Liane Pereira de Meneses

Bachelor degree in Biomedical Sciences

Evaluation of sub-, supercritical fluids as solvents for extraction and impregnation of polyhydroxyalkanoates

Dissertation for the degree of Master in Biotechnology

Supervisor: Doctor Alexandre Babo de Almeida Paiva,
LAQV-REQUIMTE, FCT-UNL

Co-supervisor: Doctor Maria Filomena Andrade de Freitas,
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President: Professor Carlos Alberto Gomes Salgueiro
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Ao Zé da Mota.

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Abstract

Polyhydroxyalkanoates (PHA) are a class of biobased and biodegradable polymers with potential application on different areas, depending on their composition and specific properties that range from thermoplastics to elastomers. However, PHA development is still limited by several factors, including its extraction procedures that rely on organic solvents, such as chloroform. On the other hand, it is important to develop applications of the polymers that further increase their commercial value.

This work included two parts: (1) the use of hot compressed water (HCW) as an eco-friendly solvent for the pre-treatment of mixed microbial culture (MMC) biomass for the extraction of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)); (2) production of medium-chain-length PHA (*mcl*-PHA) and its impregnation with ibuprofen using supercritical carbon dioxide (sc-CO_2).

The results showed that using HCW at 150 °C, with a water flow rate of 10 mL/min during 30 min, it was possible to increase the polymer content of the samples from 66% to 77%. To further purify the obtained samples, a combination of HCW with digestion of the cellular residues with hypochlorite was experimented. With the combination of the two methods it was possible to decrease the hypochlorite concentration five times than when using only the hypochlorite step, while obtaining a higher purity of 82%. The main problem of applying the HCW step was the decrease of the molecular weight (Mw) from $3.0x10^5$ to $0.3x10^5$ g/mol and the increase of the polydispersity index.

Bioreactor fermentation of *Pseudomonas chlororaphis* was performed to obtain *mcl*-PHA, using glycerol as carbon source. Besides PHA, two other products were obtained, namely, an exopolysaccharide (EPS) and phenazine. The *mcl*-PHA had a crystallinity of 13%, melting temperature (T_m) of 44.6 °C, glass transition temperature (T_g) of -50.4 °C and a Mw of $1.3x10^5$ g/mol, which are in accordance to what is said in literature.

sc-CO_2 was used to impregnate ibuprofen in the *mcl*-PHA. The maximum impregnation yield obtained was 93.3 mg/g (at 200 bar for 3 h at 40 °C). The controlled release tests showed that the ibuprofen is released from the *mcl*-PHA with a controlled profile.

Keywords

Polyhydroxyalkanoates; Hot Compressed Water; Purification; *Pseudomonas chlororaphis*; Supercritical Carbon Dioxide; Impregnation.

Resumo

Os polihidroxialcanoatos (PHA) pertencem ao grupo dos polímeros biodegradáveis de origem biológica com aplicação nas mais diversas áreas de acordo com a sua composição e características específicas que variam desde termoplásticos a elastómeros. Contudo, o desenvolvimento dos PHA continua limitado por diversos fatores incluindo os processos para a sua extração que utilizam solventes orgânicos, como é o caso do clorofórmio. É também importante desenvolver aplicações para os polímeros que aumentem o seu valor comercial.

Este trabalho incluiu duas partes: (1) a aplicação de água subcrítica como solvente verde no pré-tratamento de biomassa de culturas mistas para a extração de poli-(3-hidroxibutirato-*co*-3-hidroxivalerato) (P(3HB-*co*-3HV)); (2) a produção de um PHA de cadeia média (*mcl*-PHA) e sua impregnação com ibuprofeno usando dióxido de carbono supercrítico (sc-CO_2).

Os resultados obtidos mostraram que utilizando água subcrítica a 150 °C com um caudal de água de 10 mL/min, durante 30 min foi possível aumentar o conteúdo em PHA da amostra de 66% para 77%. De forma a tentar obter um polímero puro combinou-se o método da água subcrítica com uma extração com hipoclorito. A combinação dos dois métodos permitiu usar cinco vezes menos hipoclorito do que quando se usa apenas a extração com hipoclorito, obtendo um polímero com 82% de pureza. A maior desvantagem da combinação dos dois métodos foi a diminuição que provocam no peso molecular do polímero, de $3.0x10^5$ para $0.3x10^5$ g/mol e a diminuição do índice de polidispersão.

Fez-se uma fermentação em biorreator de *Pseudomonas chlororaphis* a partir de glicerol para obter *mcl*-PHA. Para além do PHA obteve-se outros dois produtos, sendo eles exopolissacarídeo (EPS) e fenazinas. O *mcl*-PHA apresenta cristalinidade de 13 %, temperatura de fusão de 44.6 °C, temperatura de transição vítrea de -50.4 °C e um peso molecular de $1.3x10^5$ g/mol, valores que estão de acordo com o que está descrito na literatura.

Usou-se sc-CO_2 para impregnar ibuprofeno no *mcl*-PHA atingindo-se um rendimento máximo de impregnação de 93.3 mg/g (a 200 bar durante 3 h a 40 °C). Os testes de libertação controlada permitiram verificar que a libertação do ibuprofeno do *mcl*-PHA apresenta um perfil característico de libertação controlada.

Palavras chave

Polihidroxialcanoatos; Água Subcrítica; Purificação; *Pseudomonas chlororaphis*; Dióxido de Carbono Supercrítico; Impregnação.

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3HD	3-hydroxydecanoate
3HDd	3-hydroxydodecanoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
3HTd	3-hydroxytetradecanoate
CDW	Cell Dry Weight
DSC	Differential Scanning Calorimetry
EPS	Exopolysaccharide
GC	Gas Chromatography
HCW	Hot Compressed Water
HPLC	High Performance Liquid Chromatography
***mcl*-PHA**	Medium-Chain-Length Polyhydroxyalkanoates
Mn	Average Molecular Number
MMC	Mixed Microbial Culture
Mw	Average Molecular Weight
NPCM	Non-PHA Cell Mass
OD	Optical Density
P(3HB)	Poly-(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly-(3-hydroxybutyrate-*co*-3-hydrovalerate)
p_c	Critical Pressure
PDI	Polydispersity Index
PE	Polyethylene
PHA	Polyhydroxyalkanoates
PP	Polypropylene
QS	Quorum Sensing
rpm	Rotation Per Minute
sc-CO_2	Supercritical Carbon Dioxide
SCF	Supercritical Fluid
***scl*-PHA**	Short-Chain-Length Polyhydroxyalkanoates
SCW	Supercritical Water
T_c	Critical Temperature
T_{deg}	Degradation Temperature

T_g	Glass Transition Temperature
TGA	Thermogravimetric Analysis
T_m	Melting Temperature
X_c	Degree of Crystallinity
XRD	X-ray Diffraction

Chapter 1

Introduction and Motivation

1.1 Plastics

Plastics are present in almost everything we do in our daily routine. From home appliances to automobiles, everything is made of plastic. Plastics are widely used because they can be manipulated to whatever shape and strength wanted. Conventional plastics are produced as a by-product of the oil industry. Considering the European use of oil for its different applications, only 4 to 6% are directed to plastic materials production. Looking at Figure 1.1 it can be seen that from 2005 to 2015, the world plastic production has gradually increased, but at an European level the amount of plastics produced has been stable for several years (European Bioplastics, 2016).

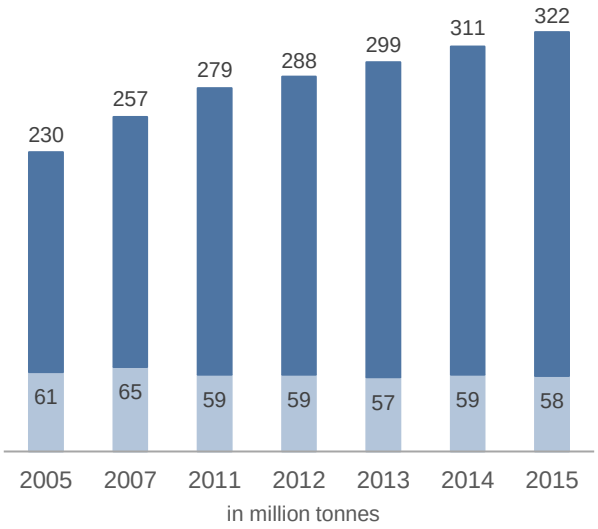

Figure 1.1 – Evolution of World (■+■) and Europe (■) plastics production over the last 10 years. The entire bar represents world production and the lighter part represents only the European production. Data from Europe is related to EU 28 State Members plus Norway and Switzerland. Adpated from Plastics Europe, 2016.

The main problem with plastics is their degradability, or on this case the lack of it. Plastics became one of the main pollutants on Earth, and they affect both land and seas. Back in 1960, plastics represented less than 0.5% of municipal solid waste on the United States; by 2010, this number had risen to 12.4% and only 8.2% of these plastics were recovered (Philp et al., 2013). About 40% of the produced plastics are disposed in landfills and as they are not biodegradable they accumulate in those areas. In addition to the plastics in landfills, there is a great percentage that is discarded into the marine environment, accumulating and comprising marine life (Reddy et al., 2003).

1.2 Bioplastics

With the problems associated to conventional plastics, work was put on to develop new materials with the appealing characteristics of plastics' – Bioplastics.

Bioplastics can either be (1) biobased, when the product is derived from biomass (corn, sugarcane or cellulose); (2) biodegradable, when the plastic is degradable in the environment in a sustainable time frame; or (3) both, when the plastic fits in both categories (European Bioplastics, 2013). Examples of each of the groups of bioplastics can be seen in Figure 1.2. The category of biobased and biodegradable plastics includes polylactic acid (PLA), starch blends and polyhydroxyalkanoates (PHA). PHA have many interesting characteristics that can even compete in the market with some oil derived plastics, such as polyethylene (PE) and polypropylene (PP), since they resemble those plastics in their physical properties.

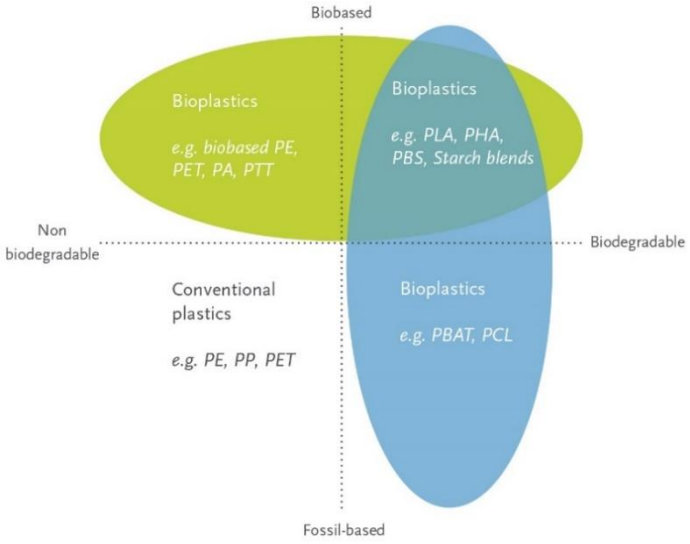

Figure 1.2 – Representation of the different families of plastics either conventional plastics and bioplastics. The green shadow shows biobased plastics, the blue shadow shows biodegradable and the part where they overlap shows the biobased and biodegradable plastics. Retrieved from European Bioplastics, 2013.

The increase on bioplastics' production would mean increasing resource efficiency by reusing raw material that would be disposed. Besides this, with less dependence on mineral resources the greenhouse gases emission would decrease. Annually, 320 million tonnes of plastic are produced. Bioplastics are still representing only 1% (4.2 tonnes) of this number. Figure 1.3 shows the global production capacity of bioplastics. The predictions indicate that the production of bioplastics will grow up to 6.1 million tonnes by 2021. As it can be seen, the biodegradable plastics represent a much smaller fraction of the bioplastics produced globally. Within the biodegradable section, PHA represents only 1.6% of the bioplastics produced. By 2021, PHA production is expected to get four times higher than it was by 2016 when the first PHA plant started-up in Europe.

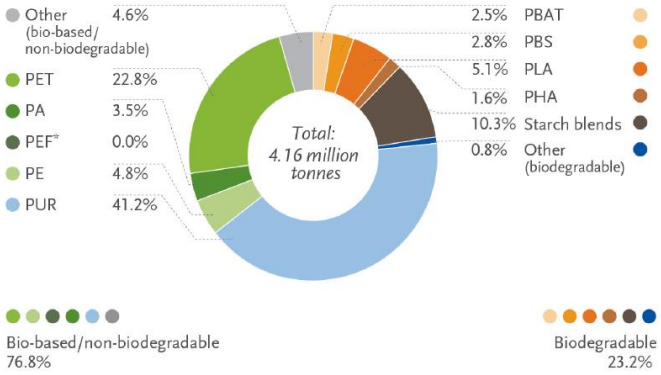

Figure 1.3 – Global production capacity of bioplastics in 2016. Retrieved from European Bioplastics, 2016.

The main disadvantage of PHA that keeps this polymer from being widely used is its price since it is still three to four times higher than the price of conventional oil-derived plastics (Kourmentza et al., 2017). This difference is mainly due to the price of the carbon sources and the costs of the downstream processes. Using pure carbon sources such as glucose (0.49 US$/kg) is more expensive than using carbon-rich residues such as cheese whey (0.07 US$/kg) (Salehizadeh and Van Loosdrecht, 2004). So, the price of the final product is highly influenced by the price of the main substrate and to overcome this problem some measures have been taken. These measures include using industrial and agricultural wastes as carbon sources and using mixed microbial cultures (activated sludge, for example) to avoid the need to work in sterile environment. Reducing these costs and increasing the production yields should help decrease the market price of PHA and make them compatible in the plastics market, not only for their characteristics but also for their price (Anjum et al., 2016; Salehizadeh and Van Loosdrecht, 2004).

1.3 Polyhydroxyalkanoates

By definition, PHA are polyesters of various hydroxyalkanoates (Reddy et al., 2003) that can be either produced by plants or bacteria in single or mixed cultures (Salehizadeh and Van Loosdrecht, 2004). The use of plants is uncommon because they can only take low PHA production yields, since higher yields limit their growth (Verlinden et al., 2007). However, bacteria can accumulate up to 90% of their dry weigh in PHA, by naturally storing carbon and energy by means of PHA accumulation in intracellular granules under conditions of excess of carbon with limitation of other nutrients (nitrogen, phosphorous or oxygen) (Reddy et al., 2003; Verlinden et al., 2007).

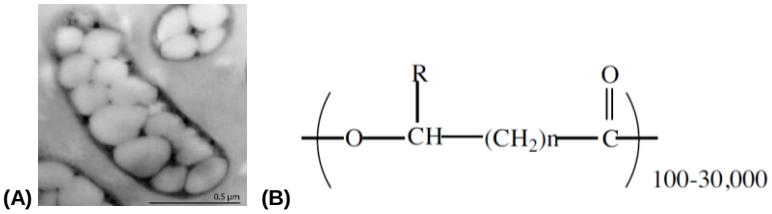

Figure 1.4 – (A) Bacterial cell with PHA granules (Madkour et al., 2013). (B) General PHA structure where **R** represents the side chain of the monomer and **n** represents the number of carbon atoms on the main chain (Anjum et al., 2016).

Structurally, PHA can be divided into two groups according to the number of carbon atoms in the chain: short-chain-length (*scl*-PHA) or medium-chain-length (*mcl*-PHA) (Anjum et al., 2016; Koller et al., 2013; Madkour et al., 2013). The general structure of PHA is represented in Figure 1.4. They can be classified as homopolymers, when composed by one monomer, or heteropolymers when composed by two or more monomers (Anjum et al., 2016). Some examples of heteropolymers are the copolymers poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)), poly-(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) (P(3HHx-*co*-3HO)), poly-(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HHx)) or poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HV-*co*-3HHx)) (Anjum et al., 2016). The produced monomers depend on the bacterial strain, the carbon source and PHA synthases substrate specificity (Anjum et al., 2016; Reddy et al., 2003). The major PHA monomers produced in bacteria are listed in Table 1.1.

PHA production can be performed by either single or mixed microbial cultures (MMC). The choice between them relies on diverse factors since there are advantages and disadvantages associated to which one. Single microbial cultures usually have better productivity and higher PHA content (about 80% of cellular dry weight (Salehizadeh and Van Loosdrecht, 2004)), and the outcome is a simple, well-defined polymer, since there is only one type of bacteria producing it. Single cultures can produce an enormous variety of PHA ranging from *scl*-PHA to *mcl*-PHA (Cruz et al., 2016). The biggest problem of using single cultures is the need to work under sterile conditions (Agustín Martinez et al., 2015). The need to use pure carbon sources has been overcome since carbon-rich wastes have successfully used for PHA production by single cultures (Cruz et al., 2016, 2015; Morais et al., 2014).

On the contrary open MMC, such as activated sludge, make the process economically more attractive since they are easier to control, do not require the need to work under sterile conditions and improve the use of carbon rich wastes like agro-industrial wastes (Agustín Martinez et al., 2015; Salehizadeh and Van Loosdrecht, 2004; Samorì et al., 2015). MMC usually accumulate lower PHA contents (less than 20%) and to increase the bacterial PHA content there is a cellular growth phase followed by a PHA accumulation phase. PHA production by MMC is usually a two or three-stage process and this represents a disadvantage when compared to single cultures on which the whole process is performed in one reactor. A major disadvantage of using MMC is that these only allow to produce *scl*-PHA, namely P(3HB), P(3HB-*co*-3HV) and sometimes the polymers can have the incorporation of 3-hydroxyhexanoate (3HHx) in smaller fraction (around 1%) (Salehizadeh and Van Loosdrecht, 2004; Serafim et al., 2008; Verlinden et al., 2007).

Table 1.1 – Major monomers in bacterial PHA (with n=1). Retrieved from Anjum et al., 2016.

Side Chain	Monomer		
Hydrogen	3-hydroxypropionate	3HP	*scl*-PHA
Methyl	3-hydroxybutyrate	3HB	
Ethyl	3-hydroxyvalerate	3HV	
Propyl	3-hydroxyhexanoate	3HHx	*mcl*-PHA
Butyl	3-hydroxyheptanoate	3HH	
Pentyl	3-hydroxyoctanoate	3HO	
Hexyl	3-hydroxynonanoate	3HN	
Heptyl	3-hydroxydecanoate	3HD	
Octyl	3-hydroxyundecanoate	3HUD	
Nonyl	3-hydroxydodecanoate	3HDD	

In MMC, cells are exposed to carbon rich media where they grow until the desired cellular concentration is achieved. In this type of culture, PHA production is induced by an intracellular limitation: when the cells are exposed to a growth medium with low nutrient concentrations they alter their physiology. When the carbon concentration increases, the cells are obligated to alter their physiology once more and since it is easier to adapt to PHA production than to growth, the cells start to synthetize PHA. This process of fermentation is called “feast and famine” (Verlinden et al., 2007). Substrates for the first step in PHA synthesis can be obtained from a variety of sources, such as wastewaters, fruits, oil rich effluents and apple pomace. These substrates are rich in complex biomolecules that require degradation into smaller biodegradable organic compounds that the acidogenic and acetogenic bacteria can then utilise in their metabolism to generate volatile fatty acids (VFA), that are the precursors to PHA formation (Reddy et al., 2003).

Production with single cultures, as it has been referred, is performed under sterile conditions. Bacterial strains such as *Cupriavidus necator* or *Pseudomonas aeruginosa* cannot grow and synthetize PHA at the same time (Tan et al., 2014). For these strains, the PHA production has to be performed in two phases. The first phase comprises the cellular growth, where a carbon rich source and a nutrient source are supplemented to the growth medium. Then, on a second phase,

PHA production is induced by limitation of one nutrient (most commonly, nitrogen or phosphorus) on the medium and the carbon source remains in excess. This limitation directs the cellular metabolism for PHA production. However, some bacterial strains, i.e. *Pseudomonas putida*, can synthetize PHA during exponential growth phase at high rates (Sun et al., 2007).

1.3.1 Short-chain-length PHA

scl-PHA are comprised of monomers with three to five carbon atoms and their main characteristics are their stiffness and brittleness (Anjum et al., 2016; Koller et al., 2013), characteristics mainly found in thermoplastics, being competitors to PE and PP (Muhr et al., 2013). Table 1.2 shows a comparison between PP and some PHA. This group of plastics usually shows high melting temperature (T_m) and degree of crystallinity (X_c), 60-80% (Anjum et al., 2016; Koller et al., 2013). Their glass transition temperature (T_g) is also high, around 0 °C, and they have high molar masses (between $5x10^4$ and $1x10^6$ g/mol) (Koller et al., 2013).They can be produced by a variety of microbial strains including *C. necator*, *P. hydrogenovora*, *Comamonas acidovorans,* among others (Anjum et al., 2016).

