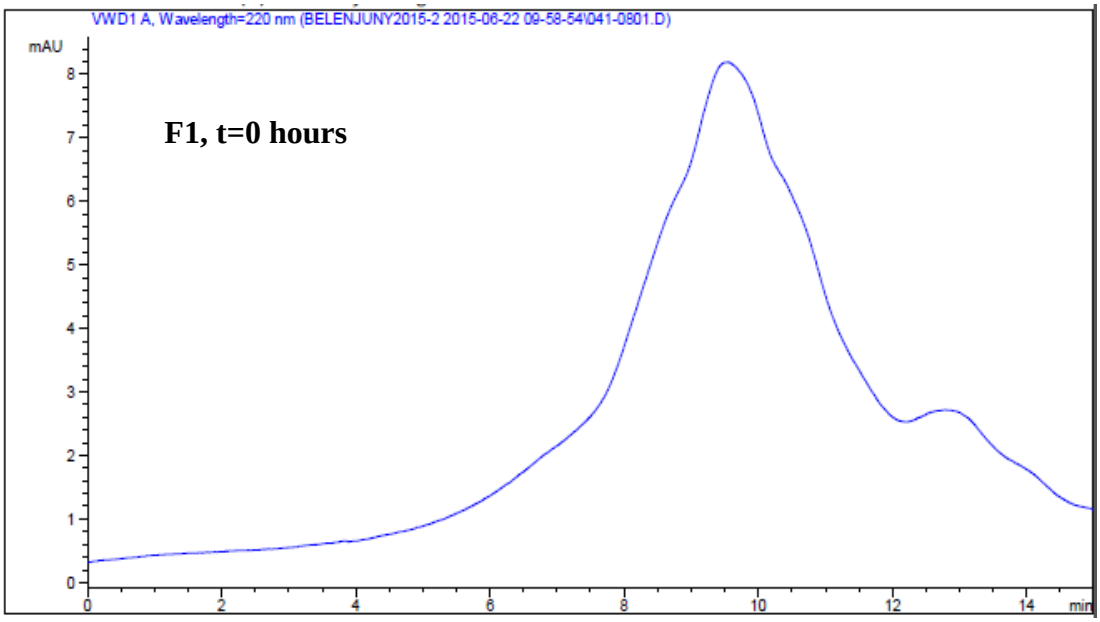


Figure C-7. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 0 hours of fermentation. Column details as previously explained.

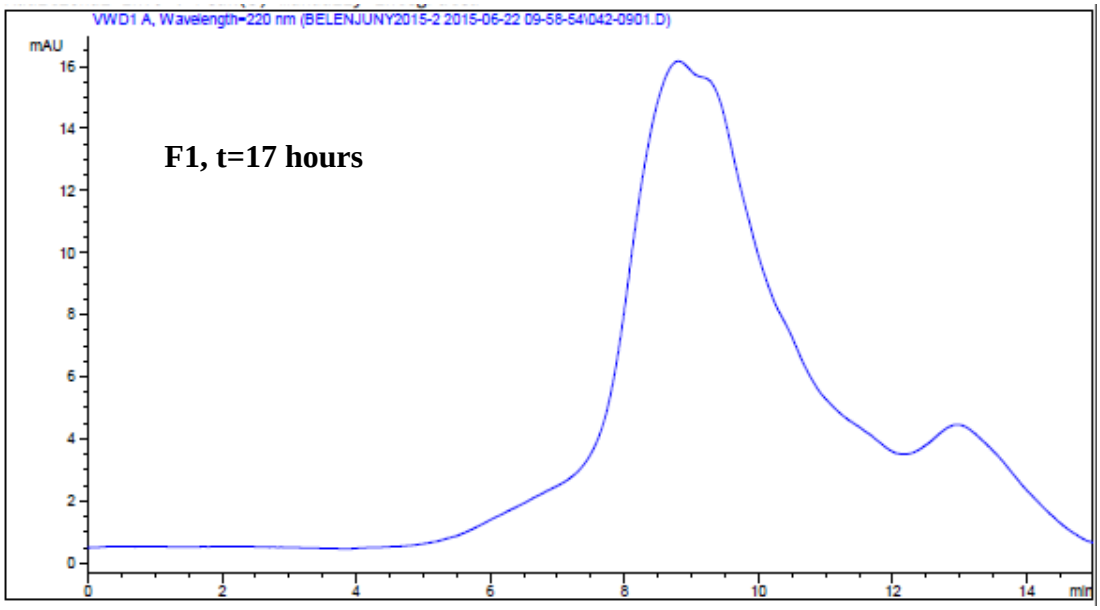


Figure C-8. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 17 hours of fermentation. Column details as previously explained.

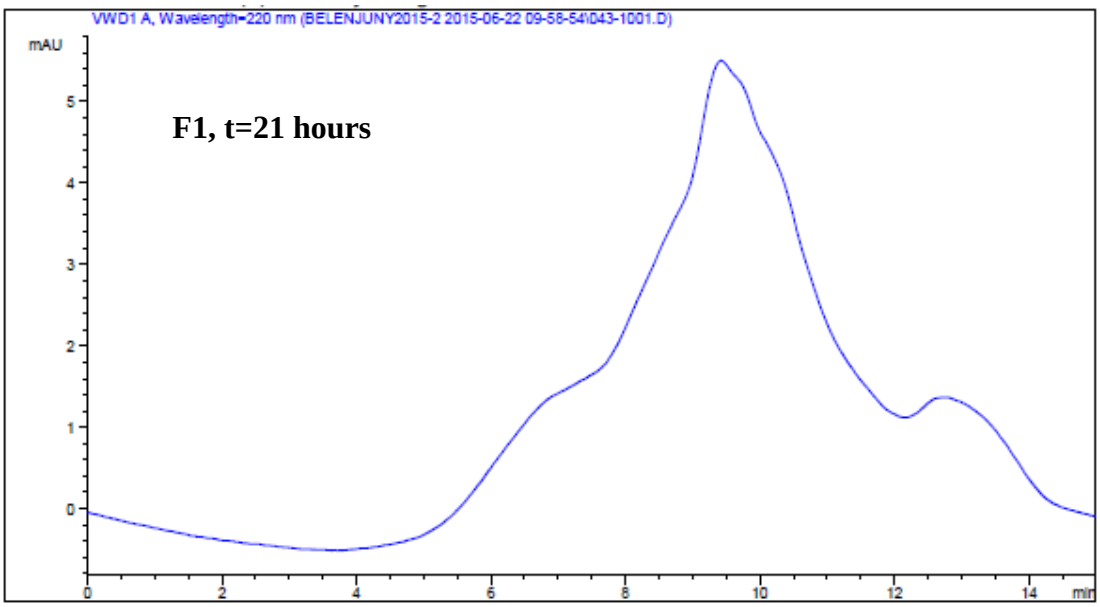


Figure C-9. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 21 hours of fermentation. Column details as previously explained.

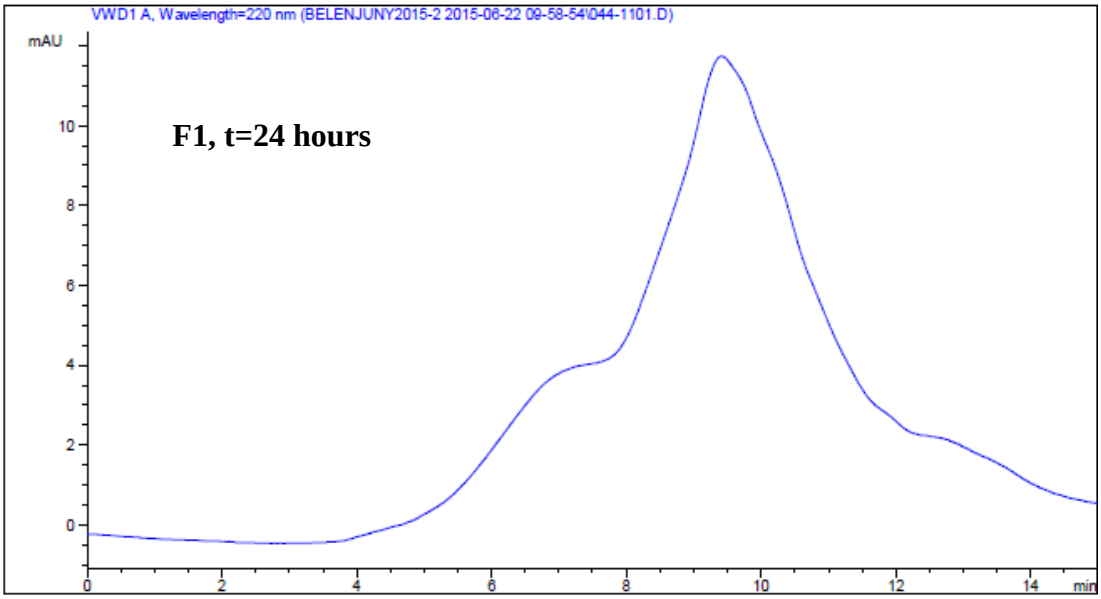


Figure C-10. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 24 hours of fermentation. Column details as previously explained.