The homopolymer P(3HB) has a crystalline structure, and is extremely stiff and brittle. These properties can be altered by the incorporation of another monomer with P(3HB), producing a copolymer. One example is the P(3HB-*co*-3HV) copolymer, which shows lower X_c and T_m than P(3HB), is less stiff and has different mechanical properties (such as higher elongation to break and flexibility). The specific characteristics of the copolymer depend on the percentage of 3-hydroxyvalerate (3HV) incorporated into the polymer (Anjum et al., 2016).

Table 1.2 – Comparison between the physical properties of different *scl*-PHA and conventional plastics (Anjum et al., 2016; Verlinden et al., 2007).

	T_m (°C)	**T_g (°C)**	**X_c (%)**	**Tensile Strength (MPa)**	**Extension to Break (%)**
P(3HB)	173-180	5-9	60	40	3-8
P(3HB-co-3HV)	145-150	-1	56	1.5	50
P(3HB-co-3HHx)	52	-4	34	20	850
PP	176	-10	50-70	38	400

1.3.2 Medium-chain-length PHA

mcl-PHA is a sub-class of PHA with monomers comprised of six to fourteen carbon atoms (Koller et al., 2013). This type of plastics have low T_m, around 60 °C, low T_g, around -40 °C, and have very low X_c, between 25 and 40% (Anjum et al., 2016; Koller et al., 2013). The main difference between them and *scl*-PHA is the lower degree of crystallinity that makes them much more elastic and flexible materials and provide them a larger variety of applications (Anjum et al., 2016; Reddy et al., 2003), such as glues, rubbers or adhesives (Muhr et al., 2013). *P. putida* was the first *mcl*-PHA producer strain to be discovered and has been widely investigated. Other *Pseudomonas* strains such as *P. aeruginosa*, *P. resinovorans* or *P. fluorescens* are also known to be *mcl*-PHA producers (Muhr et al., 2013).

1.4 Extraction methods

One of the main problems in the industrial implementation of PHA relies in its biosynthesis since PHA are accumulated in intracellular granules which makes it difficult to separate them from non-PHA cell mass (NPCM) or residual biomass composed by polypeptides, phospholipids, DNA, RNA and peptidoglycans (Koller et al., 2013). Different approaches on how to separate PHA from NPCM have been developed but they all rely on the same factors: (1) producer microbial strain, (2) type and intracellular amount of PHA, (3) biopolymer final application and (4) impact on PHA molar mass (Koller et al., 2013).

Prior to extraction, biomass can be submitted to pre-treatments to weaken the cellular structures and facilitate PHA extraction and purification. These strategies can involve heat, solvents or even mechanical processes, and have to be applied accordingly to the strain characteristics (Koller et al., 2013; Madkour et al., 2013).

The most used method for PHA extraction is extraction by solvent. A variety of organic solvents have been used for PHA recovery, especially for *scl*-PHA, such as chloroform, 1,2-dichloroethane, methylene chloride and even cyclic carbon esters. Some attempts to use nonhalogenated solvents, such as acetone, have also been tried and showed potential when applied to *mcl*-PHA since they have enough solubility in this group of solvents. Solvent extraction relies on the fact that only the polymer and the lipids of the NPCM can be dissolved in the organic solvent. In order to get a purified polymer both the lipids and the solvent have to be separated from the polymer so there is a second step. This purification step involves the use of an anti-solvent that can be ethanol, methanol or water, for all PHA types, or hexane, ether or acetone for *scl*-PHA (Koller et al., 2013; Madkour et al., 2013). This method has shown to provide pure polymers with low impact on PHA molar mass (Koller et al., 2013). The biggest problems with solvent extraction and subsequently PHA precipitation are the use of organic solvents such as chloroform for the extraction and the amount of solvent that is needed. For the extraction, at laboratory scale this problem is overcome by using a closed system like a Soxhlet apparatus, but for the precipitation step the anti-solvent volume must be ten times the PHA solution volume. As for the industrial scale this method is not feasible and alternative strategies have been developed trying to aim that purpose (Koller et al., 2013; Madkour et al., 2013).

NPCM disruption methods were developed for industrial purposes. These methods are different than the ones described above because they aim to remove the NPCM part of the cells, since it is significantly smaller than the PHA share, and release the PHA granules. Various methods can be used for disruption, such as chemical (sodium hypochlorite, surfactants, chelate-hydrogen peroxide, acidic or alkaline digestion), enzymatic or mechanical (high pressure homogenization, ultrasounds, bead mill). The action of the disruption agents turns the hydrophilic elements of NPCM into substances that can be solubilized by water, hence releasing the PHA granules to be recovered by centrifugation, filtration or settling (Koller et al., 2013; Madkour et al., 2013).

Strategies such as the application of supercritical fluids, namely supercritical carbon dioxide (sc-CO_2), air classification, dissolved-air flotation, cell fragility and spontaneous PHA release from the cells by recombinant strains have also been developed but they all show drawbacks either being their costs, yield or degree of purity of PHA obtained (Koller et al., 2013; Madkour et al., 2013). Table 1.3 shows a comparison between the extraction methods mentioned above.

Table 1.3 – Comparison of costs, recovery yields, PHA purity and impact on PHA between some of the most common extraction processes.

Method	Cellular strain	Recovery yields	PHA purity	Impact on PHA	Reference
Halogenated Solvents	*Alcaligenes eutrophus*	High	High	Low	(Ramsay et al., 1994)
Non-halogenated Solvents	*Burkholderia sacchari*	93-97%	98%	Low	(Rosengart et al., 2015)
Chemical Disruption (Hypochlorite digestion)	*Alcaligenes eutrophus*	-	97-98%	High	(Ramsay et al., 1990)
Enzymatic Disruption	*Cupriavidus necator*	93%	94%	Low	(Neves and Müller, 2012)
Mechanical Disruption (High-pressure homogenization)	*Methylobacterium* sp V49	98%	95%	Low	(Ghatnekar et al., 2002)
Supercritical Fluids	*Ralstonia eutropha*	89%	-	-	(Hejazi et al., 2003)
Air Classification	*Ralstonia eutropha*	85%	>95%	Low	(Koller et al., 2013)

1.5 Supercritical Fluids

Since the 1970s that supercritical fluids (SCF) have been gaining a lot of attention for their environmental friendly characteristics that allow them to replace organic solvents in many different applications such has natural products extraction and fractionation, impregnation, particle formation, biomass gasification or supercritical water oxidation (Brunner, 2010; Nahar and Sarker, 2005).

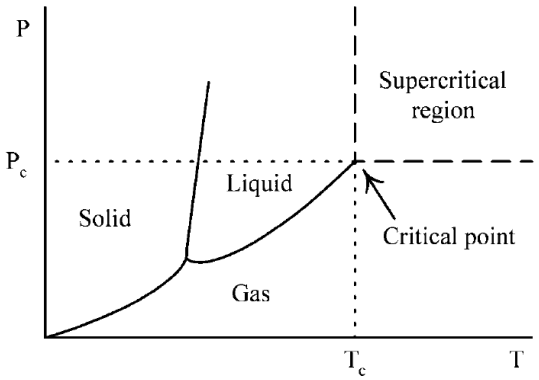

Figure 1.5– Phase diagram of a pure substance. Retrieved from Duarte et al., 2009.

As it can be seen in the phase diagram of a pure substance (represented in Figure 1.5), up until the critical point, the substance can be defined either as a solid, a liquid or a gas, but above its critical point phase separation becomes indistinguishable. Above the characteristic temperature and pressure of this point, the substance enters the supercritical region where it becomes a homogeneous fluid (Duarte et al., 2009; Nahar and Sarker, 2005). A SCF shows low viscosity, like a gas, high density, like a liquid, and almost non-existent surface tension. These properties provide interesting transport properties in condensed phases (Brunner, 2010; Nahar and Sarker, 2005). The critical temperatures (T_c) and critical pressures (p_c) of different solvents are shown in Table 1.4. As it can be seen, the T_c of liquids and gases can be very different and this is an important aspect to consider when choosing a solvent to be used as supercritical fluid.

Table 1.4 - Critical conditions of different solvents. Adapted from Mchugh and Krukonis, 1994.

	T_c, °C	**p_c, bar**
Carbon Dioxide	31	74
Ethane	32	49
Propane	97	43
Isopropanol	235	48
Ammonia	133	113
Water	374	221

One of the most used SCF is sc-CO_2 that has been used to replace organic solvents such as *n*-hexane or chloroform in processes of separation or purification, like caffeine extraction from coffee beans or tea leaves. Comparing to organic solvents, it is advantageous to use SCF for extraction processes because they are cheaper, do not contaminate the target of extraction, are manageable and are also easier and safer to dispose after use (Nahar and Sarker, 2005). Water

has also been a target to be used as SCF but the critical point of water is at high temperature and pressure so water has been mostly used as a subcritical fluid (Asl and Khajenoori, 2013).

1.5.1 Sub and supercritical Water

Water is considered a green solvent since it is part of our surroundings and it is an harmless solvent (Kruse and Dinjus, 2007). For these reasons, water has been a target to be used as solvent for many purposes. Like any SCF, supercritical water (SCW) is defined as water at temperature and pressure conditions above its critical point (T_c=374 °C, p_c=221 bar). SCW acts as non-polar solvent being miscible with organic solvents and incapable of keeping salts soluble. This fluid has low viscosity and high diffusivity rate. Since SCW is a highly reactive fluid it is mainly used for supercritical water oxidation (SCWO), for the destruction of persistent wastes, like the ones present in wastewaters or sludges. The SCW technology has some disadvantages associated, like the high corrosive potential of the fluid itself, the salt deposition, and also the high energy requirement (Bermejo and Cocero, 2006; Brunner, 2009a). Due to these problems, water is also used as a subcritical fluid because in that region it also shows interesting properties and can be used in many applications.

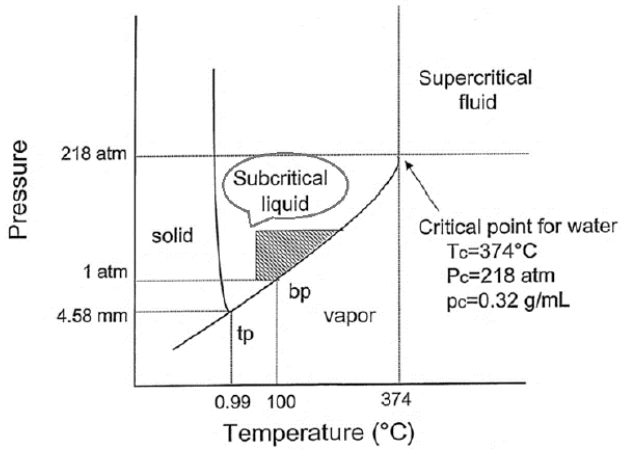

Figure 1.6 – Phase diagram of water as function of pressure (atm) and temperature (°C). Triple point (tp), boiling point (bp) and critical point are pointed out and subcritical region is indicated (Asl and Khajenoori, 2013).

Subcritical Water or Hot Compressed Water (HCW) is defined as water at high temperatures (above 100 °C) and high pressure that keeps its liquid form. In HCW the ionic product increases up to three orders of magnitude (from $K_w=10^{-14}$ mol^2L^{-2} at 25 °C to $K_w\approx10^{-11}$ mol^2L^{-2} at 300 °C) turning water into a catalyst for acid/base reactions. The dielectric constant decreases with temperature consequently decreasing the solubility of ionic molecules and increasing for hydrophobic molecules. This gives water the ability to be miscible with many organic compounds something that is not possible with water at ambient conditions. Finally, water viscosity and density are also reduced at higher temperatures improving its mass transfer capacity. Combining all these properties water becomes the solvent and reactant in hydrolysis reactions, that are usually catalysed by inorganic acids (Asl and Khajenoori, 2013; Brunner, 2009b; Kruse and Dinjus, 2007; Möller et al., 2011). Reactions are dependent of the solvent properties of water, temperature and pressure. Temperature and pressure can be adjusted to the reaction

requirements but the solvent properties of water are dependent of pressure and temperature (Möller et al., 2011). HCW has been used to a variety of applications such as wheat straw hydrolysis for production of added value products such as glucose and xylose (Abdelmoez et al., 2014) and extraction of antioxidants from grape pomace (Aliakbarian et al., 2012; Pedras et al., 2017).

1.5.2 Supercritical Carbon Dioxide

Carbon dioxide has been used as a SCF for many applications. There are many advantages in using this fluid as a substitute for other solvents in processes like extraction or purification of substances. As shown in Table 1.4 the critical point of CO_2 is established at relative ambient temperature (T_c = 31 °C) and at a pressure that is not extremely high (p_c = 74 bar). Besides having an easy to achieve critical point, CO_2 has other associated advantages since it is widely available and not expensive, it is non-toxic, non-flammable and non-corrosive. CO_2 is also considered a safe solvent considering that its separation and recovery from the products is much easier when compared to organic solvents, for instance. Control of the pressure is enough to recover the CO_2 that can be recirculated and reutilized therefore not contributing to the greenhouse gas emissions (Duarte et al., 2009; Nahar and Sarker, 2005).

sc-CO_2 has high density, like a liquid, and low viscosity, like a gas as it shown in Table 1.5. These properties combined turn this SCF into a quite effective and selective solvent. The ability to solubilize compounds is related to the density of sc-CO_2 and this characteristic can be adjusted by changing the temperature and pressure without falling out the critical region. Another way to improve the solvating power of sc-CO_2 is by adding modifiers to the SCF, for example methanol that increases de polarity of the extraction media (Nahar and Sarker, 2005).

Table 1.5 – Comparison between density (kg/m^3), viscosity (cP) and diffusivity (mm^2/s) of Gas, SCF and Liquid. Retrieved from Nahar and Sarker, 2005.

	Density (kg/m^3)	**Viscosity (cP)**	**Diffusivity (mm^2/s)**
Gas	1	0.01	1 – 10
SCF	100 – 800	0.05 – 0.1	0.001 – 0.1
Liquid	1000	0.5 – 1.0	0.001

As reviewed by Brunner, 2010, sc-CO_2 can be used in many different industrial applications such as extraction of essential and edible oils like sesame oil. It is also applied to treatment of cork used for wine bottle stoppers or the treatment of spent rubber tires so that waste rubber granules can be reutilized. Besides its applications in extraction and purification, sc-CO_2 is also applied in drying processes to produce aerogels, on polymers' impregnation and in dyeing.

1.6 Motivation

With growing environmental concern and diminishing mineral oil resources, green alternatives to well established processes have started to be developed. One of the strategies is the production of bioplastics such as PHA. These biologically synthetized thermoplastics have very similar properties to the ones found in mineral oil based plastics, such as PP or PE (Madkour et al., 2013; Reddy et al., 2003). PHA can be synthesized by a variety of bacteria and substrates and are biocompatible, biodegradable and non-toxic plastics (Madkour et al., 2013; Reddy et al., 2003).

Despite the improvements in the technology, the application of PHA in industrial scale is still limited due to high production costs (Madkour et al., 2013). One of the steps that still represents a problem in PHA industrial production is the recovery and purification process. The PHA are accumulated intracellularly, and every method to extract them ends up releasing cellular compounds that get mixed with the biopolymer, making the purification step more challenging (Madkour et al., 2013). Most strategies used for PHA purification involve the use of large amounts of organic solvents and high energy requirements (Koller et al., 2013; Madkour et al., 2013; Samorì et al., 2015).

Depending on their type and specific characteristics, PHA can have different applications. Their biocompatible and biodegradable characteristics allow PHA to be used in medical applications, such as sutures, stents, patches or even controlled release systems (Verlinden et al., 2007).

This work encompassed two main goals. The first goal was to develop a method for *scl*-PHA purification using a green solvent, namely HCW. The second goal was to produce *mcl*-PHA with *Pseudomonas chlororaphis* and, afterwards, impregnate it with ibuprofen using sc-CO_2.

Chapter 2

P(3HB-*co*-3HV) extraction using Hot Compressed Water as Pre-treatment

2.1 Introduction

Over the past two decades, a lot of attention has been given to PHA and intensive work has been developed to produce it as a viable competitor to mineral oil-based plastics. Production processes have been optimised and the costs of production have already decreased significantly. However, the downstream processes for separation and purification of the polymers from the cells still represents a problem since PHA extraction methods rely mostly on organic solvents with hazardous characteristics. Moreover, cellular disruption methods use solvents that are hard to remove from the obtained polymer thus resulting in polymers with low purity levels. Efforts have been made to develop greener methods for PHA extraction with high yields and purity, as the ones that can be achieve with the use of organic solvents. Some attempts to use supercritical fluids, namely sc-CO_2, have been tried but did not reach the desired PHA yield or purity (Hejazi et al., 2003).

It has been explained that to release PHA from the surrounding biomass the cells have to rupture since PHA is produced as intracellular granules. Microbial cell membranes are mostly comprised by phospholipids and proteins. PHA granules membrane also consists of lipids and proteins (Koller et al., 2013). With the knowledge that HCW is a hydrolysis catalyst, it can be used to hydrolyse these compounds from the PHA surrounding biomass, hence being a green solvent for PHA extraction. The membrane's proteins can be hydrolysed into oligomers and amino acids, their building blocks (Brunner, 2009b). Phospholipids can also be hydrolysed, but depending on where the hydrolysis takes place they can be split into different compounds. One example is the phospholipids hydrolysis into free fatty acids and lysophospholipid or hydrolysis to diacylglycerol (DAG) and organic phosphate (Anthonsen et al., 1999; Shah et al., 2017). The optimisation of the HCW hydrolysis conditions is extremely important because PHA are also hydrolysable since the monomers are linked together through ester bonds (Verlinden et al., 2007). It is important to find the conditions of pressure, temperature and water flow rate that are able to hydrolyse and extract the cellular membrane without affecting polymer.

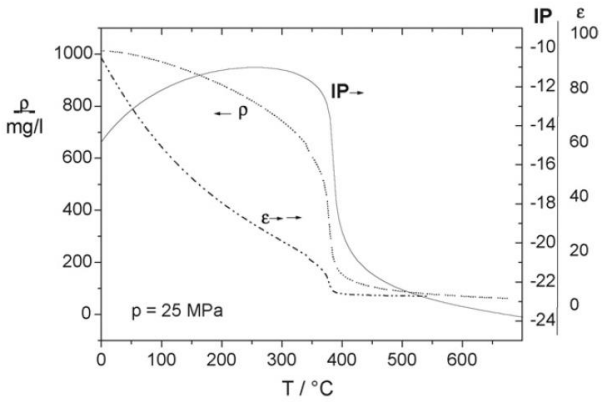

Figure 2.1- Properties of water at high pressure (25 MPa) and high temperature. The properties shown are: Dielectric constant (ε), Density (ρ) and Ionic Product (IP) (Kruse and Dinjus, 2007).

Figure 2.1 shows how the properties of water at high pressure change. In the temperature range between 130 and 200 °C, water has its density slightly decreased when compared to water at ambient pressure and temperature (around 900 mg/L). The dielectric constant of HCW is also lower and sets its value around 40, which is the reason for water to be turned into a medium able to dissolve some organic compounds. In the range of temperatures described, the ionic product of water is higher than for water at ambient conditions, -12 instead of -14 (Kruse and Dinjus, 2007).

This work focused on the extraction of the copolymer P(3HB-*co*-3HV) from MMC dried cells using HCW as pre-treatment and characterisation of the obtained polymers in terms of their composition, physical properties and molecular mass distribution.

2.2 Materials and Methods

2.2.1 PHA production

Biomass with accumulated PHA used for this work was provided by BioEng group and produced in collaboration with Sumol+Compal (Portugal). PHA was produced from waste apple pulp by a MMC (activated sludge) in a three-stage process. The first step was the conversion of the carbon source (waste apple pulp) into organic acids and ethanol, which took place in a 100 L upflow anaerobic sludge blanket (UASB) reactor. Then, in the second step, the obtained acids were feed to the mixed culture in a Feast and Famine mode, providing a way to select the PHA accumulating species from the non-accumulating ones. This process took place in a 100 L sequencing batch reactor (SBR). Then, the selected bacteria were transferred to a 10 L stirred tank reactor (STR) for the PHA accumulation stage, where they received pulse feeding of the fermented waste fruit pulp and converted it to PHA. At the end of the run, to prevent PHA degradation, the biomass was acidified to pH between 1 and 2 and stored at 4 °C.