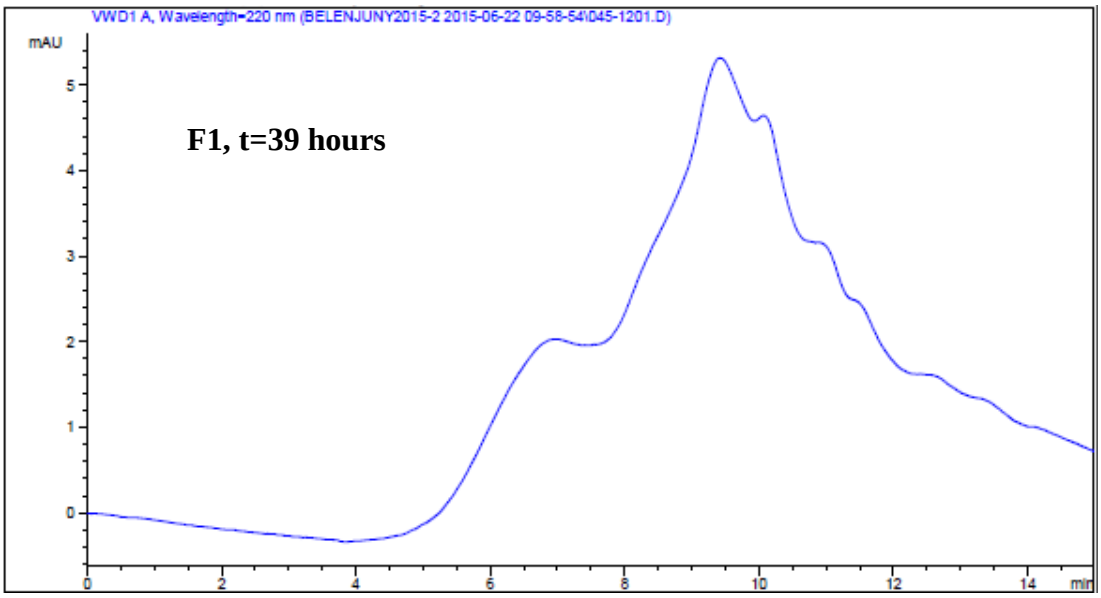


Figure C-11. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 39 hours of fermentation. Column details as previously explained.

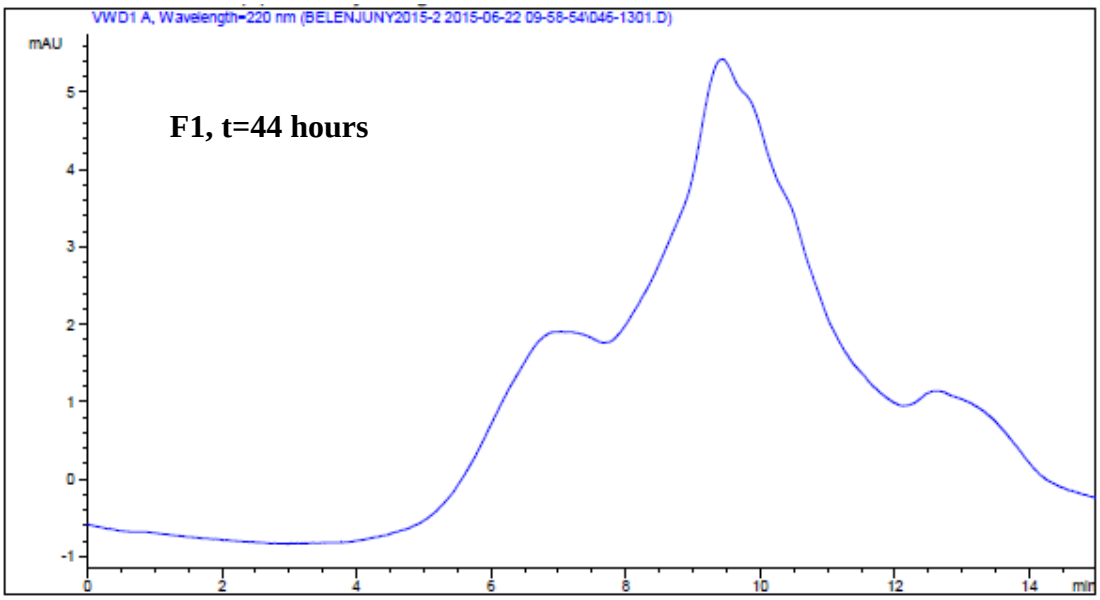


Figure C-12. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 44 hours of fermentation. Column details as previously explained.

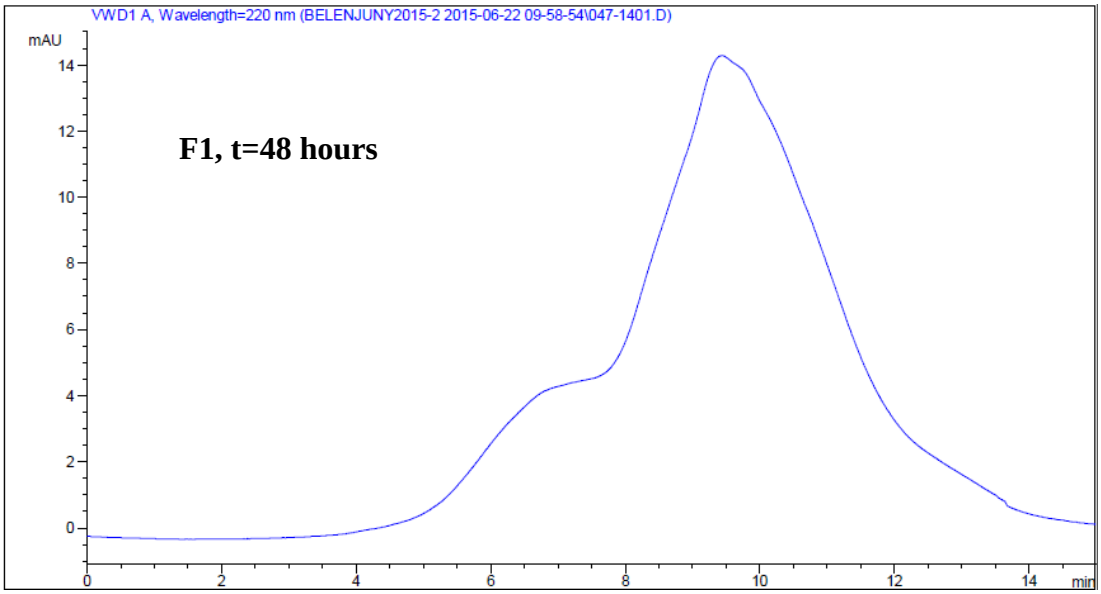


Figure C-13. Chromatogram showing the analysis of the culture medium sample from Fermentation 1 at 48 hours of fermentation. Column details as previously explained.

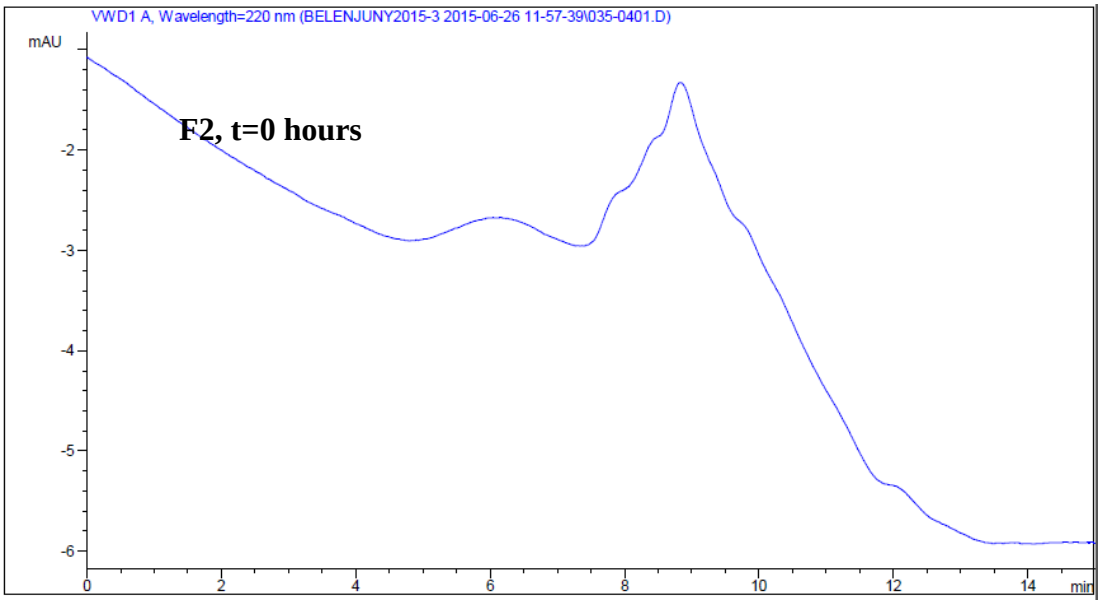


Figure C-14. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 0 hours of fermentation. Column details as previously explained.

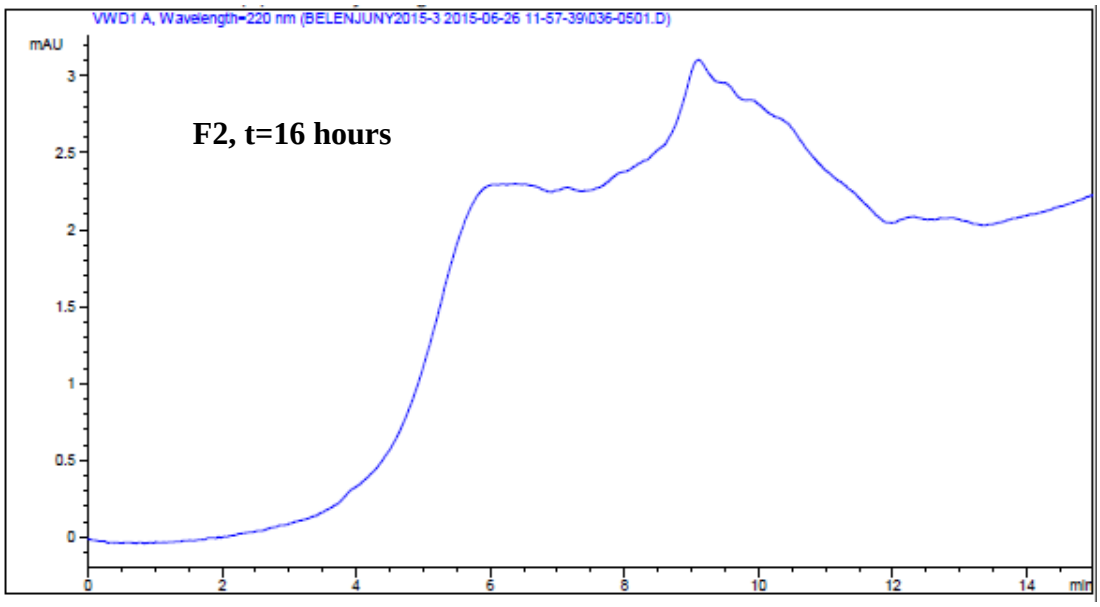


Figure C-15. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 16 hours of fermentation. Column details as previously explained.

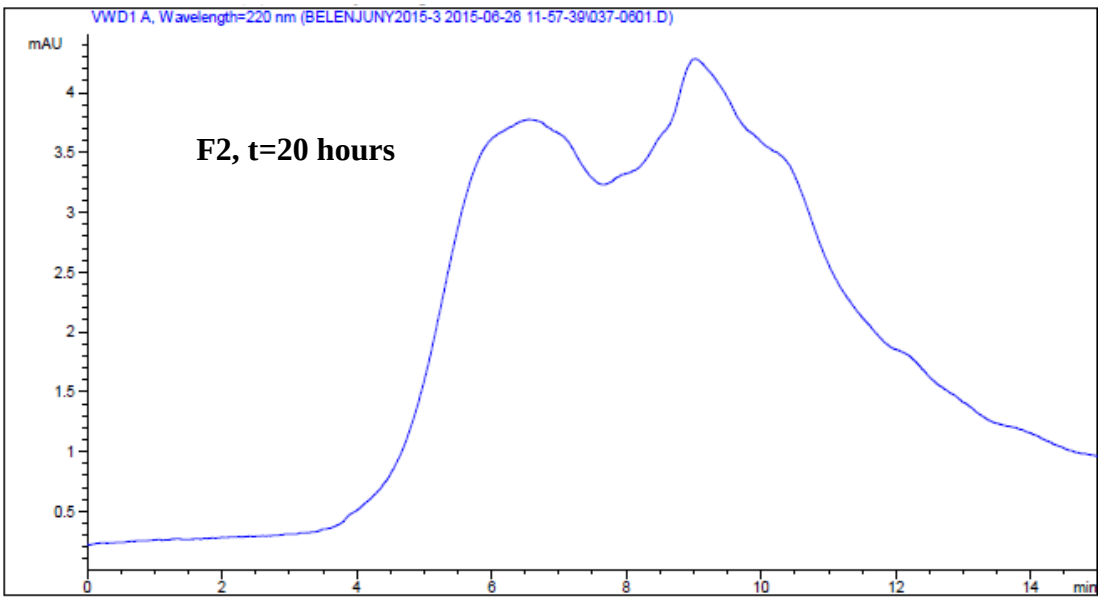


Figure C-16. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 20 hours of fermentation. Column details as previously explained.

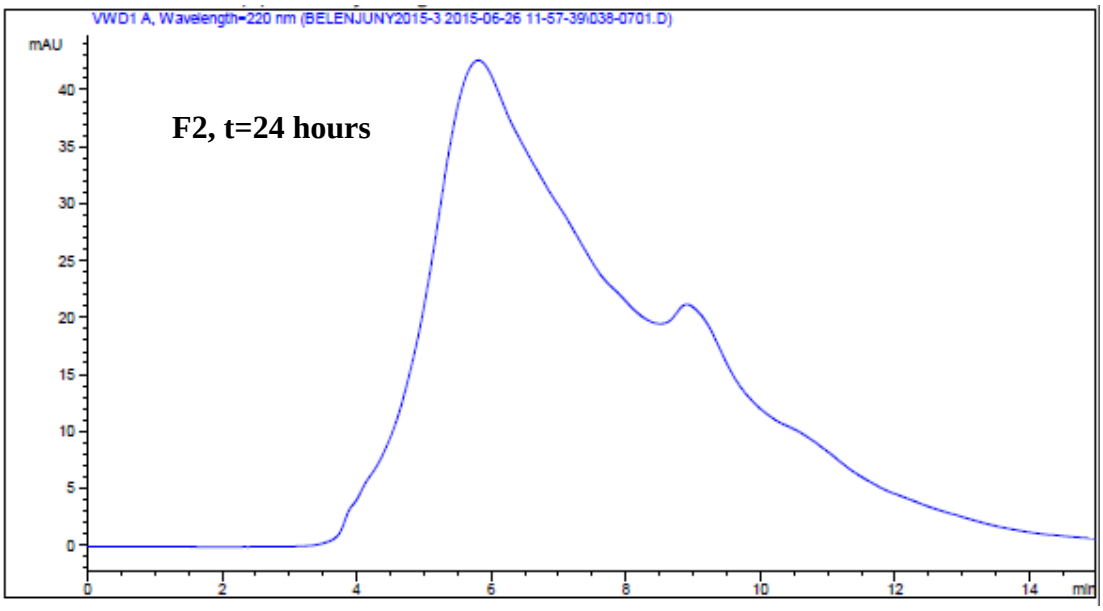


Figure C-17. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 24 hours of fermentation. Column details as previously explained.

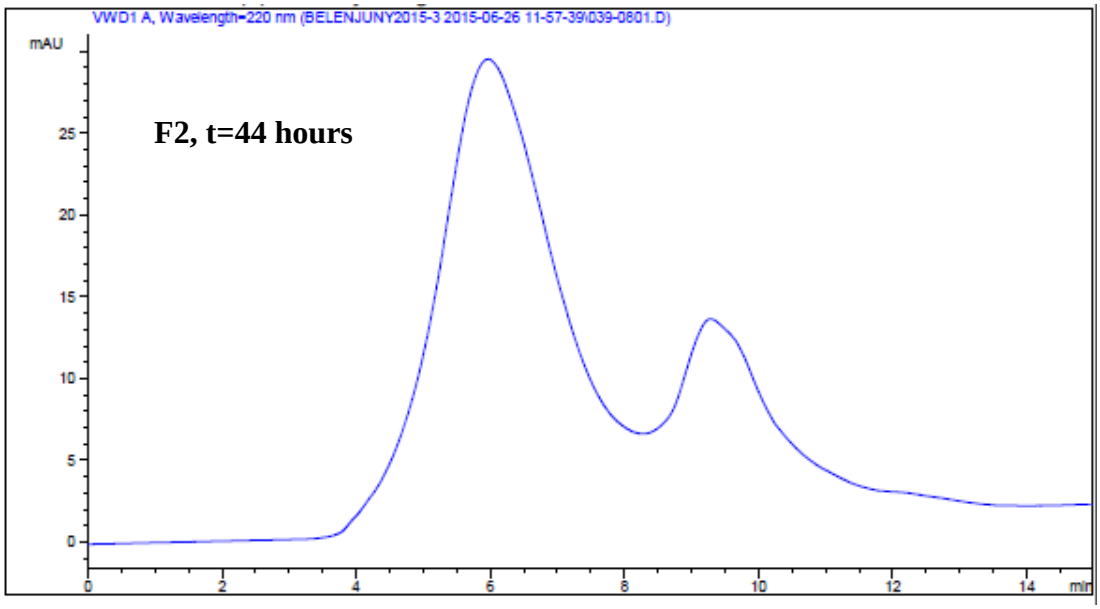


Figure C-18. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 44 hours of fermentation. Column details as previously explained.

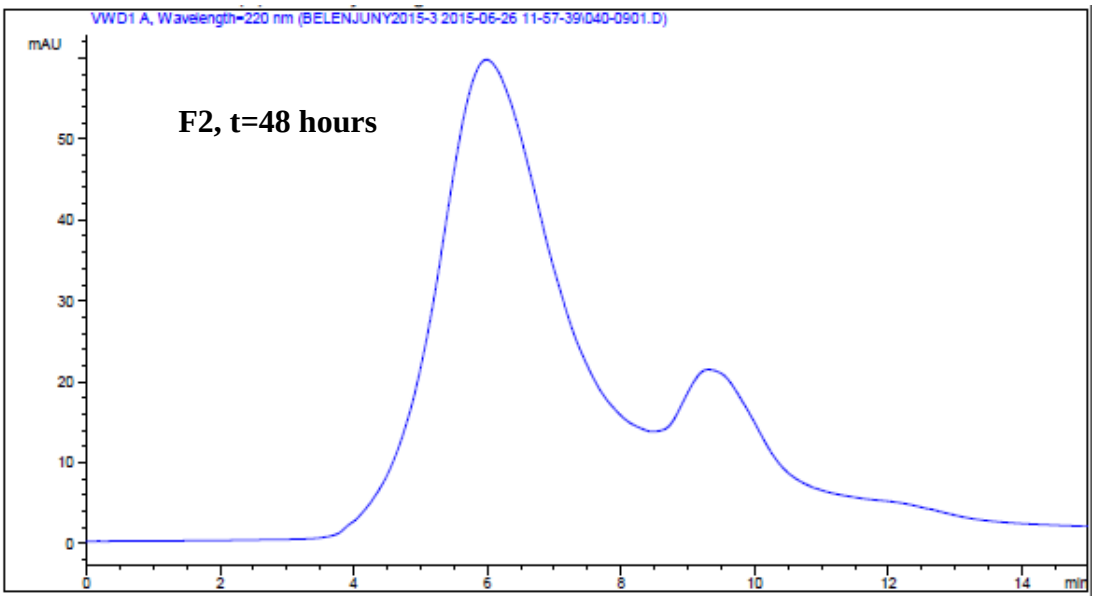


Figure C-19. Chromatogram showing the analysis of the culture medium sample from Fermentation 2 at 48 hours of fermentation. Column details as previously explained.