Previously acidified biomass was neutralised with 170 mL/L sodium hydroxide (NaOH, 5 M). Following neutralisation, the biomass was centrifuged (8000 rpm, 10 minutes, 20 °C; Sigma 4-16KS). The supernatant was discarded, while the pellets were washed with deionised water and centrifuged again under the same conditions. The pellets were collected, frozen and lyophilised for 48 hours at -50 °C and 0.8 mBar. The dried biomass was then stored in closed plastics bags at -20 °C and used for PHA content quantification and for HCW extraction assays.

2.2.2 PHA extraction

2.2.2.1 Soxhlet extraction with chloroform

PHA was extracted from 5 g of dried biomass using Soxhlet extraction with 250 mL of chloroform, at 85 °C for 24 h. After the extraction, the polymer solution was left in a fume hood overnight for evaporation of part of the chloroform. The polymer was then precipitated by mixing in cold ethanol (10 times the chloroform volume) that was continually stirred. The precipitated polymer was decanted and dried in the oven at 40 °C until constant weight.

To quantify the percentage of polymer extracted from biomass, Equation 2.1 was used. In this equation m_PHA corresponds to the mass of polymer recovered after soxhlet extraction and m_sample corresponds to the mass of dried cells or extraction residue used for the extraction.

$$\%\mathrm{PHA} = \frac{\mathrm{m\_PHA}}{\mathrm{m\_sample}} \times 100 \qquad \text{(Equation 2.1)}$$

2.2.2.2 Extraction with hypochlorite

A biomass sample (0.200 g) mixed with 5 mL of hypochlorite (42 g/L), was digested for 3 h, under agitation (200 rpm) at room temperature. After that, the samples were centrifuged (8000 rpm, 10 min, 20 °C; Sigma 4-16KS) and pellets were washed in deionised water (5 mL) until pH was neutral. Finally, the polymer was freeze dried.

To calculate the purity of the samples obtained from the purification with hypochlorite, Equation 2.2 was used. In this equation %PHA is the polymer content of the sample used, m_initial the initial mass used for the purification and m_final the mass of PHA obtained after the purification.

$$\%\text{purity} = \frac{\%\text{PHA} \times \text{m\_initial}}{\text{m\_final}} \times 100 \qquad \text{(Equation 2.2)}$$

2.2.3 PHA treatment with HCW

The dried biomass, treated as described in section 2.2.1, was subjected to HCW hydrolysis. The apparatus used is shown in Figure 2.2. Water is pumped into the reactor at a pre-defined flow rate through a high-pressure tube. Before entering the reactor, water passes through a heating section consisted of a ¼ inch (outer diameter) stainless steel tube heated by a heating cord. Afterwards water passes through a porous filter (100 µm) before entering the reactor. The pressure of the entire system is maintained by a Back Pressure Regulator (BPR).

The reactor is a stainless steel 51 cm long cylinder, with an external diameter of 5 cm and an internal diameter of 2.6 cm. It was filled with biomass between two layers of glass spheres and placed inside an electric oven that controls reaction temperature. To start the process the water pump was turned on and the system was pressurized. When the pressure reached 80 bar the heating cord and the oven were turned on. Along the experiment the temperature of water exiting the reactor increased at a constant rate until the desired maximum temperature was reached. At this point the system was let at constant temperature for 30 – 60 min. Water exiting the system was collected in fractions when specific temperatures were reached. After that period the apparatus was turned off and let to cool down, then the reactor was disassembled and its content (residue) was collect and dried in an oven at 60 °C for 24 h.

The tested final temperatures were 130 °C, 140 °C, 150 °C, 165 °C, 180 °C and 200 °C. At all tests the water flow rate was kept at 10 mL/min and the pressure was set for 80 bar.

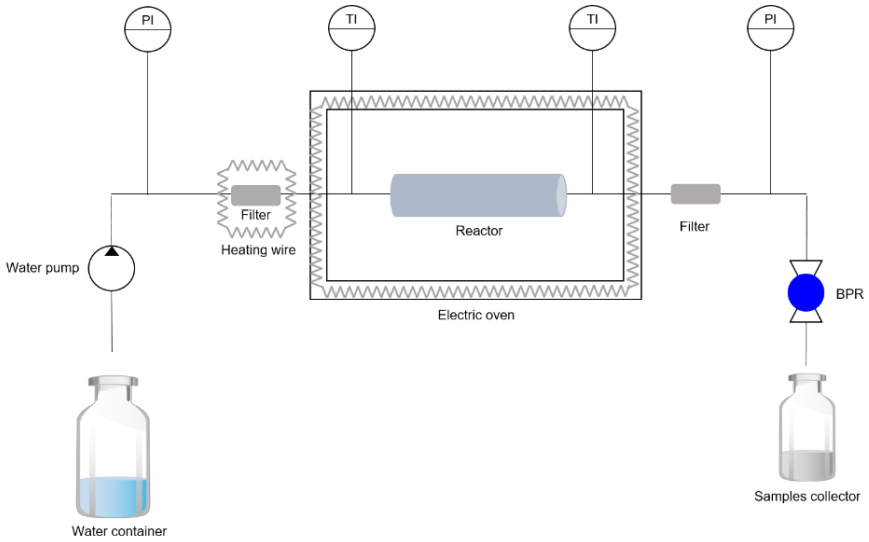

Figure 2.2 – Schematic representation of the HCW apparatus. PI represents the pressure indicators and TI represents the temperature indicators.

The percentage of biomass solubilised by HCW was calculated by Equation 2.3, where m_initial stands for the initial mass placed inside the reactor and m_final stands for the mass of the residue recovered from the reactor after the hydrolysis treatment.

$$\%\text{Solubilised Biomass} = \frac{\text{m\_initial} - \text{m\_final}}{\text{m\_initial}} \times 100 \qquad \text{(Equation 2.3)}$$

The polymer content in the biomass subjected to the different tested HCW hydrolysis conditions was quantified by soxhlet extraction with chloroform and purification as described in section 2.2.2.1., except for the fact that after precipitation in cold ethanol, the mixture was left in the freezer (-20 ºC) overnight for complete polymer precipitation. The purified polymers were recovered by centrifugation (8000 rpm, 15 minutes, 4 °C; Sigma 4-16KS) and dried in an oven at 40 °C until constant weight.

2.2.4 PHA purification with hypochlorite

The HCW hydrolysis at 150 °C (30 minutes) was selected as pre-treatment to evaluate its efficiency in improving PHA extraction using hypochlorite. The HCW treated samples (0.200 g, dried residue) were mixed with 5 mL of hypochlorite in different concentrations (Table 2.1) and extracted as described in section 2.2.2.2.

Table 2.1 – Bleach concentrations used to extract PHA from samples previously treated with HCW.

Concentration (g/L)	Bleach Volume (mL)	Water volume (mL)
42	5	0
25.2	3	2
16.8	2	3
8.4	1	4
4.2	0.5	4.5
1.68	0.2	4.8
0.84	0.1	4.9

2.2.5 PHA Characterisation

2.2.5.1 Composition

To determine the polymer's composition, gas chromatography (GC) analysis was performed with a method similar to the one described by Cruz et al., 2015. Samples consisted of 4-5 mg of HCW treated biomass (previously crushed with a tube mill) or 2 mg of purified polymer extracted with chloroform. Hydrolysis was performed with 1 mL 20% (v/v) sulphuric acid in methanol solution and 1 mL heptadecane in chloroform (1 g/L), at 100 °C for 3.5 hours. After the hydrolysis, 1 mL deionised water was added. After separation of the organic and aqueous phases, the organic phase, with the resulting methyl esters, was transferred to vials and analysed in a chromatograph 430-GC Bruker equipped with a Restek column (Crossbond, Stalbilwax). Analysis was performed at a constant 14.5 psi pressure, using Helium as carrier gas. The calibration curve was made with copolymer of P(3-HB-*co*-3-HV) (Sigma-aldrich, 88 mol% 3HB, 12 mol% 3HV) dissolved in chloroform with heptadecane (1 g/L) in concentrations ranging from 0.053 to 6.750 g/L. Heptadecane acted as intern standard.

2.2.5.2 Molecular mass distribution

To determine the polymer average molecular weight (Mw), molecular number (Mn) and polydispersity index (PDI), Size Exclusion Chromatography (SEC) was performed. For this analysis 15 mg polymer samples were dissolved in 15 mL of chloroform for 18 hours at room temperature. The samples were then filtrated with glass fibre filters (47 mm, PALL). The analysis was performed in a Waters Millenium system with chloroform as eluent with a rate of 1 mL/min. This analysis was performed by Doctor Christian Grandfils in CEIB, Université de Liège, Belgium.

2.2.5.3 Thermal Properties Determination

To determine T_g, Differential Scanning Calorimetry (DSC) analysis was performed using a differential scanning calorimeter DSC 131 (Setaram, France). The samples were placed in aluminium crucibles and analysed in the temperature range between -90 and 120 °C, with heating and cooling speeds of 10 °C/min.

To determine the T_m and degradation temperature (T_{deg}), Thermogravimetric Analysis (TGA) with DSC above ambient temperature was performed using a thermogravimetric equipment Labsys EVO (Setaram, France). Samples were placed in aluminium crucibles and analysed in the temperature range between 25 and 500 °C, at 10 °C/min.

For both analyses 0.0100 g samples of polymer were used.

2.2.5.4 Degree of crystallinity Determination

The crystallographic structural analysis was carried out by X-ray diffraction (XRD) using Benchtop X-ray Diffractometer RIGAKU, model MiniFlex II system with Cu X-ray tube with radiation over the 2θ range of 10-90 °C at a scan rate of 1 deg/min.

All samples were in powder form.

2.3 Results and Discussion

2.3.1 PHA characterisation

Biomass was produced by a MMC from waste apple pulp in three-stage process, including anaerobic digestion of the waste apple pulp, selection of PHA accumulator bacteria and, finally, a PHA accumulating stage. The stoichiometric parameters for this production are not available since the production process was not included in this work. The dried biomass was used for PHA extraction using different methods such as soxhlet extraction with chloroform and hypochlorite extraction. Soxhlet extraction with chloroform was used as a standard method to quantify the PHA content in the biomass and obtain the polymer without altering its properties. With this extraction 66% of polymer were recovered and this value was considered as the polymer content of the cells.

The PHA sample extracted with chloroform was characterised in terms of its composition, molecular mass distribution, thermal properties and degree of crystallinity. The results are shown in Table 2.2. By GC analysis, it was possible to determine that the polymer was a HB/HV co-polymer with a 3HV content of 17%. The chromatogram obtained from GC analysis is shown in Appendix 1 (Figure A.1). Similar 3HV contents (between 14 and 19%) were reported in literature (Albuquerque et al., 2011; Duque et al., 2014; Martínez-Abad et al., 2016; Reddy et al., 2009). It is known that 3HV content has impact on polymer thermal properties and on its degree of crystallinity (Anjum et al., 2016). In Table 2.2 it is possible to see that even for a small range of 3HV content (15 to 19%), T_m can change between 147 and 168 °C. The Mw of this PHA is in the same order of magnitude of values described for PHA produced by MMC from real feedstocks ($2.15x10^5$ and $6.46x10^5$ by Albuquerque et al., 2011). The PDI is low (1.27), indicating that even though the polymer was produced by a MMC, there is almost uniform length of the polymer's chains (Duque et al., 2014). The crystallinity degree of the polymer was not calculated, however the diffractogram obtained is similar to examples found in literature for P(3HB-*co*-3HV) (Cha et al., 2016; Galego et al., 2000). The diffractogram is shown in Appendix 2 (Figure A.2) and the high peaks indicated that a part of the polymer has a crystalline structure.

The sample extracted with hypochlorite was only characterised in terms of its 3HV content and molecular mass distribution. The 3HV content was not affected by hypochlorite, so it is the same for both extractions performed (17% 3HV). Extracting PHA with methods based on hypochlorite digestion is known to have impact on the PHA's Mw, there are even examples on which the PHA Mw was reduced by 50% (Berger et al., 1989). The Mw of polymer sample extracted with hypochlorite ($3.17x10^5$ g/mol) was similar to that of the polymer extracted with chloroform ($3.03x10^5$ g/mol). This indicates that the extraction with hypochlorite did not affect the Mw of P(3HB-*co*-3HV), as it would be expected. The PDI was not affected and is the same as the obtained for the polymer extracted with chloroform (1.27).

Table 2.2 – Characterisation of the P(3HV-*co*-3HB) produced from waste apple pulp and extracted from the original biomass by Soxhlet extraction with chloroform and with hypochlorite compared to examples from literature. The characterisation includes 3HV content of the PHA, thermal properties and molecular mass distribution (n.a. – not available).

Extraction method	**HV content (%)**	**T_m (°C)**	**T_{deg} (°C)**	**Mw (x10^5, g/mol)**	**PDI**	**Reference**
Soxhlet with chloroform	17.0	151.7	282.9	3.03	1.27	This study
Hypochlorite digestion	17.0	-	-	3.17	1.27	This study
Chloroform	19.0	168.2	277.8	2.5	1.3	(Duque et al., 2014)
Chloroform	15.0	147	247	6.45	2.3	(Albuquerque et al., 2011)
Hypochlorite digestion	18.3	171.0	n.a.	3.6	1.7	(Martínez-Abad et al., 2016)
Hypochlorite digestion	14.0	177.3	n.a.	6.3	n.a.	(Reddy et al., 2009)

2.3.2 PHA treatment with HCW

HCW was used as hydrolysis medium and catalyst for the purification of P(3HB-*co*-3HV) through the simultaneous hydrolysis and extraction of the remaining cell material. For the HCW assays biomass was treated as described before.

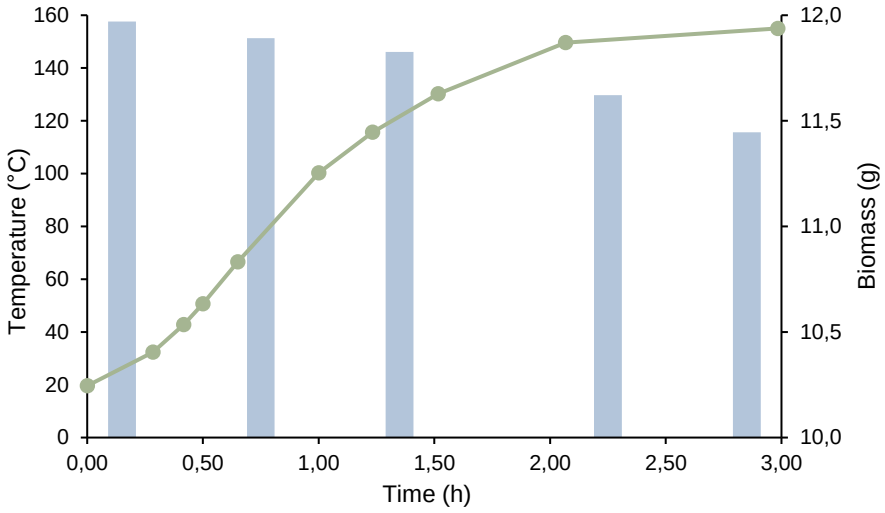

Figure 2.3 – Heating profile of the HCW system (line) of an extraction with final temperature of 150 °C and biomass loss in the reactor during the different heating phases (bars represent the biomass that remained in the reactor).

Figure 2.3 represents the heating profile of an assay with final temperature of 150 °C. The process of heating the equipment, and consequently water, to the desired temperature was slow. At first water passed through the biomass at room temperature (18 to 20 °C) for few minutes to confirm that there were no leaks on the system. At that point the system was pressurized and the heating process began. The heating rate on the first hour of this assay was 1.34 °C/min, and the system was heated from room temperature to 100 °C. Afterwards the system was heated from 100 °C to the final specific temperature at a rate of 0.74 °C/min.

Table 2.3 shows the conditions of temperature and time of extraction of all the hydrolysis performed. For all the experiments, the water flow rate was kept constant, at 10 mL/min. The efficiency of the hydrolysis was measured by the percentage of biomass solubilised and by the polymer content on the biomass that remained in the reactor. The assay at 200 °C was completely destructive so there are no results for this temperature.

The polymer content on the original biomass was 66%, so the extractions at 150 °C and 165 °C seem to have the highest improvement in polymer content, being 77% and 75%, respectively. At 165 °C the solubilised biomass was 23% which is higher than for the extraction at 150 °C (12%). However, at 165 ºC the polymer content of the sample was only 75%, indicating that a part of the PHA was probably degraded by HCW. The results obtained for the hydrolysis at 150 °C indicate that at this temperature the best results were obtained for the extraction of the cellular material without polymer loss.

After concluding that 150 °C was the best temperature to perform the HCW treatment, the reaction time was increased from 30 min to 60 min. However, the increase on the solubilised biomass was not significant (from 12% to 13%).

Table 2.3 – Different conditions of time and temperature extraction with HCW performed and respective percentages of solubilised biomass and polymer content. The values of polymer content were calculated after Soxhlet extraction with chloroform.

T (°C)	Time (min)	Solubilised Biomass (%)	Polymer content (%)	HV content (%)	T_m (°C)	T_{deg} (°C)	Mw (x10^5, g/mol)	PDI
Original P(3HB-*co*-HV)		-	-	17.0	151.7	282.9	3.03	1.27
130	30	16	57	17.0	150.1	277.9	1.15	5.09
140		11	69	-	-	-	0.50	7.97
150		12	77	17.0	147.1	294.7	0.50	6.01
165		23	75	16.0	140.6	294.1	0.23	7.35
180		25	51	15.0	127.2	290.9	0.07	3.9
200		98	-	-	-	-	-	-
150	60	13	77	16.0	143.9	292.6	-	-

As it has been referred to quantify the polymer remaining on the sample of biomass treated with HCW, the PHA was extracted by soxhlet extraction with chloroform and purified. Pictures of polymer samples extracted at three different temperatures (150 °C, 165 °C and 180 °C) are shown in Figure 2.4 and it is visible that the polymer suffered some alterations. The purified samples were than used to study the polymer's thermal properties, degree of crystallinity and molecular mass distribution (Table 2.3). The characteristics of the original polymer, obtained from biomass without any treatment are also shown in Table 2.3.

As the HCW temperature increased T_m tended to decrease. For the extraction at 150 °C, 60 min, the T_m was lower than for the extraction of 30 min, indicating that even though there was no loss of polymer during the hydrolysis its properties are different. T_{deg} also changed, however at 150 °C, 30 min, it was higher than the original polymer's. The main difference between the original polymer and the samples obtained from biomass treated with HCW was in the Mw. P(3HB-*co*-3HV)'s Mw was highly affected by HCW conditions, being reduced about three times at 130 °C, one order of magnitude at 140 °C, 150 °C and 165 °C, and two orders of magnitude at 180 °C. The PDI value increased comparatively to the original polymer for all the HCW temperatures tested. PHA's Mw is related to its mechanical and thermal properties, having high Mw is what makes PHA suitable substitutes for conventional plastics. If the polymer chains are smaller the polymer is less resistant to mechanical stress, hence being more susceptible to damage (Reddy et al., 2003; Salehizadeh and Van Loosdrecht, 2004). The diffractograms obtained from XRD are shown in Appendix 2 (Figure A.3 to Figure A.7) and the pattern of diffraction of all the samples is similar, indicating that there was no significant alteration of the degree of crystallinity of the polymer after being exposed to HCW hydrolysis.

Although with HCW at 150 °C there was no evident loss of PHA, the Mw values obtained show that P(3HB-*co*-3HV) was affected by HCW. The Mw decrease is evident at all the temperatures tested and is worse for higher temperatures. The PHA monomers are linked together by ester bonds (Verlinden et al., 2007), therefore HCW should be able to hydrolyse the polymer. It was expected to find conditions that would hydrolyse the cell material without affecting the polymer, however, that was not possible and all the conditions tested the polymer some extent of the polymer was broken into smaller and heterogenous fractions.