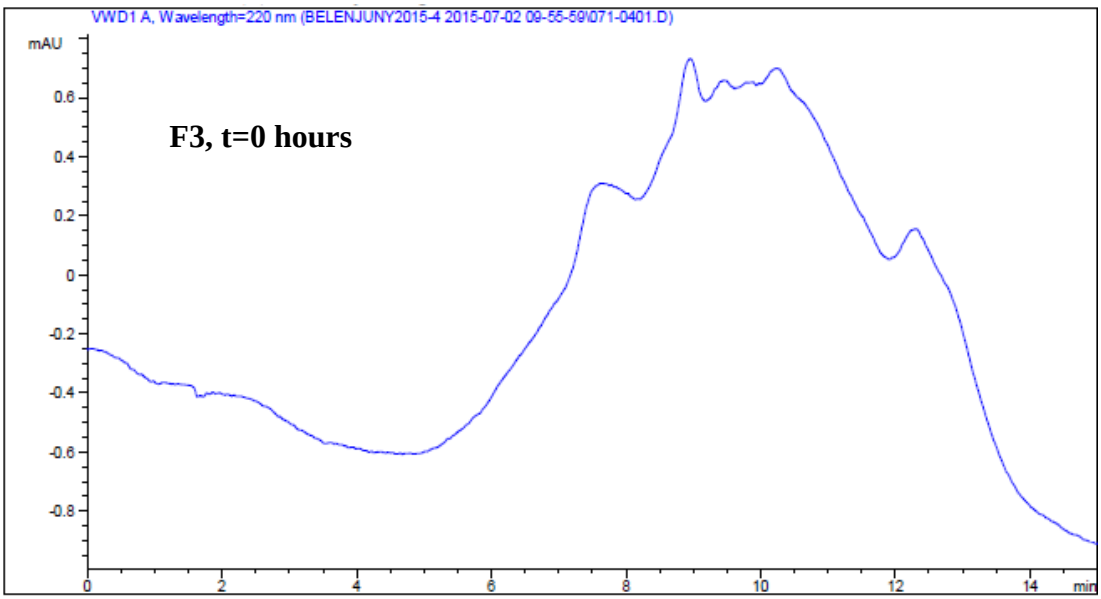


Figure C-20. Chromatogram showing the analysis of the culture medium sample from Fermentation 3 at 0 hours of fermentation. Column details as previously explained.

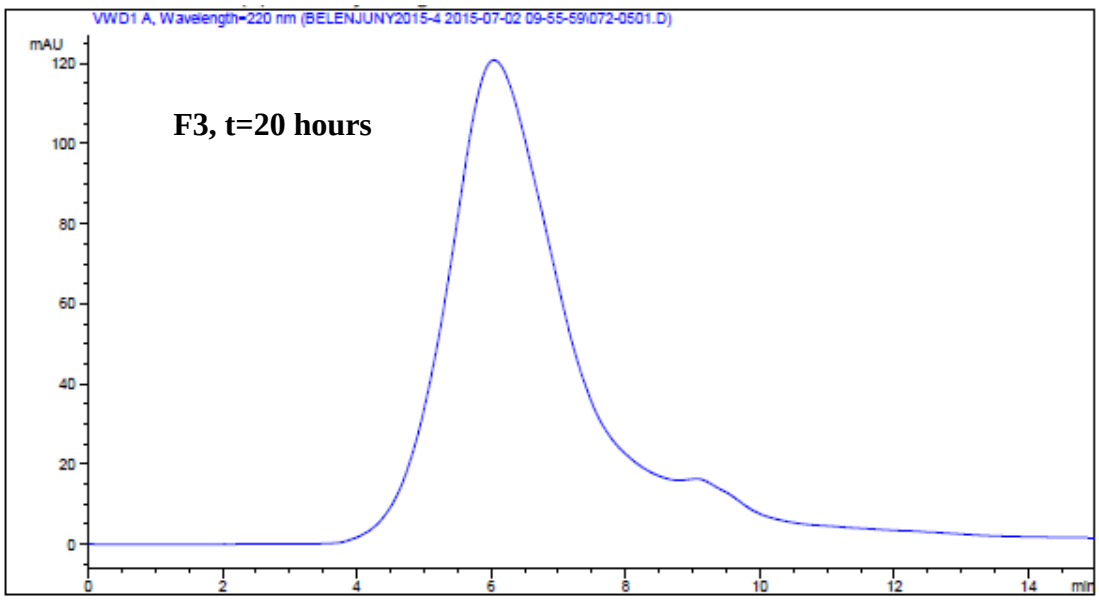


Figure C-21. Chromatogram showing the analysis of the culture medium sample from Fermentation 3 at 20 hours of fermentation. Column details as previously explained.

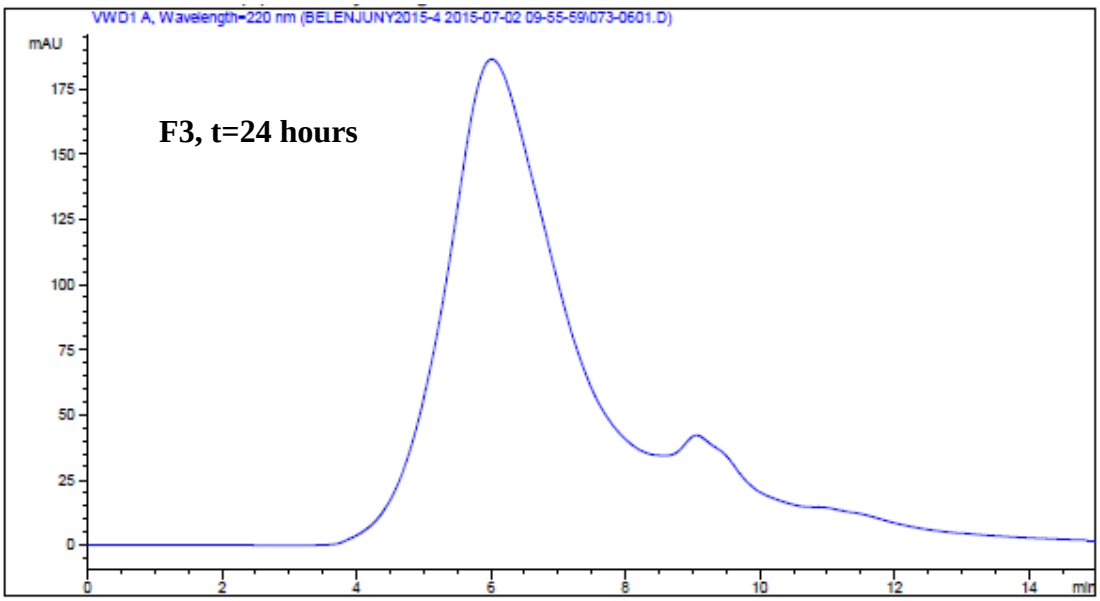


Figure C-22. Chromatogram showing the analysis of the culture medium sample from Fermentation 3 at 24 hours of fermentation. Column details as previously explained.

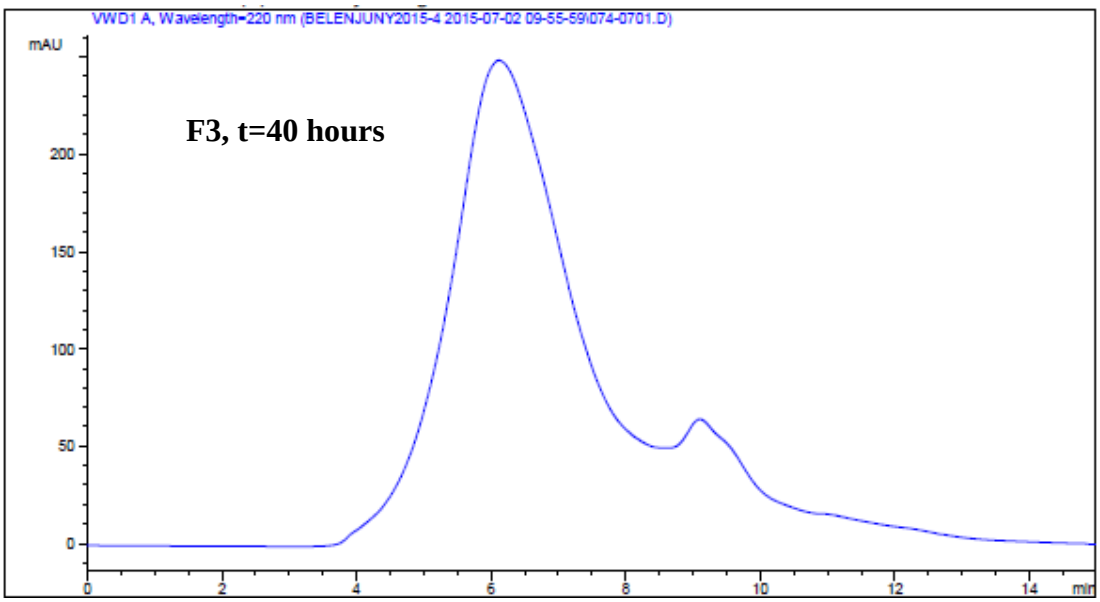


Figure C-23. Chromatogram showing the analysis of the culture medium sample from Fermentation 3 at 40 hours of fermentation. Column details as previously explained.

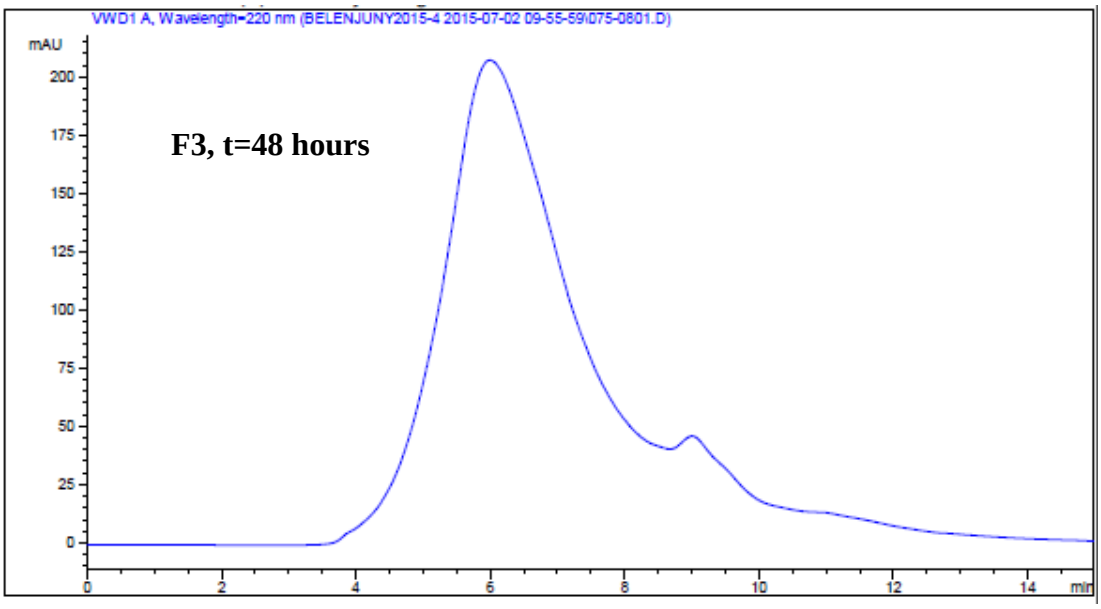


Figure C-24. Chromatogram showing the analysis of the culture medium sample from Fermentation 3 at 48 hours of fermentation. Column details as previously explained.

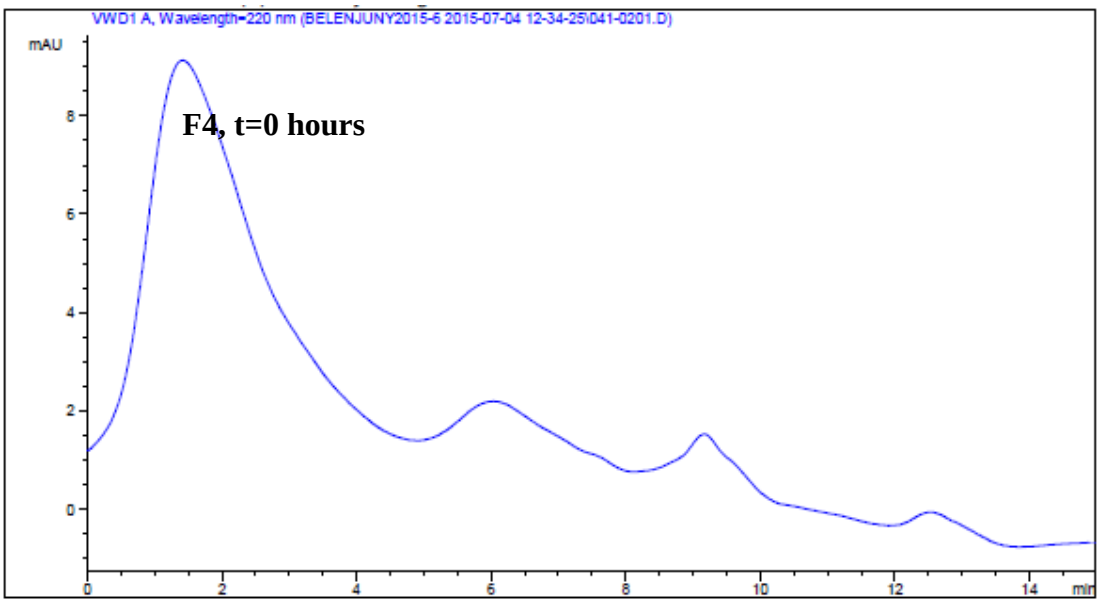


Figure C-25. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 0 hours of fermentation. Column details as previously explained.

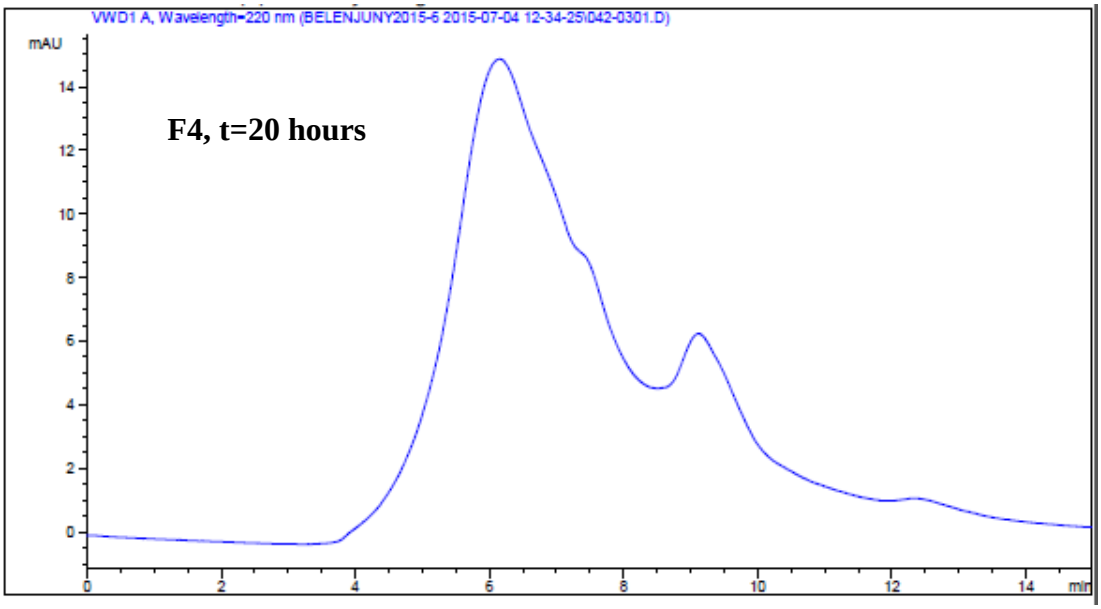


Figure C-26. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 20 hours of fermentation. Column details as previously explained.

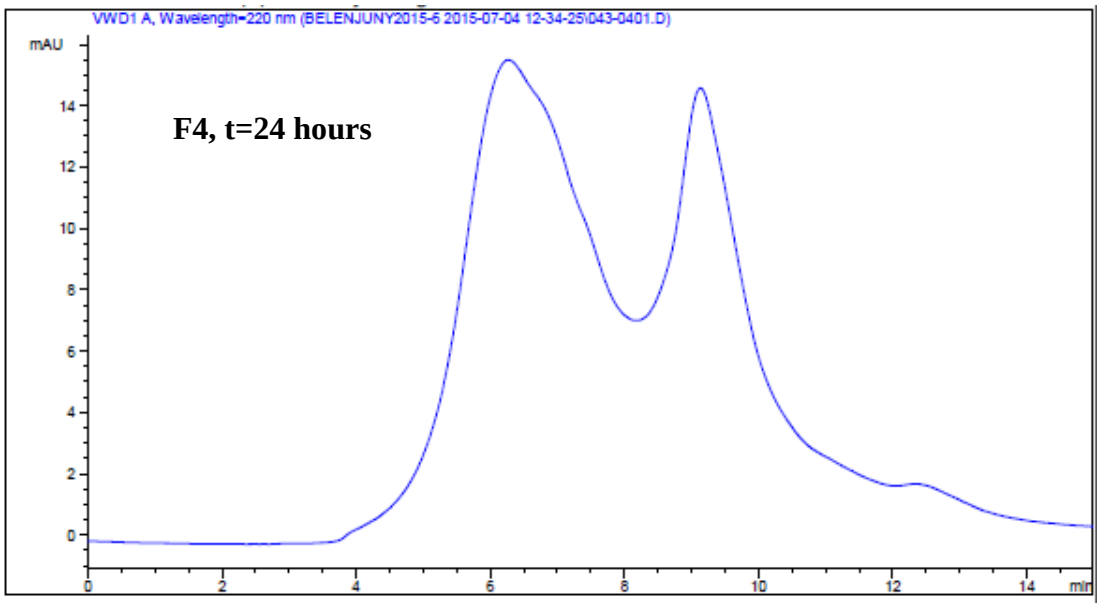


Figure C-27. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 24 hours of fermentation. Column details as previously explained.