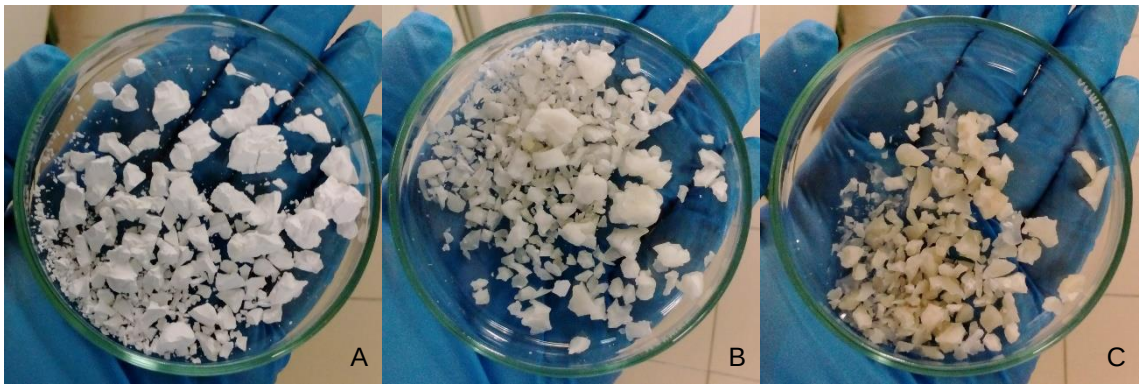

Figure 2.4 - Pictures of the P(3HB-*co*-3HV) obtained after the HCW hydrolysis of biomass. A – HCW 150 °C; B – HCW 165 °C; C – HCW 180 °C.

2.3.3 Extraction of PHA from HCW treated biomass using hypochlorite

Since it was not possible to extract and purify the P(3HB-*co*-3HV) only with HCW treatment, it was proposed to use HCW as a pre-treatment. HCW is effective in breaking and degrading cellular compounds hence leaving the polymer more accessible for extraction. With this knowledge, it was expected that it would be possible to separate the cell remains from the PHA by dissolving them in a solvent. The solvents tried for this purification were water, ethanol (25%), acetone and hypochlorite. Only the hypochlorite was effective in dissolving the cell remains to a point that it was possible to recover the PHA in a pellet form. Examples of the purification of P(3HB) and P(3HB-*co*-3HV) using hypochlorite have been described by Ramsay et al., 1990, Martinez-Abad et al., 2015, among others.

The purification was performed on the biomass treated with HCW at 150 °C, with a polymer content of 77%. As control, one sample of original biomass was also purified with this method, using the highest concentration of hypochlorite (42 g/L). Table 2.4 shows the solubilised biomass for each hypochlorite concentration and the respective polymer purity. It is possible to see that the control sample had a solubilisation yield higher than all the other samples, however, with lower polymer content. This may have happened because in the HCW treated samples the cell components were already partially hydrolysed and some of them had already been extracted. Therefore, the lower solubilisation yield only reflects the lower content of extractable material.

On the samples pre-treated with HCW, the highest polymer purity (84% ± 1%) was achieved for the highest hypochlorite concentration (42 g/L). However, for the three following concentrations (25.2, 16.8 and 8.4 g/L) the same polymer purity was achieved (82%). This indicates that on biomass treated with HCW it is possible to use five times less hypochlorite concentration to purify the P(3HB-*co*-3HV) from the surrounding cell debris.

The purity degrees achieved in this work (80 to 84%) are still lower than the ones described in literature (90 to 97%). Martinez-Abad et al., 2015, described the purification of the P(3HB-*co*-3HV) using only hypochlorite extraction, as described in this work, and achieved a 90% purity degree. Some examples combined two treatment methods to increase the purity degree of the polymer. The method described by Ramsay et al., 1990, consisted on applying a surfactant to disrupt the cellular structure and removing the cellular remains with hypochlorite, hence having two purification steps and achieving a purity degree of 97%. Hahn et al., 1994, also described a method that combined two different solvents, hypochlorite and chloroform. Briefly, hypochlorite was used on a first phase to disrupt the cellular membranes. Then, chloroform was added to the mixture and dissolved the PHA that was recovered and precipitated with a non-solvent. This method yield a PHA with 97% purity degree.

Table 2.4 – Thermal properties, degree of crystallinity of the P(3HB-*co*-3HV) samples by hypochlorite extraction of HCW treated biomass at 150 °C. The control sample was obtained by hypochlorite extraction of the untreated biomass.

Hypochlorite (g/L)	Solubilised biomass (%)	Polymer Purity (%)	HV content (%)	T_m (°C)	T_{deg} (°C)	Mw (x10^5, g/mol)	PDI
42 (Control)	43 ± 2	77 ± 1	17	-	-	3.17	1.27
42	34 ± 7	84 ± 1	17	140.6	275.6	0.28	3.88
25.2	27 ± 3	82 ± 1	17	139.8	283.4	0.30	3.11
16.8	27 ± 8	82 ± 2	17	139.9	282.1	0.30	2.82
8.4	26 ± 3	82 ± 1	17	136.3	284.9	-	-
4.2	22 ± 7	81 ± 1	17	139.1	287.5	0.30	4.79
1.68	22 ± 0	81 ± 0	-	-	-	0.33	11.79
0.42	14 ± 8	80 ± 1	17	135.9	290.4	0.30	7.46

Even though the purities achieved with the combination of HCW and hypochlorite purification were not as high as the ones described by Ramsay et al., 1990, and Hahn et al., 1994, they were still higher than the purity achieved with hypochlorite purification alone. The combination of the two methods showed promising results, however it would have to be optimised to achieve higher purity degrees.

The main problem with using the combination of the HCW and hypochlorite methods is the damage that they caused on P(3HB-*co*-3HV). The HCW treatment alone had a severe effect on the Mw and PDI and affected the polymer's thermal properties such as T_m and T_{deg}, however in a smaller extent (Table 2.3). Considering the sample digested with 16.8 g/L of hypochlorite, it is notable that the combination of the two methods caused a higher effect on the polymer's thermal properties, decreasing the T_m by 7 °C compared to the polymer obtained directly after HCW at 150 °C, and almost 12 °C when compared to the original polymer (Table 2.4). T_{deg} is less affected, and in this sample, is equal to the original polymer's, however it is different than the T_{deg} of the polymer obtained directly after HCW at 150 °C. The Mw is similar within all the samples recovered with hypochlorite. It is lower than the Mw of the sample obtained from HCW at 150 °C (0.5x10^5 g/mol) but stays within the same order of magnitude. The PDI changes considerably for all the samples, ranging from 2.82 to 11.9. The samples digested with higher concentrations of hypochlorite have their PDI around 3, but the samples digested with lower hypochlorite concentrations have higher PDI, indicating that polymer's chain length in these samples has a wide dispersity. The diffractograms obtained from XRD are shown in Appendix 2 (Figure A.8 to Figure A.13) and the pattern of diffraction of all the samples is similar, indicating that there was no significant alteration of the degree of crystallinity of the polymer after being purified with the combination of these methods.

2.4 Conclusions and Future Work

In conclusion, the characterisation of the P(3HB-*co*-3HV) produced by MMC from waste apple pulp showed that the polymer shows thermal properties and molecular mass distribution that are comparable to other polymers produced in similar conditions.

Using HCW hydrolysis to purify PHA did not prove to be an effective method. HCW is a good hydrolysis medium and catalyst and, therefore is able to hydrolyse the ester bonds that link the PHA monomers together. The decrease on the PHA Mw is notorious and with such low Mw the polymer no longer shows the characteristic that make it comparable to the conventional plastics, PP and PE. The purification of the polymer with hypochlorite after the treatment with HCW proved to be effective when compared to the extraction performed on biomass without treatment. This method caused a slightly decrease on the polymer Mw, but nothing compared to the effect that HCW itself has caused.

In the future, it would be interesting to evaluate the mechanical properties of the polymer samples obtained from HCW treatment. With the high decrease on PHA Mw that was verified the mechanical properties of the polymer should be different, since lower Mw tends to make the PHA less resistant to mechanical stress.

The treatment with hypochlorite should also be performed in different conditions to improve the solubilisation of the cell remains and further increase the purity degrees achieved to an extent that is similar to the examples found in literature.

Chapter 3

Bioreactor production and products characterisation

3.1 Introduction

Members of the *Pseudomonas* genus are known for their ability to produce multiple secondary metabolites, including bioactive metabolites such as antibiotics. *Pseudomonas chlororaphis* is a non-pathogenic bacterium widely used as plant growth-promoting rhizobacteria (Chen et al., 2015). This species was first described in 1894 by Palleroni but with further investigation has been divided into three subspecies, on which *P. chlororaphis* subs. *aurantiaca* is included (Peix et al., 2017). This strain is able to produce three products with high interest: phenazines (Chincholkar and Thomashow, 2013), *mcl*-PHA (Ashby and Foglia, 1998; Muhr et al., 2013) and an exopolysaccharide (EPS) (Fett et al., 1996).

Phenazines are a characteristic of *Pseudomonas* sp., considered the one of the best phenazine producers. Phenazines are heterocyclic nitrogenous compounds that can act as antibiotics, antitumor, antiparasitic or even antiviral agent (Chincholkar and Thomashow, 2013; Laursen and Nielsen, 2004). *P. chlororaphis* can produce at least three types of compounds of the phenazine family: (1) phenazine-1-carboxylic acid (PCA); (2) 2-hydroxyphenazine-1-carbolylic acid and (3) 2-hydroxyphenazines. Phenazine biosynthesis relies on the phz gene whose expression is regulated by nutritional, metal and environmental parameters. Nevertheless, the most important factor for phenazine biosynthesis is cellular density, since it is regulated by quorum sensing (QS). The fermentative process for phenazine production is not completely understood, but higher yields have been described for 20% dissolved oxygen (DO) (Chincholkar and Thomashow, 2013).

Pseudomonas sp. are known to include several strains able to accumulate *mcl*-PHA from fatty acids (Ashby and Foglia, 1998). *P. putida* was the first strain described to do so, being a widely investigated organism for *mcl*-PHA production (Muhr et al., 2013). *P. chlororaphis* has been described as able to accumulate *mcl*-PHA (Solaiman et al., 2006) and has even been cultivated using waste carbon sources, such as palm kernel oil (PKO) (Yun et al., 2003) and saturated biodiesel fraction (SFAE) from animal waste (Muhr et al., 2013). *mcl*-PHA have very interesting properties, like their low crystallinity (around 25%) and low melting point (between 40 and 60 °C) (Rai et al., 2011), that have made them suitable to be used as resins, latexes, glues or rubbers (Muhr et al., 2013; Solaiman et al., 2006).

EPS are biopolymers produced by a wide range of bacterial strains, including several *Pseudomonas* species and *P. chlororaphis* is no exception. Bacteria produce EPS as a protective strategy, since they are involved in biofilm formation and in the host-pathogen interaction. Due to their physical properties, EPS have also been shown to have applicability in various industries, such as food, textile, cosmetic or pharmaceutical (Fett et al., 1996; Kumar et al., 2007). Depending on the producer strain, EPS can be produced from a variety of carbon sources that will affect the composition and yield of polymer, while the nitrogen source also has some effect on both those parameters (Kumar et al., 2007).

Co-production of *mcl*-PHA and phenazines by *P. chlororaphis* subs *chlororaphis* was reported by Muhr et al., 2013, but it was not investigated in great detail even though the interest of phenazines is stated. The ability of *P. chlororaphis* to produce EPS was described by Fett et al., 1996, on work using the *P. chlororaphis* NRRL B-2075 strain. There are also examples of the production of lipopolysaccharides (LPS) by *Pseudomonas chlororaphis* subsp. *aureofaciens* (Varbanets et al., 2015). Despite knowing the bacterium's ability to produce more than one product, most works were focused on the production and characterisation of only one of them. Therefore, this work focused on the co-production of *mcl*-PHA, EPS and phenazines from glycerol by *P. chlororaphis* subs *aurantiaca* DSM 19603 and characterisation of those products.

3.2 Materials and Methods

3.2.1 Bacterial strain and cultivation media

The bacterial strain used in this work was *Pseudomonas chlororaphis* subs *aurantiaca* DSM 19603 that was cryopreserved in 20% (v/v) glycerol at -80 ºC.

For the pre-inoculum, Luria-Bertani (LB) medium was used with tryptone, 10 g/L; yeast extract, 5 g/L and NaCl, 10 g/L. The medium was autoclaved at 121 °C for 20 minutes.

Medium E* was used for the bioreactor cultivation and inocula preparation. It had the following composition: HK_2O_4P, 5.8 g/L; KH_2PO_4, 3.7 g/L; $(NH_4)_2HPO_4$, 3.3 g/L; 10 mL/L of $MgSO_4$, 25 g/L and 10 mL/L of mineral solution diluted (1:10) containing $FeSO_4.7H_2O$, 2.78 g/L; $MnCl_2.4H_2O$, 1.98 g/L; $CoSO_4.7H_2O$, 2.81 g/L; $CaCl_2.2H_2O$, 1.67 g/L; $CuCl_2.2H_2O$ 0,17 g/L; $ZnSO_4.7H_2O$, 0.29 g/L. Glycerol (86 – 88% w/w, Scharlau) was used as the substrate, at a concentration of 40 mL/L. Medium E*, $MgSO_4$ solution, mineral solution and glycerol were autoclaved separately, at 121 °C for 20 minutes, and mixed after cooling down to avoid salts' precipitation.

3.2.2 Inocula preparation

Pre-inoculum was prepared by adding a 1 mL of the cryopreserved sample (stored at -80 °C) to 100 mL of LB medium, in a 250 mL shake flask. The shake flask was kept in an orbital shaker for 24 hours, at 30 °C and 200 rpm.

Inocula was prepared by transferring 20 mL of the pre-inoculum to each of the four 500 mL capacity shake flasks with 200 mL of medium E*. The shake flasks were kept in the orbital shaker, at 30 °C and 200 rpm, for 72 hours.

3.2.3 Fed-batch bioreactor fermentation

Cultivation of *P. chlororaphis* was performed in BioStat® B-plus bioreactor (Sartorius, Germany) with a working volume of 10 L. The assay was initiated with 8 L of Medium E* that included glycerol (40 g/L) and the inoculum (10%, v/v). The bioreactor was operated with controlled conditions of pH, 7.00±0.02, temperature, 30.0±0.5 °C, and dissolved oxygen concentration (pO_2), 30%. The air flow rate was kept constant at 4 L/min.

The pH was controlled by the addition of acid (HCl, 4 M) or base (NH_4OH, 25% v/v; or NaOH, 4 M). The pO_2 was maintained at 30% of the air saturation by automatic variation of the stirring (310 to 795 rpm) that was provided by two six-blade impellers. For foam control, anti-foam (Anti-foam Y-30 Emulsion, Sigma-Aldrich) was used.

After 22 h of fermentation the base was exchanged from NH_4OH (25% v/v) to NaOH, to imposed nitrogen limiting conditions, and a glycerol pulse (320 mL) was added to the culture.

A second bioreactor experiment was performed under the same controlled conditions described above.

Samples (15 mL) were collected periodically and centrifuged (12000 rpm, 20 min, 4 °C; Sigma 4-16KS). The cell-free supernatant was stored at -20 ºC for quantification of glycerol, ammonium, phenazines and EPS, while the cell pellets were used for biomass and PHA quantification.

3.2.4 Analytical techniques

3.2.4.1 Cellular growth

Cellular growth was monitored during the experiment by measuring the optical density of the cultivation broth at 600 nm (OD_{600nm}). The samples were diluted with deionised water so that the OD_{600nm} value was below 0.3. Deionised water was used as zero reference. All measurements were done in duplicate.

3.2.4.2 Nile Blue Staining

Nile blue staining was performed to some of the samples collected periodically from the reactor. To do so, 50 µL of nile blue were added to 0.5 mL of sample on an microtube. The microtube was then covered in tin foil and placed on an oven at 70 °C for 10 minutes. After that time, the slides were prepared and observed at the microscope under contrast light and fluorescent light.

3.2.4.3 Biomass Quantification

The cell dry weight (CDW), the dry weight of the cells per litre of fermentation broth, was determined by gravimetric method.

The cell pellets, obtained as described above, were washed once by resuspension in deionised water (5 mL) and centrifuged again under the same conditions. The washed cell pellets were frozen in liquid nitrogen and lyophilized for 48 hours at -108 °C. The CDW was obtained by weighing the lyophilized cell pellets. All samples were done in duplicated.

3.2.4.4 Glycerol Quantification

Determination of glycerol concentration was made by high performance liquid chromatography (HPLC) with a VARIAN Metacarb 87H column coupled to an infrared (IR) detector. The analysis was performed at 50 °C using H_2SO_4, 0.01 N, as eluent with a flow rate of 0.6 mL/min.

Samples for this analysis were prepared by diluting the cell-free supernatant, obtained by centrifugation of the cultivation broth as described above, in the eluent (H_2SO_4, 0.01 N), in a 1:50 proportion. All samples were filtered with using VWR centrifuge filters (0.2 µm).

As standards, glycerol (Scharlau, 86 – 88% w/w) at concentrations ranging from 0.067 to 1.072 g/L were used.

3.2.4.5 PHA Quantification and Composition

For PHA quantification, the lyophilized cell pellets were hydrolysed following the method described in 2.2.5.1 (Chapter 2). Heptadecane (1 g/L) acted as internal standard. Standards were prepared using a *mcl*-PHA with 3% 3-hydroxyhexanoate (3HHx), 17% 3-hydroxyoctanoate (3HO),

57% 3-hydroxydecanoate (3HD), 11% 3-hydroxydodecanoate (3HDd) and 12% 3-hydroxytetradecanoate (3HTd) in concentrations ranging from 0.047 to 6.053 g/L.

3.2.4.6 EPS quantification

For EPS quantification, the cell-free supernatant was dialysed against deionised water. For this process, dialysis membranes (ZelluTrans Carl Roth, MWCO 12000 - 14000) with 4 mL of supernatant, were placed in a 5 L beaker with deionised water, under constant stirring. Sodium azide (10 ppm) was added to the water to prevent biological contamination and sample degradation. The water was changed every two to three hours during the day. Before every water change a sample was collected for conductivity measurement. Dialysis was finished after six water changes, when the dialysis water conductivity was the closest to deionised water conductivity (after approximately 72 hours when conductivity was 2.63 µS/cm).

All samples were collected, frozen at -80 °C and lyophilized for 48 hours at -108 °C. After that, they were weighed for the EPS gravimetric quantification.

3.2.4.7 EPS characterisation

The EPS was characterized in terms of its sugar monomers composition. The samples were prepared by dissolving 5 mg of the lyophilized sample in deionised water (5 mL) and hydrolysing them with trifluoracetic acid (TFA) (0.1 mL, 99%), for 2 hours at 120 °C. After cooling down to room temperature, the samples were filtered using VWR centrifuge filters (0.2 µm).

Samples sugar monomers composition was determined by HPLC using a CarboPac PA10 column (Dionex) coupled with a AminoTrap column (Dionex). The analysis was performed at 30 ºC with sodium hydroxide (NaOH,18 mM) as eluent, at a flow rate of 1 mL/min. D-(-)-arabinose (99%, Sigma), L-rhamnose monohydrate (99%, Fluka), D-(+)-galactose (99%, Fluka), D-(+)-glucose anhydrous (99%, Scharlau), Sucrose (99%, Fluka), D-(-)-fructose (99%, Scharlau), D-(+)-mannose (99%, Fluka), D-(+)-trehalose dehydrate (99%, Alfa Aesan), D-glucuronic acid (98%, Alfa Aesan) and D-(+)-galacturonic acid monohydrate (97%, Fluka), were used as standards (5 to 50 ppm).

For confirmation of the identity of some of the sugar monomers detected in the samples, some of them were prepared with addition of one standard sugar (0.01 mL, 1 g/L).

3.2.4.8 Phenazines quantification

For the detection of the phenazines produced over the cultivation time, a spectrophotometric method was used, according to Bauer et al., 2016. The cell-free supernatant (1 mL) was acidified with 0.1 mL of HCl (1 M) and the phenazines were extracted with 1 mL of chloroform. The absorbance was measured 365 nm using a UV/vis spectrophotometer.

3.2.5 Calculus

The PHA volumetric productivity (r_p, g/L.day) was determined by Equation 3.1, where P stands for the PHA concentration (g/L) produced at the time t (h).

$$r_p = \frac{dP}{dt} \qquad \text{(Equation 3.1)}$$

The EPS volumetric productivity (r_p, g/L.day) was also determined by Equation 3.1, with P standing for the EPS concentration (g/L) produced at the time t (h).

$$X = CDW - PHA \qquad \text{(Equation 3.2)}$$

Active Biomass (X) was calculated by Equation 3.2 where CDW stands for the cellular concentration at a certain time and PHA stands for the concentration of PHA at that time.

Yield of biomass on substrate ($Y_{x/s}$, g/g), was determined by Equation 3.3 where x_f and x_i stand for the final and initial active biomass (g/L) and S_f and S_i stand for the final and initial concentration of glycerol (g/L).

$$Y_{x/s} = \frac{X_f - X_i}{S_f - S_i} \qquad \text{(Equation 3.3)}$$

Yield of PHA on substrate ($Y_{PHA/s}$, g/g) was determined by and Equation 3.4 where p_f and p_i stand for the final and initial PHA concentration (g/L), respectively.

$$Y_{p/s} = \frac{p_f - p_i}{S_f - S_i} \qquad \text{(Equation 3.4)}$$

Yield of EPS on substrate ($Y_{EPS/s}$, g/g) was also determined by Equation 3.4, with p_f and p_i standing for final and initial EPS concentration (g/L), respectively.