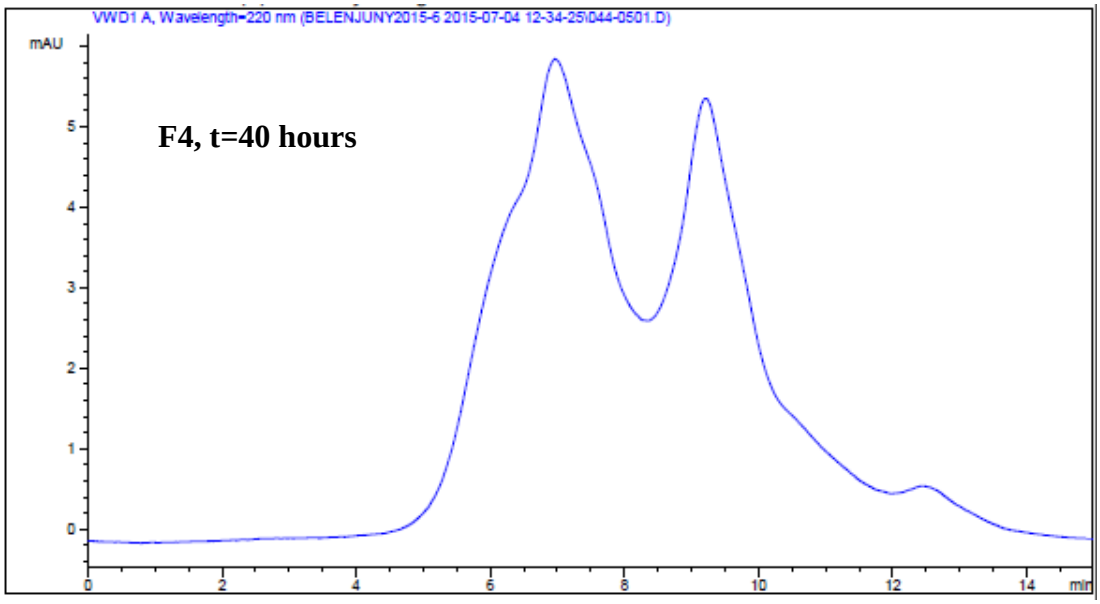


Figure C-28. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 40 hours of fermentation. Column details as previously explained.

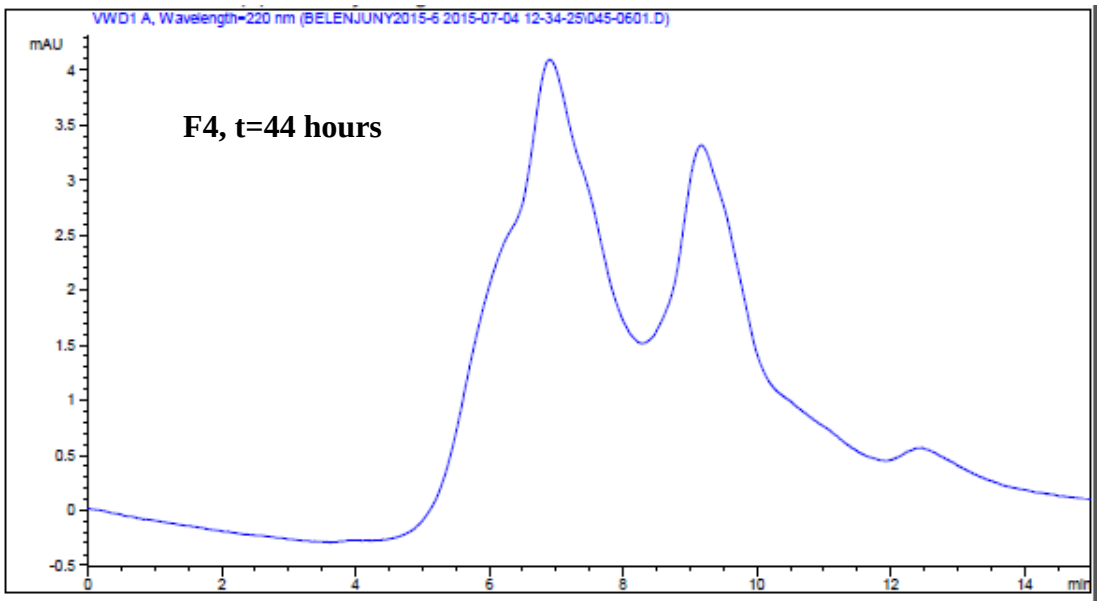


Figure C-29. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 44 hours of fermentation. Column details as previously explained.

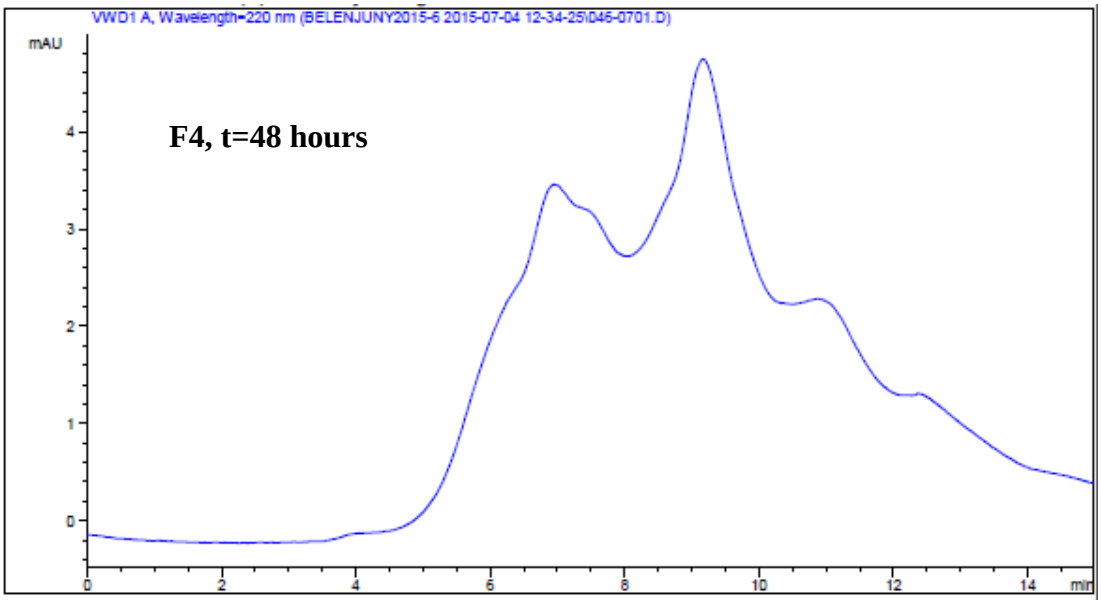


Figure C-30. Chromatogram showing the analysis of the culture medium sample from Fermentation 4 at 48 hours of fermentation. Column details as previously explained.

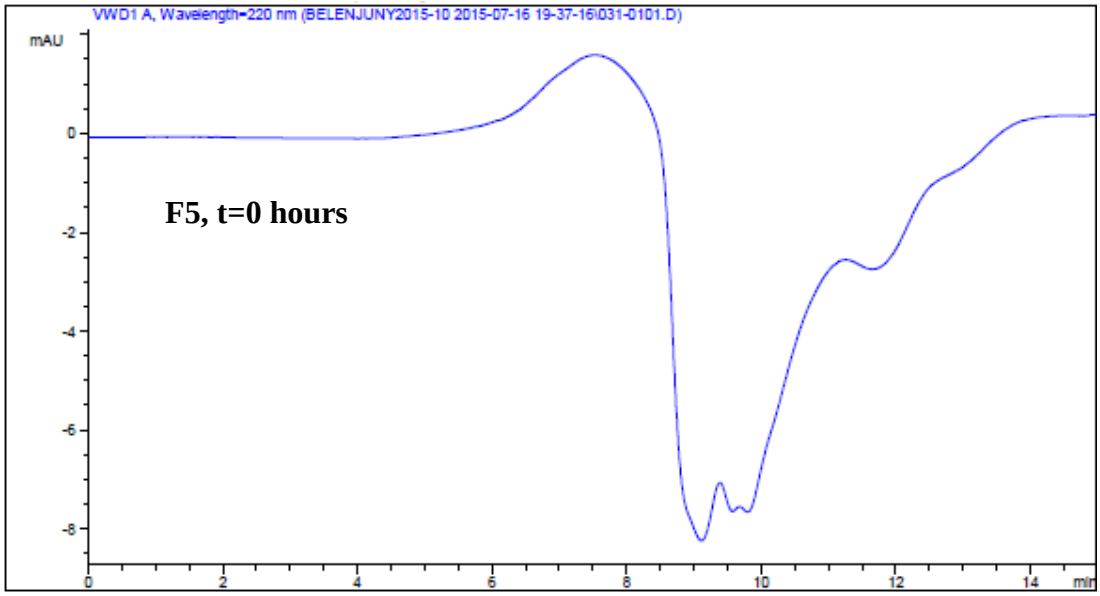


Figure C-31. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 0 hours of fermentation. Column details as previously explained.