3.2.6 PHA Extraction and purification

The culture broth collected at the end of the bioreactor cultivation was centrifuged (8000 rpm, 30 min, 4°C; Sigma 4-16KS). The cell pellets thus obtained were resuspended in deionised water and centrifuged again under the same conditions. The washed biomass was then lyophilized for 48 hours at -108 °C. Dried biomass was used for PHA extraction and purification.

The PHA was recovered from the dried cells by Soxhlet extraction using chloroform as solvent. The extraction and purification of *mcl*-PHA was performed as described in 2.2.2.1 (Chapter 2).

3.2.7 PHA Characterisation

3.2.7.1 Composition

Gas chromatography-mass spectrometry (GC-MS) analysis of purified polymer samples was performed to confirm the identity of the monomers in the polymer. Samples were prepared by

weighing approximately 3 mg of purified polymer and following the methanolysis method described in 2.2.5.1 (Chapter 2).

3.2.7.2 Molecular mass distribution

To determine the polymer's Mw, Mn and PDI, Size Exclusion Chromatography (SEC) was performed. For this analysis, 15 mg polymer samples were dissolved in 15 mL of chloroform for 18 hours at room temperature. The samples were then filtered with glass fibre filters (47 mm, PALL). The analysis was performed in a Waters Millenium system with chloroform as eluent with a rate of 1 mL/min. This analysis was performed by Dr. Christian Grandfils in CEIB, Université de Liège, Belgium.

3.2.7.3 Determination of PHA Thermal Properties

To determine the T_g and the T_m of the *mcl*-PHA produced by *P. chlororaphis*, DSC analysis was performed using a differential scanning calorimeter DSC 131 (Setaram, France). The samples were placed in aluminium crucibles and analysed in the temperature range between -90 and 120 °C, with heating and cooling speeds of 10 °C/min.

To determine the PHA's T_{deg}, TGA was also performed using a thermogravimetric equipment Labsys EVO (Setaram, France). Samples were placed in aluminium crucibles and analysed in the temperature range between 25 and 500 °C, at 10 °C/min.

For both analyses 0.010 g samples of polymer were used.

3.2.7.4 Determination of PHA degree of crystallinity

The crystallographic structural analysis was carried out by XRD using PANalytical's X'Pert PRO MRD, system with Cu K-alpha radiation over the 2θ range of 10-90 °C at a scan rate of 1 deg/min.

All samples were purified polymer.

The crystalline percentage of the polymer was calculated by the ratio of the crystalline area and the total area (Galego et al., 2000).

3.3 Results and Discussion

3.3.1 Bioreactor cultivation of *P. chlororaphis*

In the first experiments, the cultivation of *P. chlororaphis* was performed in a 10 L bioreactor with medium E* and glycerol as sole carbon source for about 50 h (reactor 1). Figure 3.1(A) shows the glycerol consumption and CDW over the entire cultivation time and Figure 3.1(B) shows the production profile of each of the polymers, PHA and EPS.

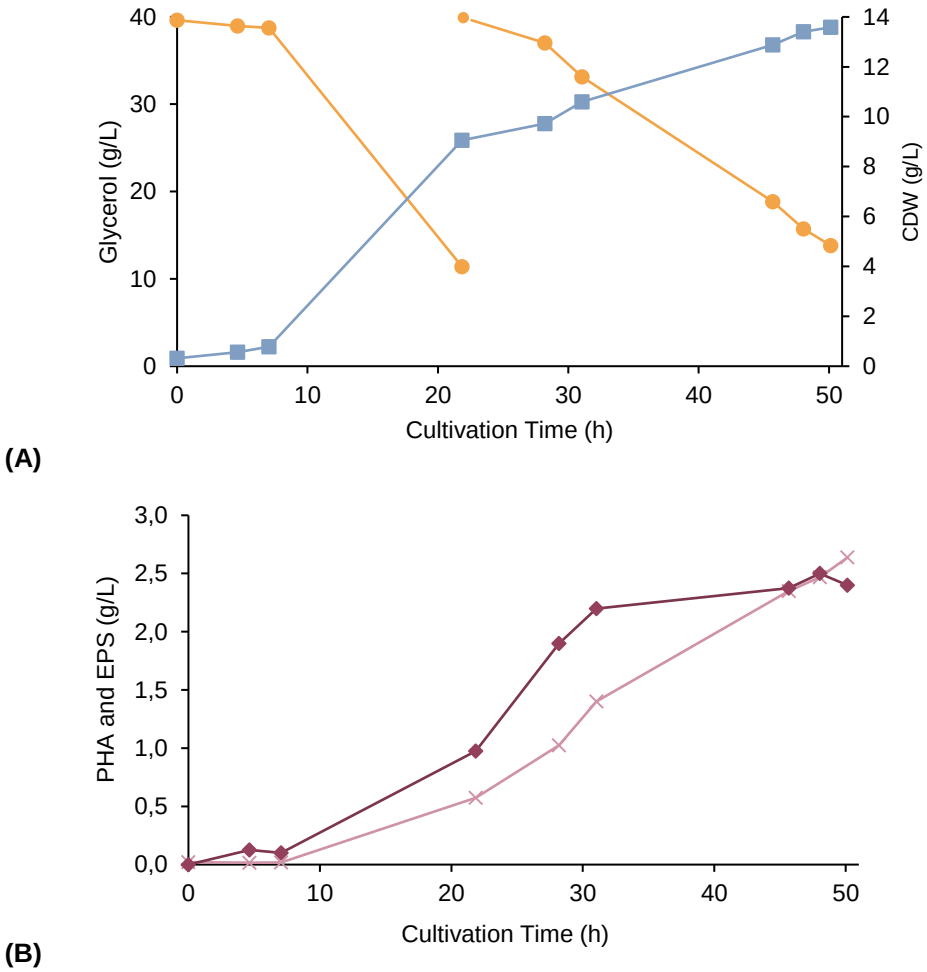

Figure 3.1 –Cultivation profile of *P. chlororaphis* during the first experiment. (A) CDW (■) and glycerol concentration (●); (B) production of PHA (x) and EPS (♦).

The lag phase had a duration of about 7 h and after that the culture entered an exponential growth phase that lasted until about 22 h of cultivation. At that time, the obtained CDW was 9.05 g/L and 28.23 g/L of glycerol were consumed, at a consumption rate of 1.29 g/L·h. The glycerol was mostly directed to cellular growth, since at that point the accumulated PHA was only 6% of the biomass content, corresponding to a concentration of 0.57 g/L. The EPS concentration was slightly higher, 0.98 g/L.

After 22 h of cultivation, a glycerol pulse was fed to the culture and the assay was prolonged for another 28 h. Over this period the cellular growth rate decreased, however the CDW still increased to a final value of 13.58 g/L. Intracellularly, PHA was accumulated to a cell content of

19%, representing a concentration of 2.64 g/L. The PHA inclusions inside the cells can be seen on Figure 3.2(B). As it has been referred and can be seen in Figure 3.1(A), the CDW kept increasing on the period between 22 and 50 h. This may be related to the fact that PHA accumulates intracellularly and contributes to the CDW. At the end of the cultivation CDW was 13.58 g/L with 19% PHA content, which corresponds to 10.95 g/L of active biomass (X). Extracellularly, EPS was produced and its concentration increased up to 2.40 g/L. After the glycerol pulse, 26.09 g/L of glycerol were consumed, at a consumption rate of 0.93 g/L·h, and it was used mostly used to produce PHA and EPS, since the cellular growth slowed down during this period.

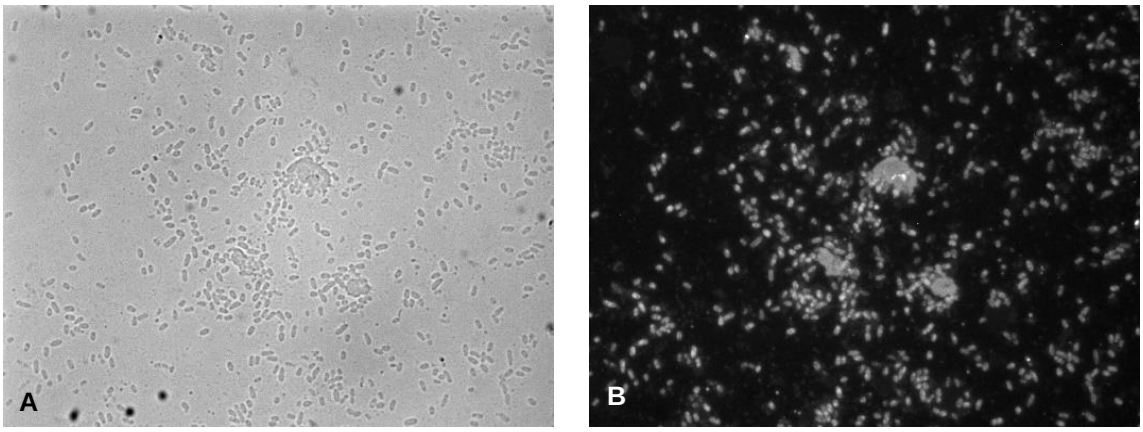

Figure 3.2 – Visualisation of the *P. chlororaphis* cells under the optical microscope (100x) at 48 h of cultivation, under phase contrast (A) and with fluorescence (B) after Nile Blue staining.

Overall, 54.32 g/L of glycerol were consumed, at an average glycerol consumption rate of 1.08 g/L·h. This provided a growth yield of 0.20 g/g. The products' yields were 0.048 and 0.044 g/g for PHA and EPS, respectively (Table 3.1). The PHA concentration was 2.64 g/L, corresponding to a volumetric productivity (r_p) of 1.26 g/L·day (Table 3.1). The maximum production of EPS was 2.40 g/L, corresponding to a r_p of 1.15 g/L·day (Table 3.1).

Figure 3.1(B) shows the production curves of both polymers. They show a similar production rate until 31 h of fermentation. On the time period corresponding to the first glycerol pulse (0 to 22 h) the polymers production was small. By the time the second glycerol pulse was added to the culture, the polymers production increased. Most of the EPS was produced on the period from around 22 and 31 h of cultivation, when its concentration was 2.20 g/L. From 31 h until the end of the cultivation the EPS kept increasing, although at a very slow rate, reaching the final concentration of 2.40 g/L. As for the PHA accumulation, from the 22 h of fermentation until the end its concentration kept increasing at an almost steady rate to a final value of 2.64 g/L. During the first 31 h, they do not seem to have impact on each other's production, however after this period the slowing down on EPS production seems allow the culture to keep accumulating PHA.

The values obtained in this study are within the ones reported in the literature for other *P. chlororaphis* strains, namely CDW between 3.3 and 30.0 g/L, with a PHA content of 12-45% (Table 3.1), Nevertheless, in those studies other carbon sources were used instead of glycerol.

Using PKO as sole carbon source, a much higher polymer content (45%) was achieved but with very low cell density (3.3 g/L) (Yun et al., 2003). On the other hand, using SFAE as sole carbon source, the achieved cellular concentration was much higher (30.0 g/L) than the achieved in this study but the polymer content of the cells was lower (12%) (Muhr et al., 2013).

Table 3.1 - Kinetic and stoichiometric parameters for both reactors performed in this study compared to examples of PHA production by other *P. chlororaphis* strains found in the literature. (n.a. – not available; values with * where estimated based on the values given in the literature).

Parameter	**Reactor 1**	**Reactor 2**	***mcl*-PHA production**	***mcl*-PHA production**
Carbon source	Glycerol	Glycerol	SFAE	PKO
Bacterial strain	*P. chlororaphis* DSM-19603	*P. chlororaphis* DSM-19603	*P. chlororaphis* DSM-50083	*P. chlororaphis HS21*
Cultivation mode	Fed-batch bioreactor	Fed-batch bioreactor	Fed-batch bioreactor	Batch bioreactor
CDW (g/L)	13.58	11.79	30.0	3.3
PHA (%)	19	19	12	45
PHA (g/L)	2.64	2.23	3.57*	1.49
X (g/L)	10.95	9.57	26.43*	1.81*
EPS (g/L)	2.40	6.10	-	-
$r_{p\ PHA}$ (g/L·day)	1.26	1.21	1.704*	n.a.
$r_{p\ EPS}$ (g/L·day)	1.15	3.33	-	-
$Y_{x/s}$ (g_{biom}/g_{subs})	0.20	0.24	0.62	0.62
$Y_{PHA/s}$ (g_{PHA}/g_{subs})	0.048	0.053	0.075	n.a.
$Y_{EPS/s}$ (g_{EPS}/g_{subs})	0.044	0.15	-	-
Reference	This study	This study	(Muhr et al., 2013)	(Yun et al., 2003)

SFAE – saturated biodiesel fractions; PKO – palm kernel oil

Along with the two biopolymers referred, the culture also produced a bright orange colour pigment, as can be seen in Figure 3.3. This colour was noticeable after about 24 h of fermentation and until the end of the assay the colour turned into a darker orange. As referred in the introduction, *Pseudomonas* sp. are known to produce pigments and coloured compounds. Phenazines are another product of *P. chlororaphis* activity that show some interesting properties and can act as antitumor, antibiotic or ant parasitic agents (Laursen and Nielsen, 2004). With this knowledge, some attempts to quantify the phenazines produced during the cultivation experiment were made. The protocol used to quantify was described by Bauer et al., 2016, and the results obtained are shown in Figure 3.4.

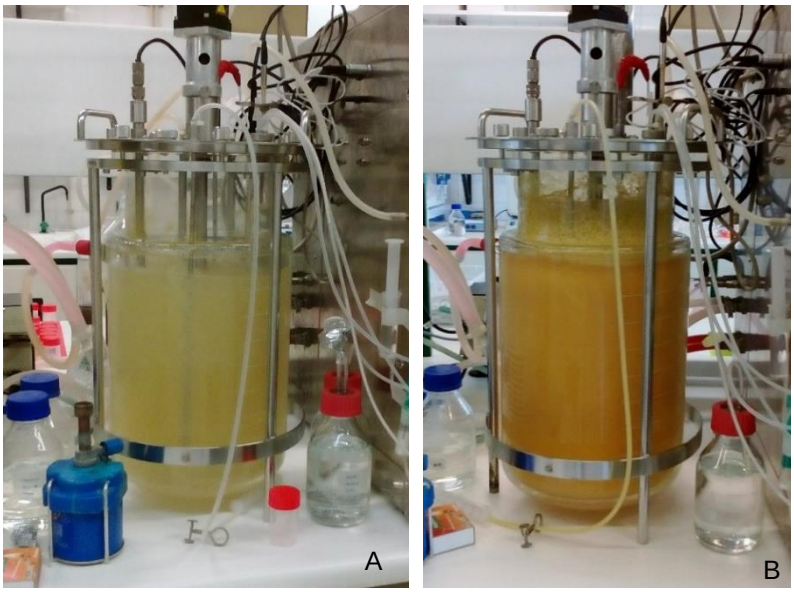

Figure 3.3 – Pictures of the bioreactor with *P. chlororaphis* culture: (A) was taken after the inoculation and (B) was taken after 24 hours.

It is known that phenazines production is regulated by QS, so the expected would be that during the exponential growth phase the production would be more significant. In fact, during cultivation, it was seen that the orange coloration appeared during this phase (Figure 3.3). The results of the absorbance reading show the same thing, during the exponential growth phase the culture intensively produced phenazines and, as the culture started to enter the stationary growth phase the amount of phenazines of the broth started to decrease. Bauer et al., 2016, described a similar pattern of phenazine production that goes along with cellular growth.

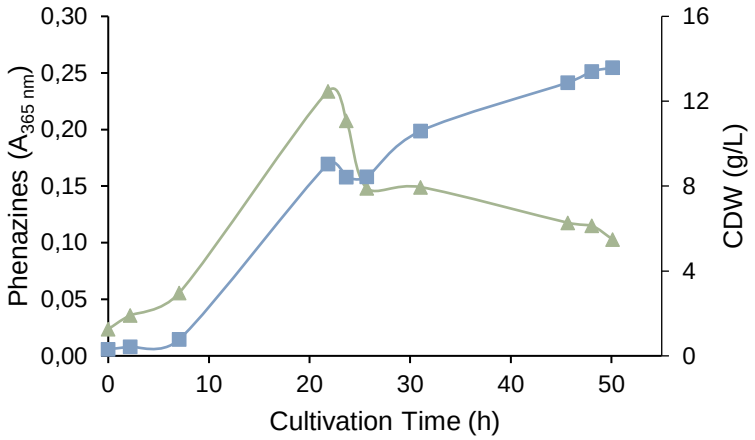

Figure 3.4 - Phenazines production profile (▲) along with the *P. chlororaphis* growth (■) during the cultivation time of the first reactor performed.

P. chlororaphis subs *aurantiaca* has been described for producing four members of the phenazines family: (1) 2-hydroxy-phenazine, (2) phenazine-1-carboxylic acid, (3) phenazine-1-carboxamide and (4) 2-hydroxyphenazine-1-carboxylic acid (Bauer et al., 2016; Morohoshi et al., 2013; Schneemann et al., 2011). It is expected that the orange pigment results of a combination of different molecules instead of being produced by only one phenazine derivate.

These results are not quantitative but show that there is dependence between cellular concentration and phenazine production. Along with the QS regulation other factors, like the carbon source, must have impact on the production of such metabolites. Similar work had already been developed on the laboratory using glycerol waste from biodiesel carbon source (Pereira, 2016) and there is ongoing work testing other carbon sources and none of them showed production of such high concentrations of phenazines like happened when using pure glycerol.

3.3.2 Optimisation of bioreactor cultivation conditions of *P. chlororaphis*

In an attempt of optimising PHA accumulation by *P. chlororaphis* from glycerol, a second bioreactor cultivation was performed (reactor 2). The bioreactor was operated under the same conditions described above with the main difference that the glycerol pulse was fed to culture earlier with the goal of stimulating the culture to produce more PHA. The results obtained for both reactors are listed in Table 3.1 and compared to other results of *mcl*-PHA production by *P. chlororaphis*.

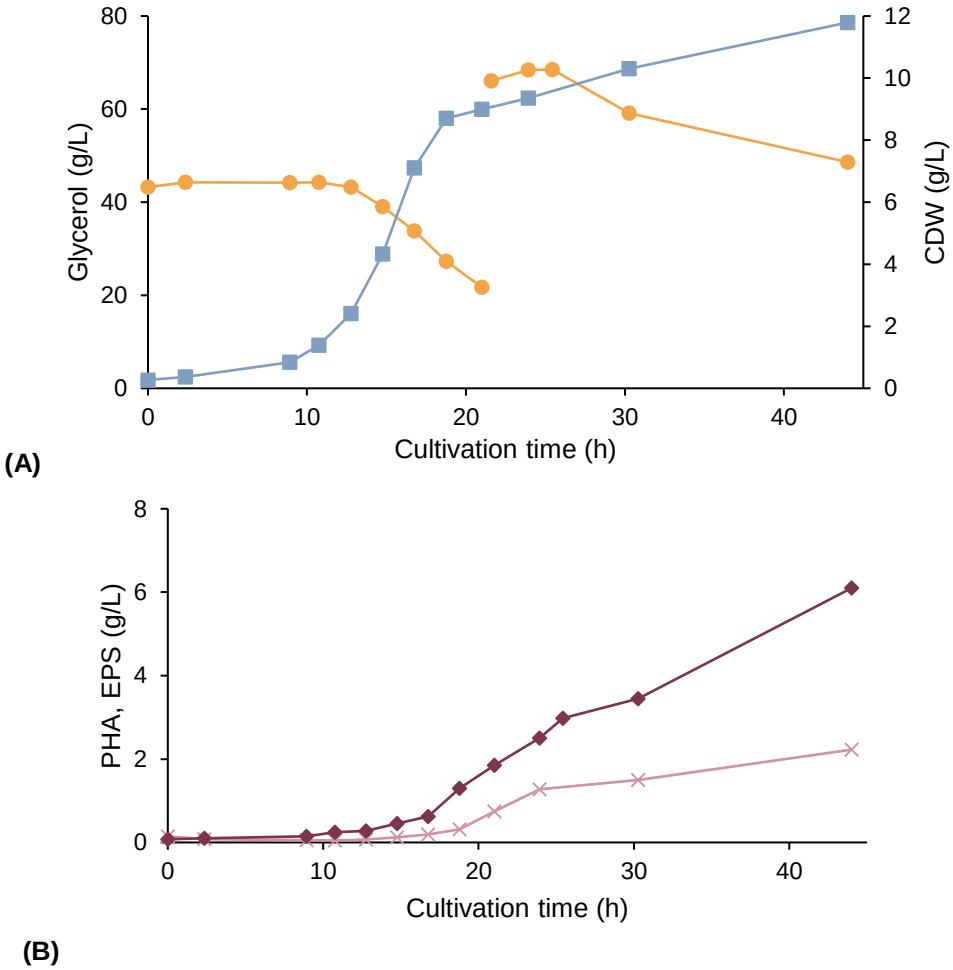

Figure 3.5 - Cultivation profile of *P. chlororaphis* during the second experiment. (A) CDW (■) and glycerol concentration (●); (B) production of PHA (x) and EPS (♦).

Figure 3.5(A) shows the growth profile of *P. chlororaphis* and the glycerol consumption for the entire duration of the assay, Figure 3.5(B) shows the production profile of PHA and EPS. The culture's lag phase lasted about 9 h and at that point the exponential growth phase started, ending after 19 h of cultivation. After 19 h, the CDW was 8.71 g/L with only 4% of PHA accumulated (1.28 g/L). The concentration of EPS was 2.50 g/L. On the period comprising the lag and exponential growth phase, 21.54 g/L of glycerol were consumed at a consumption rate of 1.02 g/L·h. During this period the consumed glycerol seems to have been mostly directed to cellular growth, since the polymers production over this period was relatively low. On the time period comprising the first glycerol pulse the CDW achieved was 9.05 and 8.71 g/L for reactor 1 and 2, respectively,

which is close. Less amount of glycerol was consumed in reactor 2 however the PHA concentration was close (0.57 g/L on reactor 1 and 0.75 g/L on reactor 2). The main difference was the EPS concentration that was higher for reactor 2 (1.85 g/L) than it was at around the same time on reactor 1 (0.98 g/L).

At around 22 h a pulse of glycerol was feed to the culture. Between 22 and 44 h the glycerol consumption was 17.48 g/L, at a rate of 0.78 g/L·h. Over this period, the CDW kept increasing even though at a slower rate, and at the end of the assay (44 h) it had increased to 11.79 g/L. By this time the cellular content of PHA had increased up to 19%, corresponding to an active biomass of 9.57 g/L. The PHA concentration was 2.23 g/L. Over the same period the concentration of EPS increased up to 6.10 g/L. Over the time period concerning the second glycerol pulse the main differences between the two reactors were evident. The amount of glycerol consumed was clearly lower on reactor 2 (17.48 g/L) than on reactor 1 (26.09 g/L) however, the CDW was similar and so was the PHA concentration. The EPS concentration, on the other hand, was significantly higher on reactor 2 (6.10 g/L compared to 2.40 g/L produced on reactor 1). On reactor 2, the culture seems to have directed its metabolism to produce EPS without affecting the amount of PHA accumulated.

Considering the 44 h of cultivation, a total of 39.02 g/L of glycerol were consumed, at an average glycerol consumption of 0.89 g/L·h. This provided a growth yield of 0.24 g/g. The products' yields were 0.05 and 0.15 g/g for PHA and EPS, respectively (Table 3.1). The PHA concentration was 2.23 g/L corresponding to a r_p of 1.21 g/L·day. The maximum EPS production was 6.10 g/L, giving a r_p of 3.33 g/L·day.

This reactor was meant to be an optimisation of the PHA accumulation by *P. chlororaphis*, by adding the glycerol pulse sooner, increasing the carbon concentration to excess values (66 g/L) (Anjum et al., 2016; Verlinden et al., 2007). The carbon excess was supposed to led the culture to produce higher amounts of PHA. Instead there was a considerable increase of EPS production which was most noticeable during the period between 21 and 44 h. Although, in this period, the EPS concentration was much higher there was no significant differences on the PHA percentage within the cells or in its overall concentration.

3.3.3 Characterisation of PHA accumulated by *P. chlororaphis*

For characterisation, the *mcl*-PHA produced by *P. chlororaphis* was extracted with chloroform since it is a solvent that causes no harm to the polymer (Koller et al., 2013). The characterisation of the thermal properties, degree of crystallinity and molecular mass distribution of two different samples of the *mcl*-PHA were analysed: an unpurified sample obtained from biomass washed only once and a purified sample obtained from biomass that was washed several times, with deionised water, until the supernatant showed no evidence of the orange pigment.

Table 3.2 – Monomeric composition of the *mcl*-PHA produced by *P. chlororaphis* from glycerol and of other *mcl*-PHA produced by different bacterial strains found in literature.

Bacterial strain	Carbon source	Monomers (%)					Reference
		3HHx	3HO	3HD	3HDd	3HTd	
P. chlororaphis DSM 19603	Glycerol	3	17	50	13	17	This study
P. chlororaphis DSM 50083	SFAE	15	51	26	5	-	(Muhr et al., 2013)
P. chlororaphis HS21(*)	PKO	5	40	37	2	5	(Yun et al., 2003)
P. citronellolis NRRL B-2504	OOD	14	43	32	12	1	(Cruz et al., 2016)
P. resinovorans NRRL B-2649	UCO	11	43	33	12	1	(Cruz et al., 2016)
P. resinovorans NRRL B-2649	OOD	19	44	28	9	1	(Cruz et al., 2016)

SFAE – saturated biodiesel fractions; PKO – palm kernel oil;
OOD – olive oil distillate; UCO – used cooking oil
(*) The polymer produced on the work of Yun et al., 2003, was composed of two more monomers: 3-hydroxytetradecenoate ($C_{14:1}$), 8% and 3-hydroxytetradecadienoato ($C_{14:2}$), 3%.

Both samples of *mcl*-PHA produced by *P. chlororaphis* from glycerol had the same composition: 50% 3HD, 17% 3HO, 17% 3HTd, 13% 3HDd and, in a much smaller fraction, 3% 3HO (Table 3.2). The monomers identification was confirmed by GC-MS analysis, the results obtained are shown in Appendix 3 (Figure A.14 and Figure A.15). These results correspond to the PHA produced on reactor 1 but the composition of the PHA produced on reactor 2 did not differ from this one. Comparing the composition obtained with other examples of *mcl*-PHA produced by *P. chlororaphis* (Table 3.3) it is possible to see that the main monomers are present but their proportion within the PHA is different. The *mcl*-PHA obtained in this work has a high content of 3HD (50%). However, the examples of *mcl*-PHA produced by *P. chlororaphis* found in literature show higher content of the 3HO monomer (51%). Another difference that is evident is that the *mcl*-PHA obtained in this work has 17% content of 3HTd and the contribution of this monomers on other PHA produced by *P. chlororaphis* is low (5%) or non-existent. It can be established that the carbon source has a great impact on the polymers' composition. When compared to *mcl*-PHA produced by other *Pseudomonas* strains (Table 3.2), such as *P. citronellolis* and *P. resinovorans* the same thing happens, since the polymer is mainly composed of 3HO (43-44%) and 3HD (28-

33%), and the other monomers have a smaller fraction. The presence of 3HTd in these polymers is almost negligible (1-4%).

Table 3.3 - Thermal properties, degree of crystallinity, Mw and PDI of the *mcl*-PHA produced by *P. chlororaphis* from glycerol and of other *mcl*-PHA produced by different bacterial strains found in literature.

Bacterial strain	Carbon source	PHA	T_m (°C)	T_g (°C)	T_{deg} (°C)	X_c (%)	Mw (x10^5, g/mol)	PDI	Reference
P. chlororaphis DSM 19603	Glycerol	Unpurified *mcl*-PHA	44.6	-50.4	284	13	1.31	1.45	This study
P. chlororaphis DSM 19603	Glycerol	Purified *mcl*-PHA	45.0	-49.9	288	27	1.25	1.45	This study
P. chlororaphis HS21	PKO	*mcl*-PHA	n.o.	-44	n.a.	n.a.	0.83	1.5	(Yun et al., 2003)
P. citronellolis NRRL B-2504	OOD	*mcl*-PHA	25.2	-14.2	n.a.	1	0.3	1.5	(Cruz et al., 2016)
P. resinovorans NRRL B-2649	UCO	*mcl*-PHA	43.3	-28.9	n.a.	7	0.4	1.3	(Cruz et al., 2016)
P. resinovorans NRRL B-2649	OOD	*mcl*-PHA	35.6	-15.8	n.a.	6	0.3	1.5	(Cruz et al., 2016)

n.a. – not available; n.o. – not observed;
PKO – palm kernel oil; OOD – olive oil distillate; UCO – used cooking oil

The two polymer samples shown in Table 3.3 were obtained from the same biomass but the purified sample had a much lower content of pigment (phenazines). For what concerns the thermal properties of the *mcl*-PHA produced by *P. chlororaphis* from glycerol, either the unpurified *mcl*-PHA or the purified *mcl*-PHA have their T_m around 45 °C and T_g is around -50 °C. This indicates that the phenazines had no significant impact on these properties. The available information for other *mcl*-PHA produced from *P. chlororaphis* is quite different, since that polymer shows no T_m. However, its T_g is within the range of values obtained in this study. As for *mcl*-PHA produced by other *Pseudomonas* it is possible to see in Table 3.3 that the T_m can change a lot even for the same producing strain, as it happens for *P. resinovorans*, where different substrates yield PHA with very different thermal properties. This information is helpful to conclude that the thermal properties of PHA are related to its monomeric composition, since the relative proportion of the monomers is the main difference between the five *mcl*-PHA presented in Table 3.3 (Cruz et al., 2016).

The X-ray diffractograms of the two *mcl*-PHA samples presented in Table 3.3 are shown in Figure 3.6. Both polymer samples show a pattern of diffraction that is characteristic of amorphous polymers, presenting a large bump within the 2ɵ=20° region (Sánchez et al., 2003) and a small peak at 2ɵ=22°. However, this peak is more accentuated for the purified polymer, indicating that the presence of phenazines may influence the crystallinity of the PHA. The fact that the *mcl*-PHA produced from *P. chlororaphis* is amorphous (X_c of 13%) is no surprise, since the *mcl*-PHA produced by other *Pseudomonas* shown in Table 3.3 have also very low crystallinity degrees. These low values are related to the diversity of monomers with high number of carbons which form the polymer and difficult the building of organized polymeric structures (Cruz et al., 2016).

The Mw of the *mcl*-PHA produced from *P. chlororaphis* is higher than the examples found in literature. Compared to the other *mcl*-PHA produced by *P. chlororaphis* the Mw is slightly higher. When compared to the *mcl*-PHA produced by other *Pseudomonas* the difference is of one order of magnitude, which is a great difference for Mw. The PDI of all the PHA shown in Table 3.3 are within the range of 1.3-1.5, so they are similar and relatively low.

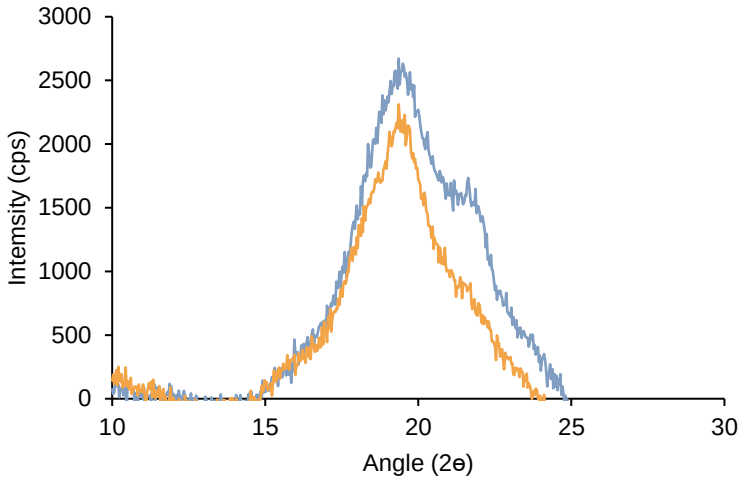

Figure 3.6 – X-ray diffractogram of two samples of the *mcl*-PHA produced by *P. chlororaphis* from glycerol. The blue line corresponds to the purified sample and the yellow line corresponds to the unpurified sample.

3.3.4 Characterisation of the EPS produced by *P. chlororaphis*

To understand the sugar composition of the EPS produced by *P. chlororaphis*, the samples were hydrolysed and analysed by HPLC. The chromatogram obtained from that analysis is shown in Figure 3.7. There were 7 major peaks on the sample, of which only four were identified: glucose that was apparently the main sugar monomer of the EPS (appearing at retention time RT=11.74 min), glucosamine (RT=9.22 min), rhamnose (RT=7.88 min) and mannose (RT=13.08 min). It was not possible to identify the other three peaks, appearing at RT=5.77 min, RT =8.41 min and RT=10.03 min, although galactose and arabinose were ruled out. There were no uronic acids present in the sample either (the chromatograms are shown in Appendix 4).

There is very limited information on EPS secreted by *P. chlororaphis* strains. Fett et al., 1996, described the production of an alginate-like polymer by *P. chlororaphis* NRRL B-2075 grown on nutrient agar-yeast extract (0.5%) dextrose medium (NDYA) with 5% dextrose. In its work the polymer was comprised by 13-33% (w/w) of mannuronate to guluronate (in a 9:1 ratio), 1-14% (w/w) neutral sugars (mannose and gulose) and 10% (w/w) hexosamines. The EPS obtained from *P. chlororaphis* in this work has similarities with the example described, such as the presence of mannose and the glucosamine (which is an hexosamine).

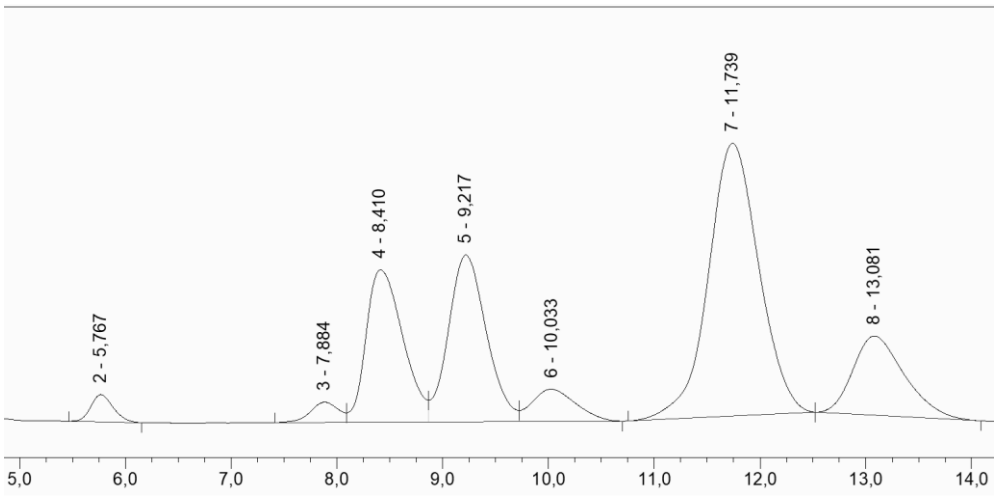

Figure 3.7 – Chromatogram of the EPS produced by *P. chlororaphis* from glycerol.

3.4 Conclusions and Future Work

In conclusion, this work helped to prove that *P. chlororaphis* uses glycerol as sole carbon source to produce *mcl*-PHA. Besides, accumulating PHA this strain was also able to convert the carbon source into an EPS and phenazines, an orange-coloured pigment. The results shown that the production of PHA and EPS is affected by the fermentation conditions. The mechanisms that make the culture direct its metabolism to produce either one or another polymer are still to be better understood, however it seems that the concentration of the carbon source is one the reasons influencing this result. More tests would be required to confirm this hypothesis.

The *mcl*-PHA produced was composed of 5 monomers: 3HHx, 3HO, 3HD (the most representative monomer), 3HDd and 3HTd. The physical and thermal characterisation of the polymer showed that it is a highly amorphous PHA with low T_m, acting almost like a glue. These properties, combined with a high degree of purity, make the *mcl*-PHA produced by *P. chlororaphis* suitable to be used in the biomedical field for the development of glues, patches or drug delivery systems.

In the future, for what concerns the *mcl*-PHA its mechanical properties should be tested on films. The production of *mcl*-PHA by *P. chlororaphis* from glycerol should also be optimised with order to try to achieve higher polymer contents. The pulse fed-batch fermentation seems to be a good strategy, however some things need to be better studied, such as the time to add the glycerol pulses or the time to remove the nitrogen source to induce limitation conditions on the culture.

The EPS produced by *P. chlororaphis* was composed of glucose, rhamnose, glucosamine and mannose. Glucose seems to represent most of the polymer, however further characterisation still needs to be done, since there are some constituents left to identify. The rheological and emulsifying properties and the ability of the EPS to form films should also tested to understand if tit is viable to proceed with its production. As for the EPS production, it would be interesting to understand under which conditions *P. chlororaphis* chooses to increase EPS production and what is the effect of that choice on the PHA production.

P. chlororaphis is best known as a phenazine producer and, in this work, the amount of phenazines produced was very high. The process to remove the pigment from the cells took much time and was dispendious so after comparing the thermal and physical properties of unpurified and purified *mcl*-PHA and confirming that the differences were minimal it was chosen to continue the work using unpurified *mcl*-PHA. The phenazines produced by *P. chlororaphis* are described to have some very interesting properties, such as antibiotic or antiviral agent. It would also be interesting to try to purify and quantify them and perhaps find areas where they can be applied.

Chapter 4

mcl-PHA impregnation with ibuprofen using sc-CO_2

4.1 Introduction

PHA are a group of biodegradable and biocompatible plastics hence showing great potential to be used on the biomedical field. Biopol (Metabolix, USA), is a commercially available copolymer of P(3HB-*co*-3HV) used mostly for packaging but also used as surgical material in the form of stiches or pins. The most used PHA in the medical field are P(3HB), P(4HB), P(3HO) and the copolymers P(3HB-*co*-3HV) and P(3HB-*co*-3HHx). They are used for various applications such as bone plates, wound dressings, prodrugs, tablets or agents for controlled release of drugs (Anjum et al., 2016).

The physical characteristics of *mcl*-PHA such as its low melting temperature give them elastomer and rubber like properties that make them versatile biopolymers and allow them to be used in special applications such as rubbers, latexes or thermo-sensitive adhesives and glues (Anjum et al., 2016; Muhr et al., 2013). As it has been referred PHA can be used for delivery of drugs in the form of tablets, implants or micro-carriers (Keshavarz and Roy, 2010). The copolymers P(3HB-*co*-4HB) and P(3HB-*co*-3HV) have been tested as drug releasing implants applied for the prevention of post-operative infections (Gursel et al., 2002; Türesin et al., 2001), and P(3HB) has been used as matrix for tablets of 7-hydroxyethyltheophylline. With the properties shown by *mcl*-PHA (glue-like behaviour and adhesive properties) this polymer can be used in the biomedical field in the form of subcutaneous patches that can be impregnated with drugs (antibiotics, non-inflammatory drugs and anti-cancer drugs) and act as drug delivery matrix (Nigmatullin et al., 2015).

So far, the impregnation of PHA was performed by solvent casting or by compression of the polymer and the drug. Gursel et al., (2002) and Türesin et al., (2012) have described the preparation of polymer rods by dissolving the polymer in chloroform, adding the active compounds to the mixture and then letting the solvent evaporate to yield a compact solid blend of drug and polymer. These formulations were applied as implants for the osteomyelitis treatment. Some tablets formulations have been created by compressing P(3HB) powder with the active compound powder. PHA have also been applied in tablets as excipients or disintegrators (Nigmatullin et al., 2015). The main problem with these methods for impregnation of active compounds is that the mechanical and thermal disturbances applied to the drug may have some impact on its therapeutic effect. Moreover, there is also the problem of using organic solvents such as chloroform for biomedical applications, where high product purity is always required (Antonov et al., 2013; Knez et al., 2011).

To overcome the problems associated to the use of organic solvents on the impregnation process, green solvents, like sc-CO_2 are now being considered as alternatives. Using sc-CO_2 for these processes carries some advantages concerning the characteristics of SCF such as high density and diffusivity and low viscosity. Furthermore, sc-CO_2 has low environmental impact, is soluble in some polymers and is easy to remove from the polymer resulting on a polymer with no solvent residues (Champeau et al., 2015; Duarte et al., 2009).

The aim of this work was to impregnate the *mc*l-PHA obtained by fermentation of *P. chlororaphis* (Chapter 3) with ibuprofen using sc-CO_2. Two different pressures were used and their effect on impregnation yield was accessed by performing controlled release tests of the impregnated PHA on phosphate buffer saline (PBS).