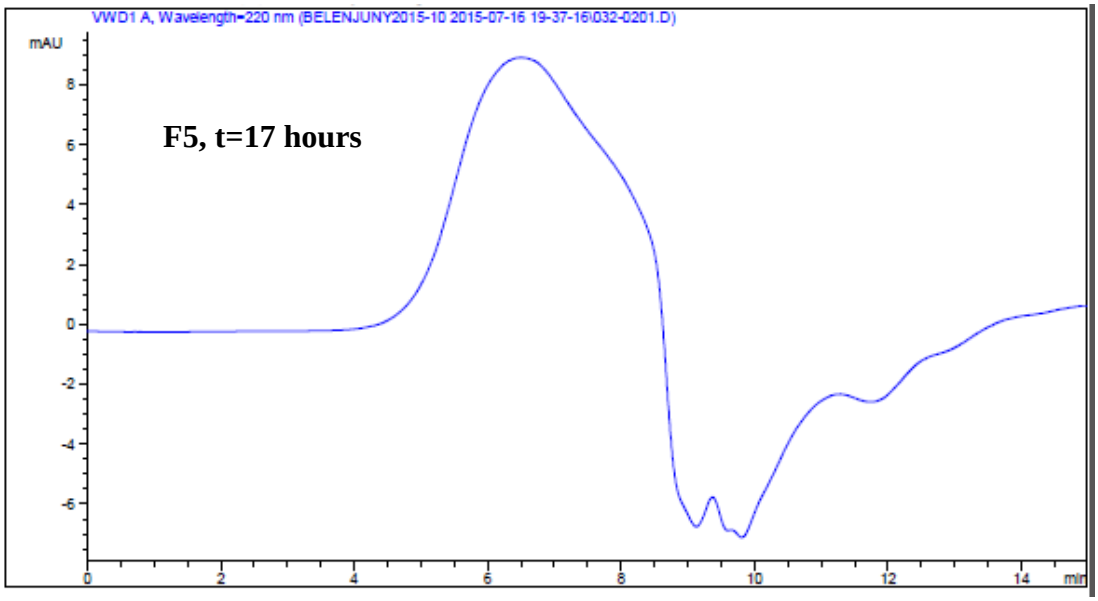


Figure C-32. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 17 hours of fermentation. Column details as previously explained.

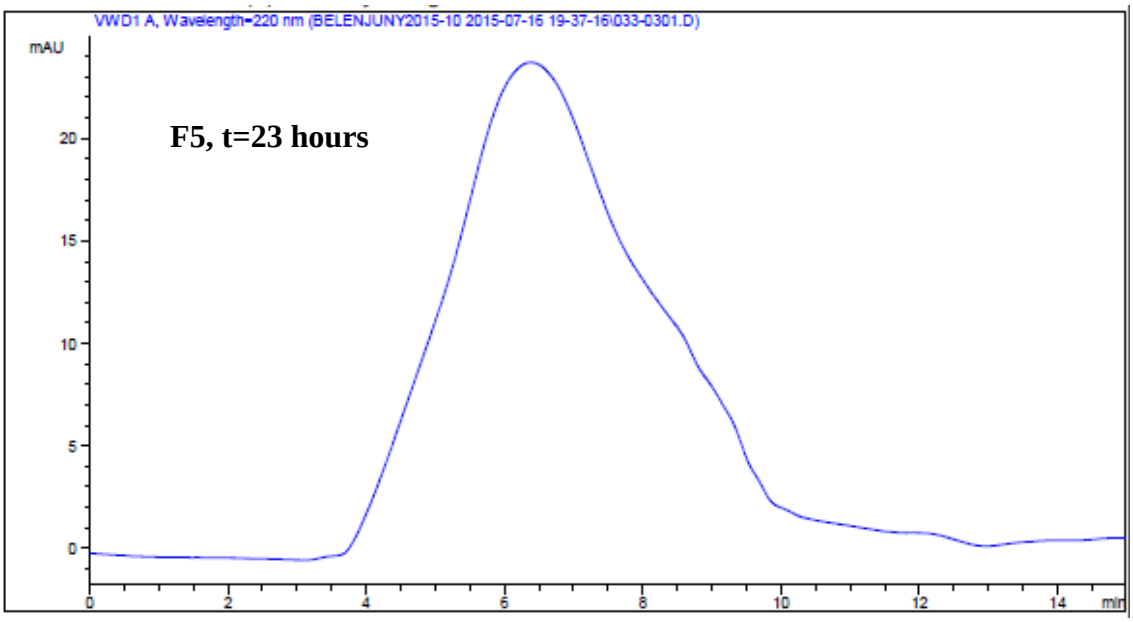


Figure C-33. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 23 hours of fermentation. Column details as previously explained.

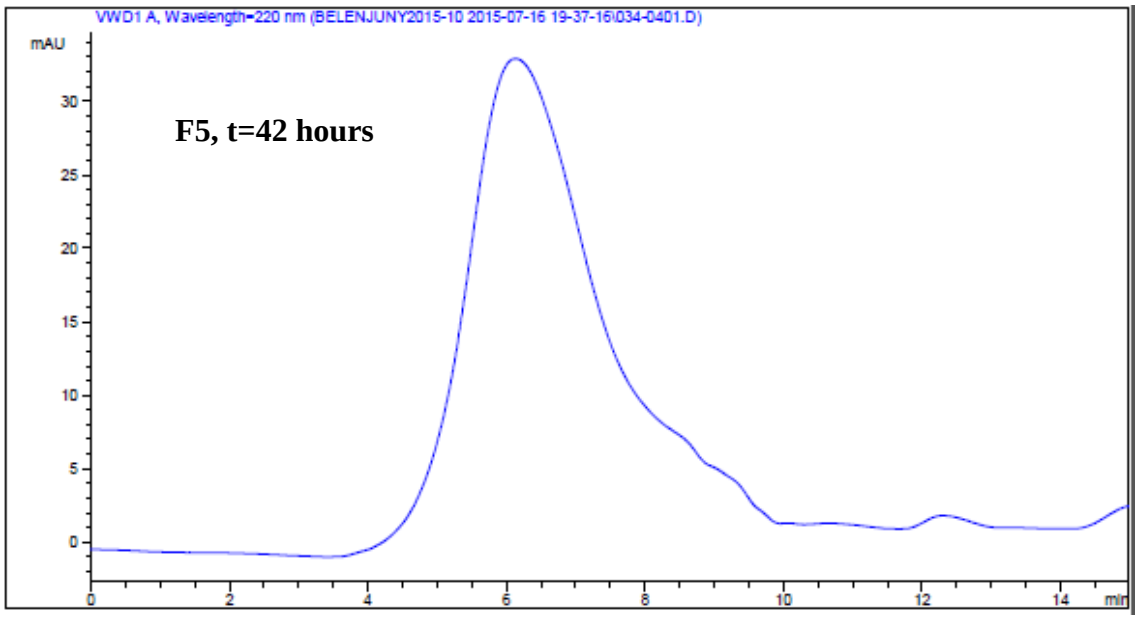


Figure C-34. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 42 hours of fermentation. Column details as previously explained.

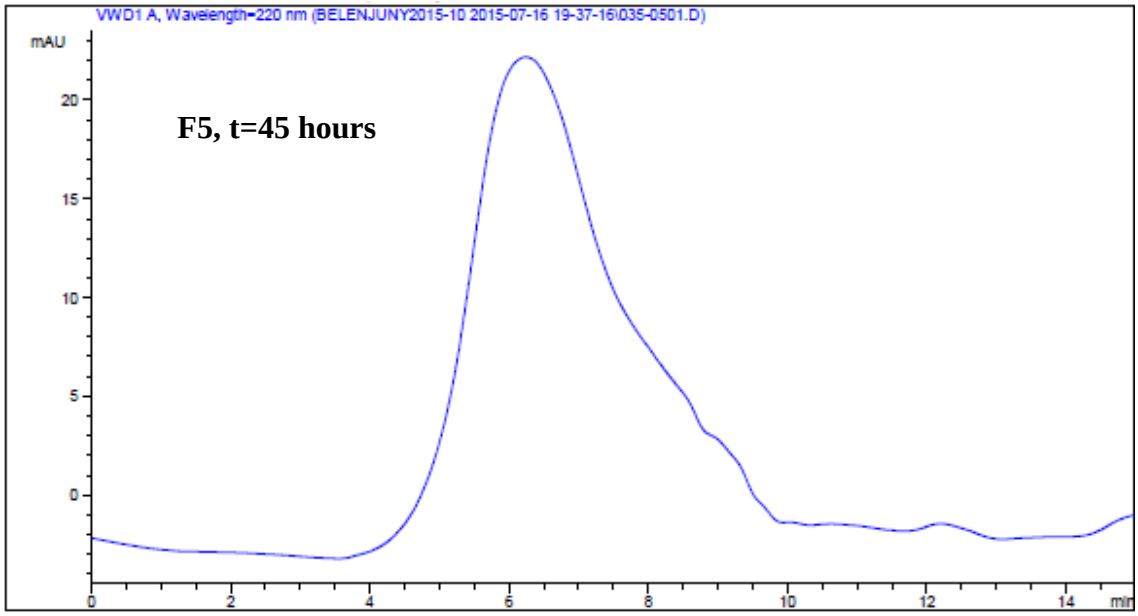


Figure C-35. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 45 hours of fermentation. Column details as previously explained.