4.2 Materials and Methods

4.2.1 PHA extraction

For the impregnation experiments, *mcl*-PHA obtained from the fermentations described in Chapter 3 was used. *mcl*-PHA was extracted from dried *P. chlororaphis* cells by soxhlet extraction with chloroform, followed by purification by precipitation in cold ethanol, as described in 2.2.2.1 (Chapter 2).

4.2.2 Evaluation of PHA cytotoxicity

To evaluate the effect of the polymer and pigment on human cells, cell viability assays were performed. Suspensions of purified and unpurified *mcl*-PHA in water were tested on two cellular strains, fibroblasts and A2730, grown on DME medium and RPMI medium, respectively.

PHA samples were dissolved in acetone and precipitated in cold water (1:10, acetone:water proportion) to form a stable polymer suspension in water. Acetone was then evaporated under air passing through a sterile air filter. Everything was performed under sterile conditions.

Each polymer suspension (1 mg/mL) was diluted in each media in 1:1 proportions and this solution was added to the cells with the following concentrations: 500, 250, 150, 100, 50, 10, 1 and 0 µg/mL. As control, the media were diluted in deionised water in the same proportions.

The cells were seeded in 96-well plates at a concentration of $0.75x10^4$ cells/well and were incubated for 24 hours at 37 °C, with 5% (v/v) CO_2 and 99% (v/v) relative humidity. After 24 h the media was replaced for fresh media supplemented with polymer in concentrations up to 500 µg/mL. Doxorubicin 0.4 µM and dimethyl sulfoxide (DMSO) 0.1% were used as intern controls. After 48 h of incubation at 37 °C, with 5% (v/v) CO_2 and 99% (v/v) relative humidity, media was removed again and CellTiter 96 Aqueous non-radioactive solution (MTS) was added to all the wells. After 1 hour of incubation, absorbance at 490 nm was measured in a microplate reader. These tests were performed with the help of Doctor Catarina Rodrigues from the Human Genetics and Cancer Therapeutics Group on Life Sciences Department, FCT-UNL.

4.2.3 Ibuprofen preparation

Pure ibuprofen was obtained from ibuprofen sodium salt. 1 g of ibuprofen sodium salt (98%, Fluka) was dissolved in 20 mL of deionised water and acidified with HCl (3 M) until pH was between 1 and 2. The aqueous solution was then separated with 10 mL of dichloromethane, and the separation was performed four times to ensure that all the ibuprofen was extracted in the organic phase. The dichloromethane solution was left to evaporate completely. The obtained white powder (pure ibuprofen) was then weighed and stored at room temperature.

4.2.4 Supercritical Fluid Impregnation

The impregnation of the *mcl*-PHA samples (0.300 g) with ibuprofen (0.300 g), using sc-CO_2, was performed in a batch apparatus. The samples and ibuprofen were loaded into the high-pressure vessel, and then heated in a bath up to 40 °C. CO_2 was liquefied in a cooling bath containing water/ethylene glycol solution and then pumped with a pneumatic metering pump (Williams, V series), to the desired pressure. The pressure inside the vessel was controlled using a pressure transducer. The tests were performed at two different pressures: 150 and 200 bar. The vessel was kept at constant pressure and temperature (40 °C) for 3 hours. After that period, the system was slowly depressurized. Figure 4.1 shows a schematic representation of the system used for the impregnation assays.

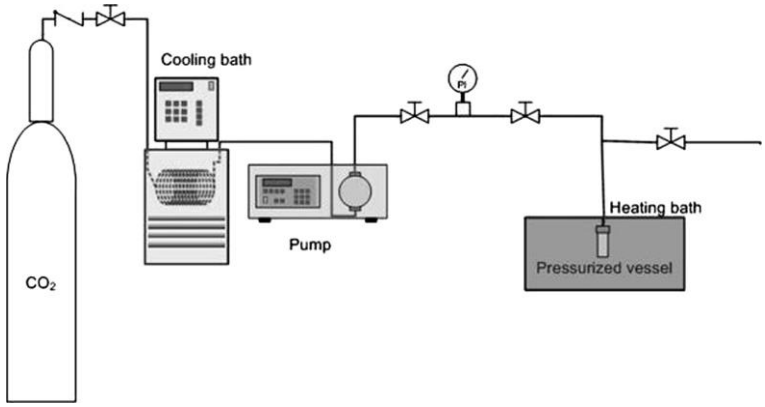

Figure 4.1 – Schematic representation of the apparatus used for the impregnation assays. Retrieved from Martins et al., 2014.

4.2.5 PBS preparation

PBS was prepared with the following composition: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4. pH was corrected for 7.4 using HCl, 3M.

4.2.6 Controlled Release Tests

The amount of ibuprofen released from the impregnated *mcl*-PHA samples was determined by spectrophotometric methods. To do so, each of the impregnated samples were immersed in 20 mL of PBS and they were placed on the orbital shaker (37 °C, 200 rpm). Samples (0.5 mL) were collected at pre-defined times of 0, 5, 10, 15, 30, 45, 60 min, 4 h, 5 h and 24 h. After each sample was collected, 0.5 mL of PBS was added to maintain the buffer volume. The ibuprofen amount on the samples was determined by measuring the absorbance in a UV/vis spectrophotometer at 265 nm. Calibration curve was made with an ibuprofen on PBS solution (1 mg/mL) with the following concentrations: 1, 0.5, 0.2, 0.1, 0.05, 0.01 and 0.001 mg/mL. PBS was used as zero reference.

4.3 Results and Discussion

4.3.1 Evaluation of *mcl*-PHA cytotoxicity

In this work, besides accumulating *mcl*-PHA, *P. chlororaphis* also produced phenazines, that are orange coloured pigments, known for their antimicrobial and antifungal properties (Laursen and Nielsen, 2004). When the *mcl*-PHA was extracted from the cells it always had a high amount of pigment associated to it. Obtaining polymer with low pigment concentration would imply washing the biomass 5 to 6 times with deionised water, a process that involves numerous centrifugations. It is known that PHA are biocompatible and non-toxic (Reddy et al., 2003) providing a good matrix for controlled drug release. However, the toxicity of the pigment was not clear neither was the combination of PHA and phenazines. To understand if the pigment or the combination of pigment and PHA would cause toxic effects, cell viability assays were performed. Figure 4.2 shows the two polymer samples used for this test.

In these assays two cellular lines were used: a healthy cellular line, fibroblasts, and a cancer cellular line, A2780. Using the cancer cell line ensured that any toxic effect would be visible, since these cells are much more sensible to stress conditions.

Figure 4.3(A) shows the cell viability of the fibroblasts grown on DMEM and subjected to different concentrations of *mcl*-PHA with high and with low concentrations of phenazine. The results show that cell viability remains high, and it is possible to assume that the pigment and polymer do not have toxic effect on the cells. Figure 4.3(B) shows the cell viability of A2780 grown on RPMI in the same conditions as fibroblasts. This cellular line also shows that the polymer and the pigment cause no harm to its viability. The results for the highest concentration of pigment are not showed because the control samples were under too much stress because their growth medium was very diluted. However, the absorbance value for the samples with the polymer were within the obtained for the other concentrations.

The results of both cellular lines reinforced the concept that PHA are non-toxic and biocompatible and helped to conclude that phenazines are also non-toxic. With these results, it was chosen to proceed with the impregnation tests using the polymer with high concentration of pigments.

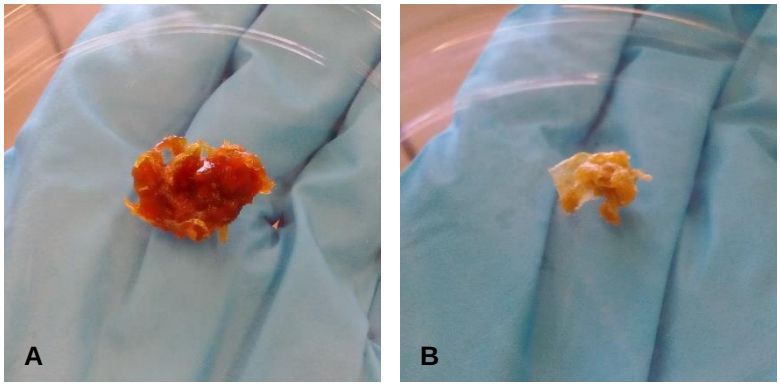

Figure 4.2 – Pictures of both samples of *mcl*-PHA used for cell viability assays. (A) is the unpurified sample and (B) is the purified sample.

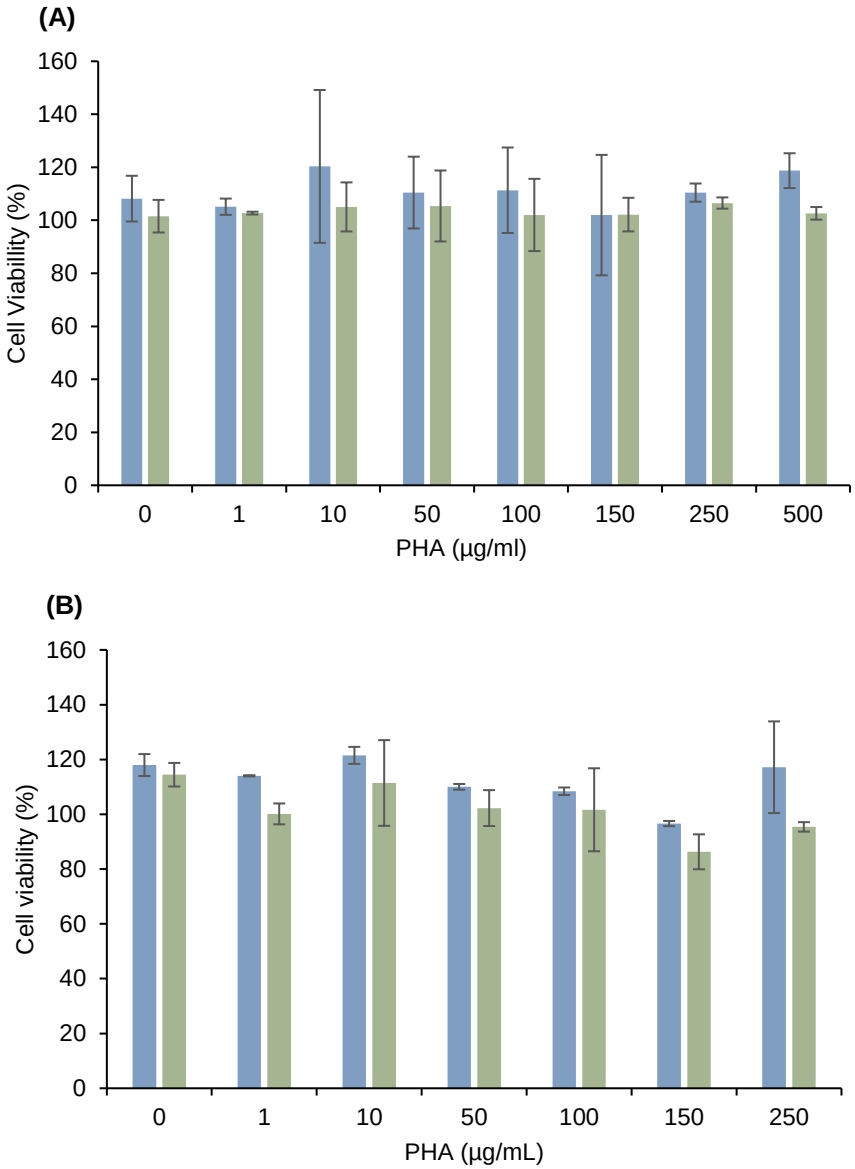

Figure 4.3 –Cell viability of (A) Fibroblasts grown on DMEM and of (B) A2780 grown on RPMI in the presence of PHA with low concentrations of pigment (■) and PHA with high concentrations of pigment (■).

4.3.2 Impregnation of *mcl*-PHA with sc-CO_2

One of the reasons that lead to choosing this PHA for this study was its T_m. The *mcl*-PHA produced by *P. chlororaphis* has a T_m of 45 °C which is relatively low and easy to achieve. The impregnation assays were conducted at 40 °C to ensure that the polymer was solid at normal pressure. sc-CO_2 at high pressures caused a melting point depression due to its solubilisation in the polymer. Therefore, at 40 ºC and at the experimental pressures, the polymer melted. This aspect is very important because with the polymer in its liquid state the mass transfer between the sc-CO_2 and the polymer was facilitated, hence resulting in a homogeneous impregnation of ibuprofen in the *mcl*-PHA. After depressurization, the polymer returned to its original solid form. To facilitate the recovery of the *mcl*-PHA after the system was depressurized, the ibuprofen was placed directly into the high-pressure vessel and the *mcl*-PHA was placed in a pre-weighed micro tube.

Figure 4.4 shows a sample of *mcl*-PHA immediately after it was removed from the high-pressure vessel. The solubility of sc-CO_2 on the polymer is evidenced by the polymer's expansion (sc-CO_2 foaming). Usually the foaming process is associated with porous formation inside the polymer that can be controlled trough depressurization rate. Polymer plasticization is also associated with sc-CO_2 solubility in polymers. To confirm that sc-CO_2 had a plasticising effect on the *mcl*-PHA, DSC analysis would have to be performed on the samples after impregnation. However, it was expected that the polymer would suffer a decrease on its T_g, since it is an amorphous polymer and amorphous polymers are more sensitive to the plasticising effect of sc-CO_2 (Yoganathan et al., 2010).

Figure 4.4 – Picture of the *mcl*-PHA recovered after an impregnation test performed at 200 bar and 40 °C during 3 h.

4.3.3 Ibuprofen Controlled Release Tests

The release of ibuprofen from *mcl*-PHA was studied over 24 h. The medium used for the release was PBS and the release was performed at 37 °C. Figure 4.5 shows the release of ibuprofen from the *mcl*-PHA samples. As it can be seen the release profile resembles a controlled release profile (Gursel et al., 2002). For both pressures tested, the initial ibuprofen release is fast. That may happen due to the decrease of solubility of ibuprofen in sc-CO_2 when the system is depressurized that leads to ibuprofen precipitation on the surface of the polymer.

The ibuprofen impregnation yields were 66.7 ± 0.7 and 93.3 ± 4.7 mg/g for 150 and 200 bar, respectively. The pressure used for the impregnation influenced the amount of ibuprofen that was dissolved inside the polymer. This is explained by the fact that ibuprofen's solubility in sc-CO_2 increases with pressure (Kuznetsova et al., 2013).

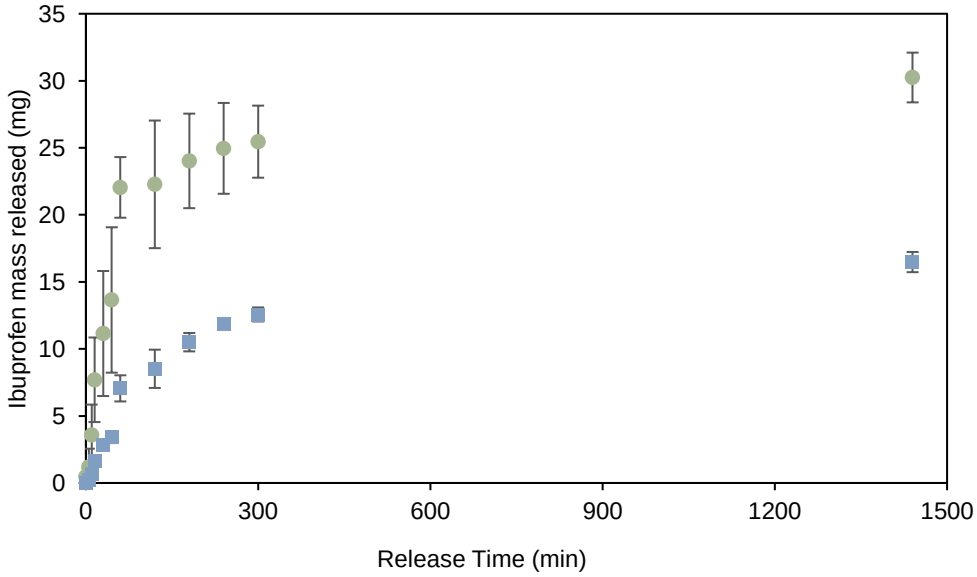

Figure 4.5 – Ibuprofen release (mg) from *mcl*-PHA on medium simulating physiologic conditions (PBS) over 24h. *mcl*-PHA was impregnated at two different pressures: 150 bar (■) and 200 bar (●)

4.4 Conclusions and Future Work

This work was a preliminary study, however it was possible to conclude that the *mcl*-PHA produced from *P. chlororaphis* can act as impregnation matrix for controlled drug delivery systems. It was also evidenced that sc-CO_2 is soluble in the *mcl*-PHA by one alteration on the polymer after impregnation: the foaming effect.

In the future, it would be interesting to study other conditions of pressure and time of impregnation and optimise the process to achieve the higher impregnation yields. The depressurisation rate should also be evaluated since it is responsible for the porous structure of the final polymer. Furthermore, models can be applied to explain the release mechanisms of ibuprofen from the *mcl*-PHA.

Chapter 5

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Chapter 6

Appendices

Appendix 1 – GC chromatogram of P(3HB-*co*-3HV)

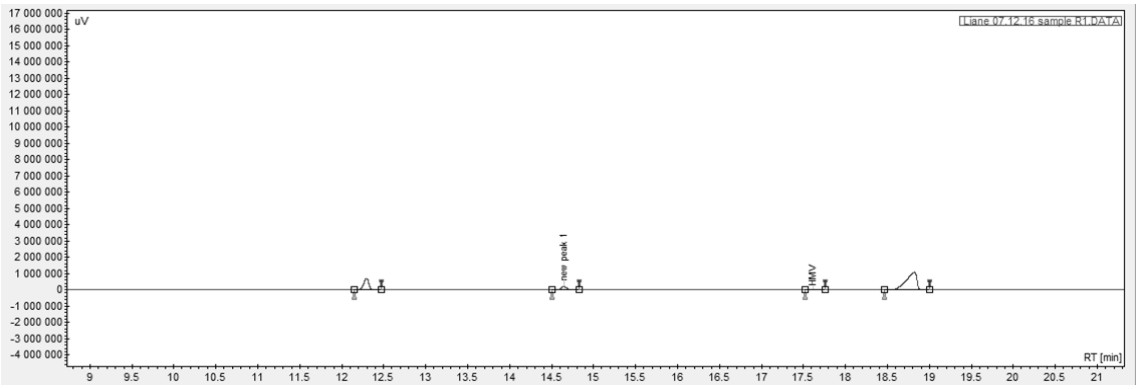

Figure A.1 – GC chromatogram of P(3HB-*co*-3HV).

Appendix 2 – XRD Diffractograms of P(3HB-*co*-3HV)

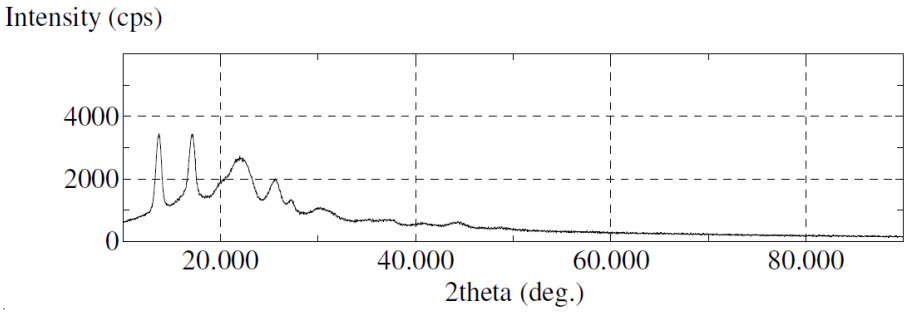

Figure A.2 - XRD of the original P(3HB-*co*-3HV).

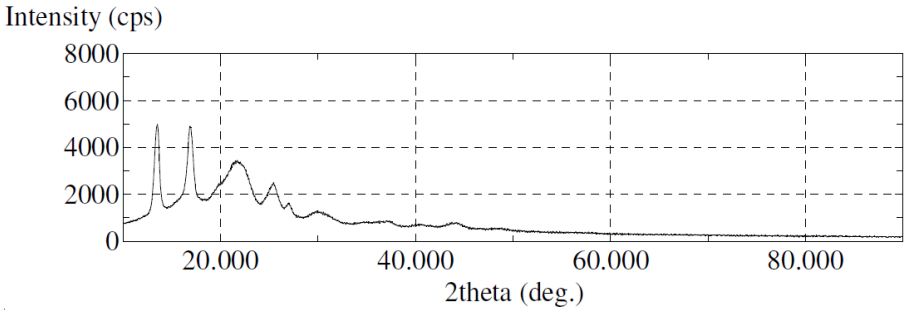

Figure A.3 - XRD of the P(3HB-*co*-3HV) obtained after HCW treatment at 130 °C.