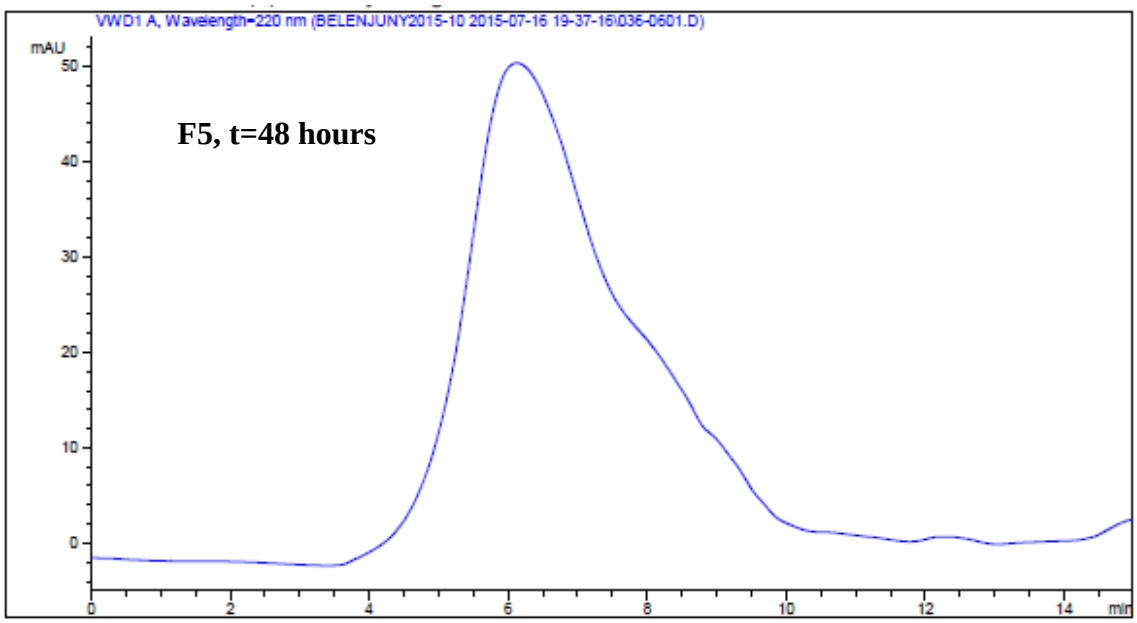


Figure C-36. Chromatogram showing the analysis of the culture medium sample from Fermentation 5 at 48 hours of fermentation. Column details as previously explained.

APPENDIX D: EXPERIMENTAL DATA

Table D-1. Cell Dry Weight experimental data for Fermentation 2.

Sample	t (h)	m_{epp} (g)	$m_{epp + pellet}$ (g)	CDW (g/l)	Standard error
1	0	1,0776	1,0652	-	
		1,0799	1,0800	0,10	
		1,0696	1,0702	0,60	
				0,35	0,3215
2	16	1,0683	1,0694	1,10	
		1,0681	1,0692	1,10	
		1,0722	1,0737	1,50	
				1,23	0,2309
3	20,5	1,0790	1,0800	1,00	
		1,0736	1,0749	1,30	
		1,0663	1,0674	1,10	
				1,13	0,1528
4	24	1,0698	1,0717	1,90	
		1,0954	1,0965	1,10	
		1,0833	1,0845	1,20	
				1,40	0,4359
5	44	1,0614	1,0640	2,60	
		1,0676	1,0698	2,20	
		1,0760	1,0780	2,00	
				2,27	0,3055
6	48	1,0784	1,0800	1,60	
		1,0854	1,0877	2,30	
		1,0706	1,0730	2,40	
				2,10	0,4359

Table D-2. Cell Dry Weight experimental data for Fermentation 3.

Sample	t (h)	m_{epp} (g)	$m_{epp + pellet}$ (g)	CDW (g/l)	Standard error
1	0	1,0683	1,0673	-	
		1,0808	1,0811	0,30	
		1,0645	1,0646	0,10	
				0,20	0,1414
2	16	1,0543	1,0566	2,30	
		1,0959	1,0970	1,10	
		1,0930	1,0949	1,90	
				1,77	0,6110
3	20	1,0694	1,0728	3,40	
		1,0596	1,0609	1,30	
		1,0680	1,0695	1,50	
				2,07	1,1590
4	24	1,0757	1,0799	4,20	
		1,0750	1,0786	3,60	
		1,0600	1,0634	3,40	
				3,73	0,4163
5	40	1,0820	1,0856	3,60	
		1,0601	1,0647	4,60	
		1,0948	1,0990	4,20	
				4,13	0,5033
6	48	1,0678	1,0721	4,30	
		1,0664	1,0700	3,60	
		1,0674	1,0715	4,10	
				4,00	0,3606

Table D-3. Cell Dry Weight experimental data for Fermentation 4.

Sample	t (h)	m_{epp} (g)	$m_{epp + pellet}$ (g)	CDW (g/l)	Standard error
1	0	1,0777	1,0787	1,00	
		1,0684	1,0690	0,60	
		1,0545	1,0550	0,50	
				0,70	0,2646
2	20	1,0549	1,0563	1,40	
		1,0665	1,0692	2,70	
		1,0798	1,0818	2,00	
				2,03	0,6506
3	24	1,0674	1,0701	2,70	
		1,0816	1,0835	1,90	
		1,0683	1,0708	2,50	
				2,37	0,4163
4	40	1,0768	1,0797	2,90	
		1,0604	1,0639	3,50	
		1,0946	1,0981	3,50	
				3,30	0,3464
5	44	1,0622	1,0660	3,80	
		1,0702	1,0745	4,30	
		1,0690	1,0728	3,80	
				3,97	0,2887
6	48	1,0884	1,0921	3,70	
		1,1100	1,1129	2,90	
		1,0680	1,0720	4,00	
				3,53	0,5686

Table D-4. Cell Dry Weight experimental data for Fermentation 5.

Sample	t (h)	m_{epp} (g)	$m_{epp + pellet}$ (g)	CDW (g/l)	Standard error
1	0	1,0712	1,0708	-	
		1,0694	1,0703	0,90	
		1,0675	1,0683	0,80	
				0,85	0,0707
2	17	1,0790	1,0813	2,30	
		1,0849	1,0870	2,10	
		1,0617	1,0640	2,30	
				2,23	0,1155
3	23	1,0840	1,0880	4,00	
		1,0831	1,0872	4,10	
		1,0889	1,0915	2,60	
				3,57	0,8386
4	42	1,0602	1,0650	4,80	
		1,0636	1,0677	4,10	
		1,0730	1,0780	5,00	
				4,63	0,4726
5	45	1,0923	1,0977	5,40	
		1,0674	1,0730	5,60	
		1,0887	1,0937	5,00	
				5,33	0,3055
6	48	1,0554	1,0621	6,70	
		1,0515	1,0581	6,60	
		1,0881	1,0948	6,70	
				6,67	0,0577

Table D-5. GPC analysis data for Fermentation 1.

Age (h)	Peak area	Concentration (g/l)	Corrected[a] (g/l)	Mn	Mw	Mp	D
0	0	0,000	0,000	0	0	0	0
16	150	0,000	0,000	0	0	0	0
21	180	0,000	0,000	40345	69670	15977	1,72
24	445	0,147	0,147	41144	94335	14599	2,29
39	250	0,062	0,062	40178	67752	34718	1,68
44	309	0,088	0,088	40017	73857	31304	1,84
48	550	0,193	0,193	43476	102100	15216	2,34

[a] Applying dilution factor.

Table D-6. GPC analysis data for Fermentation 2.

Age (h)	Peak area	Concentration (g /l)	Corrected[a] (g/l)	Mn	Mw	Mp	D
0	0	0,000	0,000	0	0	0	0
16	339	0,101	0,101	61224	161900	69452	2,64
20	522	0,181	0,181	53863	136520	56991	2,53
24	3662	1,551	1,551	61645	156390	124500	2,53
44	3600	1,524	1,829	62295	142770	105440	2,29
48	3650	1,546	1,855	61183	134410	103380	2,19

[a] Applying dilution factor.

Table D-7. GPC analysis data for Fermentation 3.

Age (h)	Peak area	Concentration (g /l)	Corrected[a] (g/l)	Mn	Mw	Mp	D
0	85	0,000	0,000	0	0	0	0
16	2600	1,088	2,176	54140	115430	98902	2,13
21	1400	0,564	2,256	52451	114320	97936	2,18
24	1756	0,719	2,878	53148	118930	100880	2,24
39	3050	1,284	5,137	53307	118500	91842	2,22
48	1977	0,816	3,264	53616	125430	102870	2,34

[a] Applying dilution factor.

Table D-8. GPC analysis data for Fermentation 4.

Age (h)	Peak area	Concentration (g /l)	Corrected[a] (g/l)	Mn	Mw	Mp	D
0	210	0,045	0,045	0	0	0	0
20	2114	0,876	0,876	49605	112330	87802	2,26
24	2105	0,872	0,872	48961	101010	77394	2,06
40	717	0,266	0,266	37393	70709	34498	1,89
44	470	0,158	0,158	32222	58718	37217	1,82
48	420	0,136	0,136	29958	71964	34947	2,40

[a] Applying dilution factor.

Table D-9. GPC analysis data for Fermentation 5.

Age (h)	Peak area	Concentration (g /l)	Corrected[a] (g/l)	Mn	Mw	Mp	D
0	133	0,011	0,006	0	0	0	0
17	1076	0,423	0,845	50883	88724	61485	1,75
23	3486	1,474	2,949	55380	143930	68733	2,60
42	3060	1,289	5,154	54846	105740	89580	1,93
45	3897	1,654	6,615	54152	101470	81028	1,87
48	6280	2,694	9,429	54197	111350	90473	2,05

[a] Applying dilution factor.