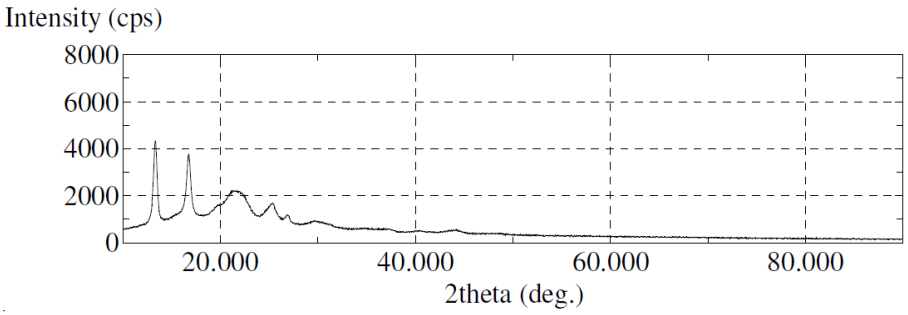

Figure A.4 - XRD of the P(3HB-*co*-3HV) obtained after HCW treatment at 150 °C for 30 min.

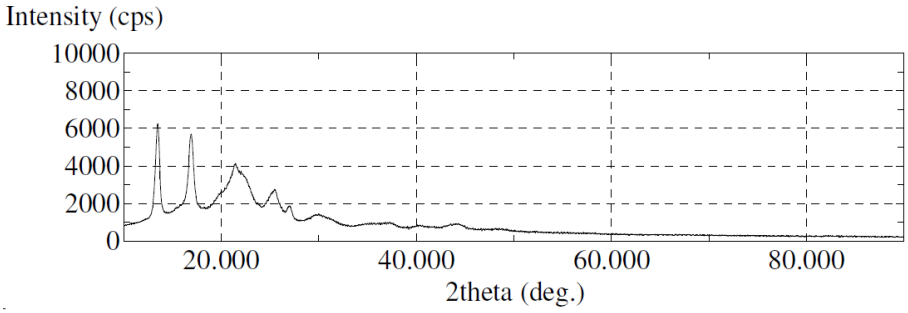

Figure A.5 - XRD of the P(3HB-*co*-3HV) obtained after HCW treatment at 150 °C for 60 min.

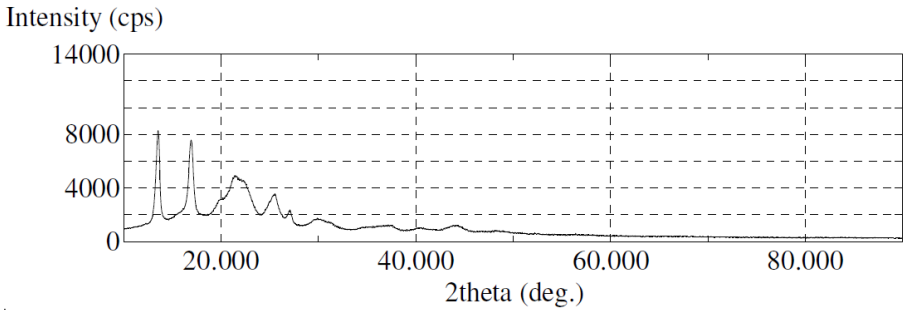

Figure A.6 - XRD of the P(3HB-*co*-3HV) obtained after HCW treatment at 165 °C.

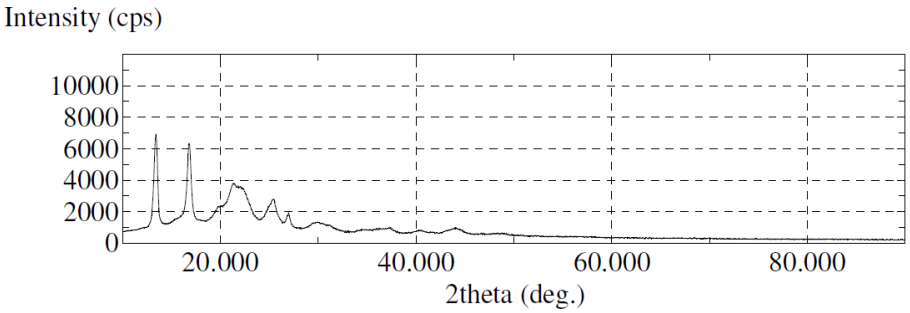

Figure A.7 - XRD of the P(3HB-*co*-3HV) obtained after HCW treatment at 180 °C.

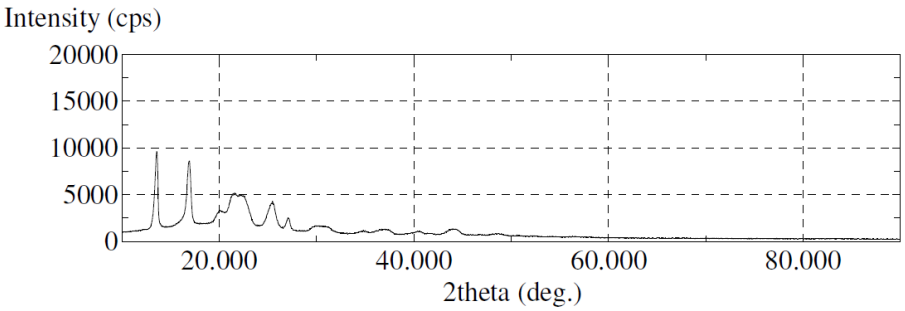

Figure A.8 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (42 g/L) after HCW treatment at 150 °C.

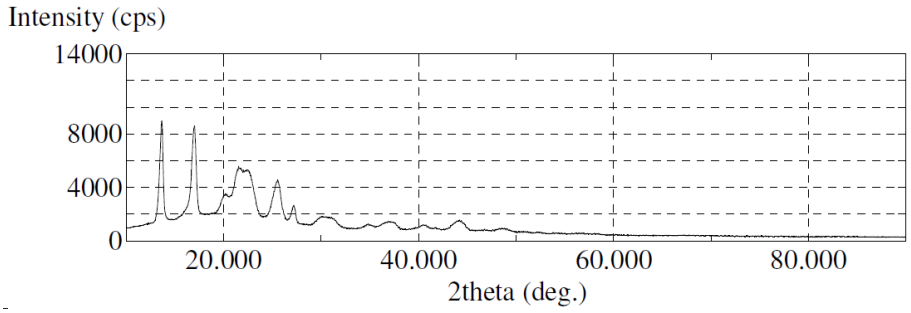

Figure A.9 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (25.2 g/L) after HCW treatment at 150 °C.

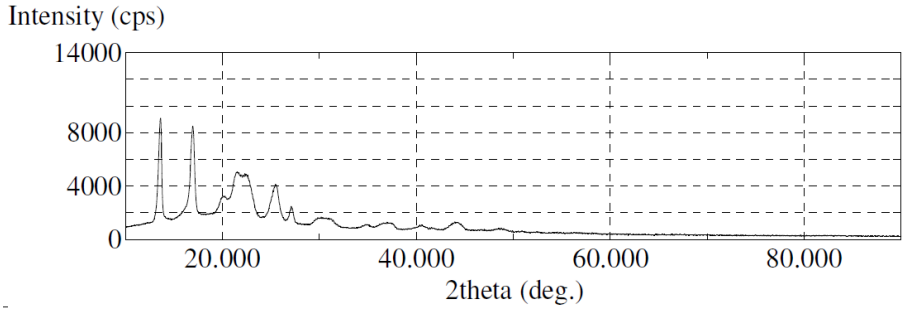

Figure A.10 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (16.8 g/L) after HCW treatment at 150 °C.

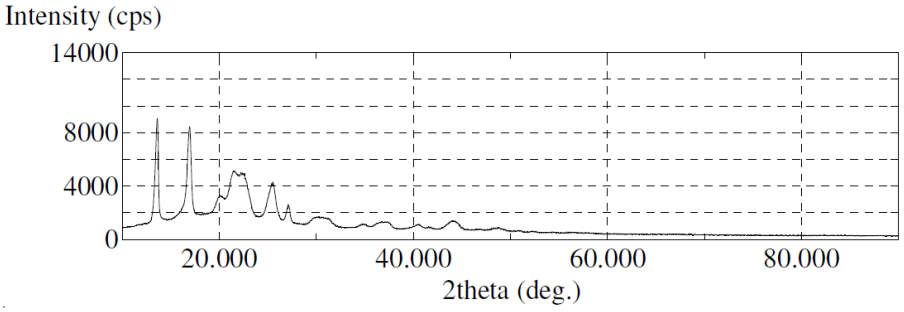

Figure A.11 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (8.4 g/L) after HCW treatment at 150 °C.

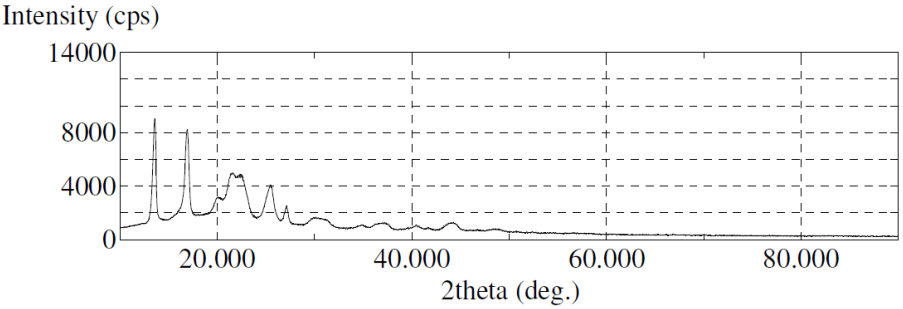

Figure A.12 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (4.2 g/L) after HCW treatment at 150 °C.

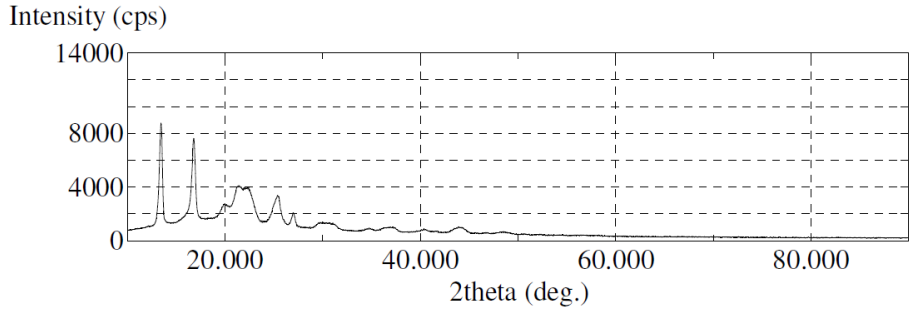

Figure A.13 - XRD of the P(3HB-*co*-3HV) obtained from hypochlorite extraction (0.84 g/L) after HCW treatment at 150 °C.

Appendix 3 – *mcl*-PHA composition

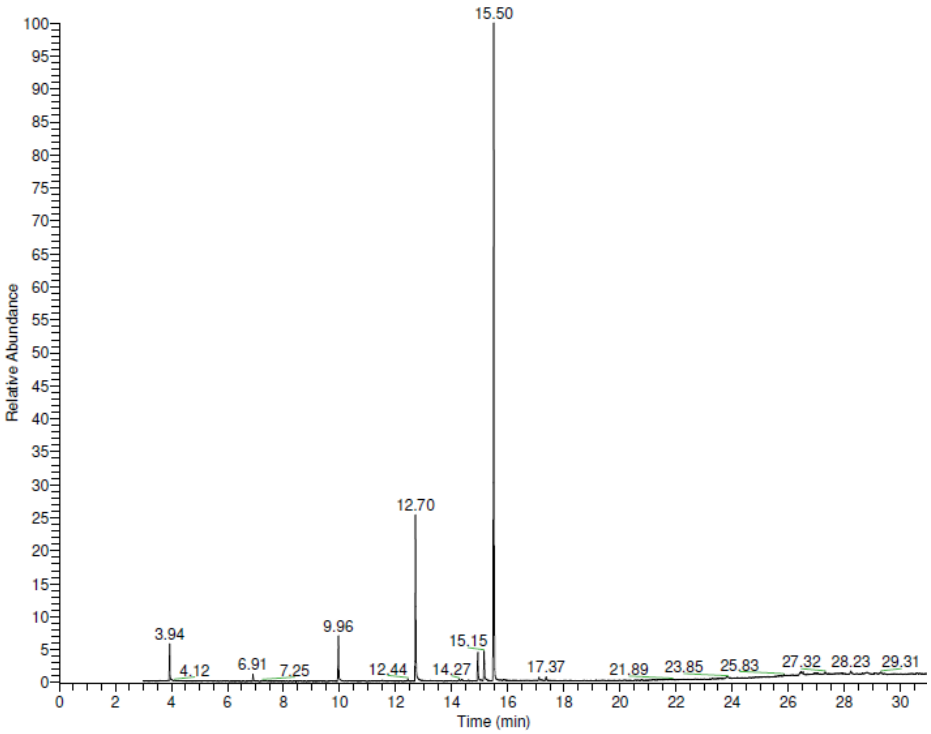

Figure A.14 - Total Ion Chromatogram of the *mcl*-PHA produced by *P. chlororaphis* obtained from GC-MS analysis.

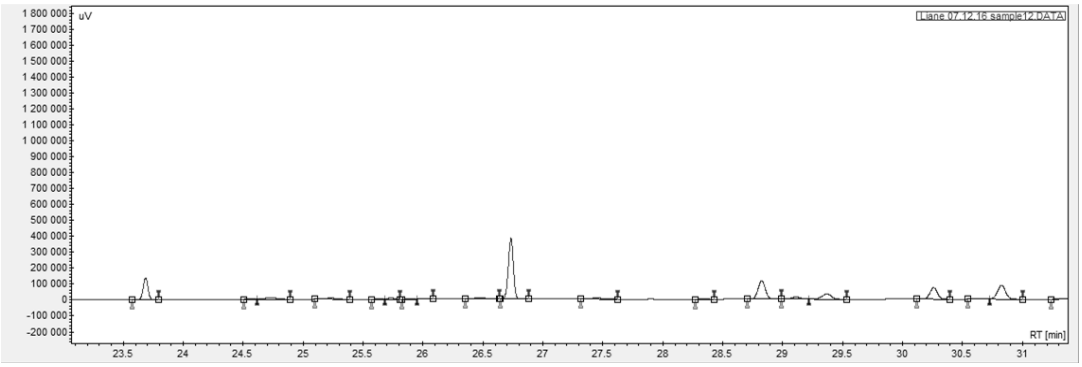

Figure A.15 - Gas Chromatogram of the *mcl*-PHA produced by *P. chlororaphis* obtained from GC analysis.

Appendix 4 – EPS composition, HPLC analysis

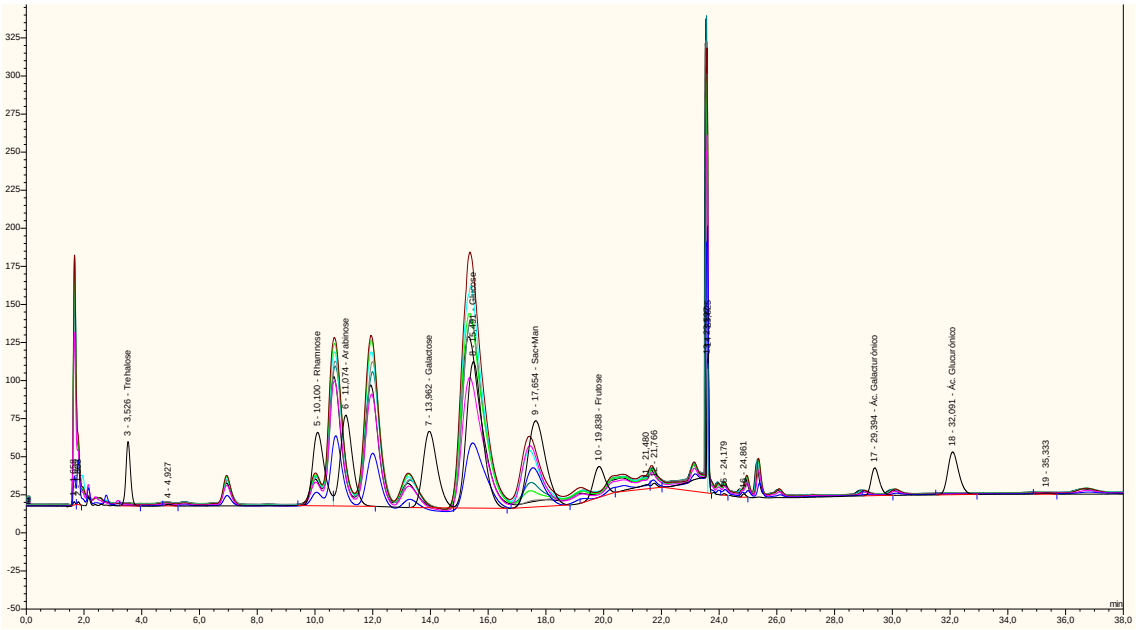

Figure A.16 - HPLC chromatogram of the EPS samples obtained from reactor 1. The black line represents the standard.

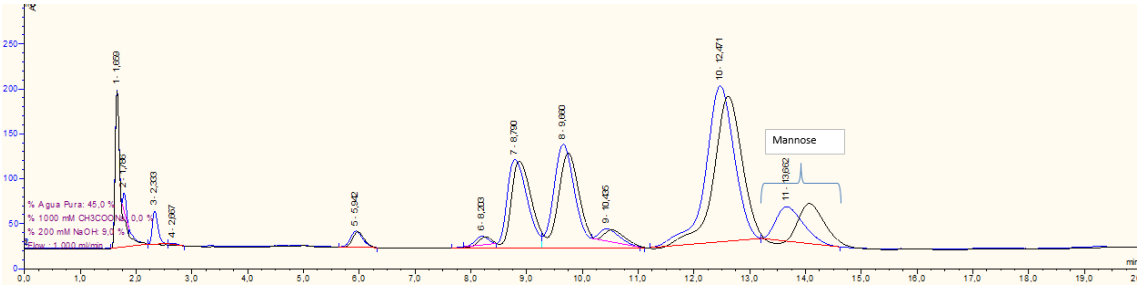

Figure A.17 - Chromatogram of the final EPS sample with mannose as added standard sugar.

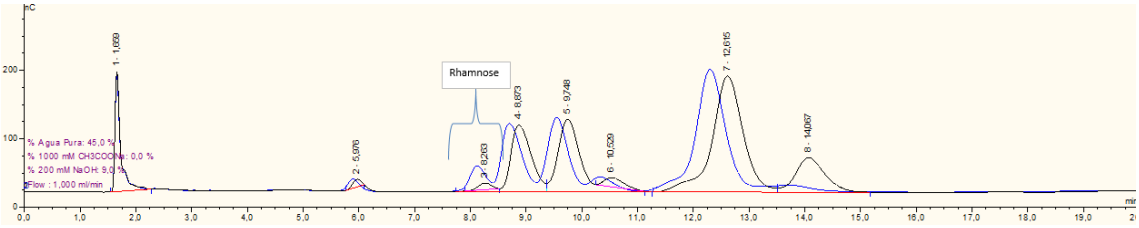

Figure A.18 - Chromatogram of the final EPS sample with rhamnose as added standard sugar.

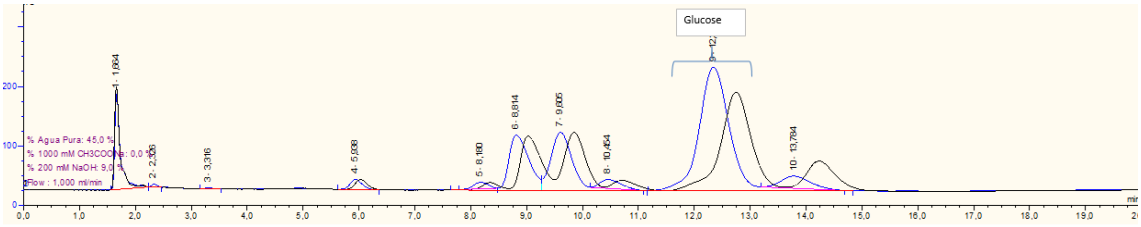

Figure A.19 - Chromatogram of the final EPS sample with glucose as added standard sugar.

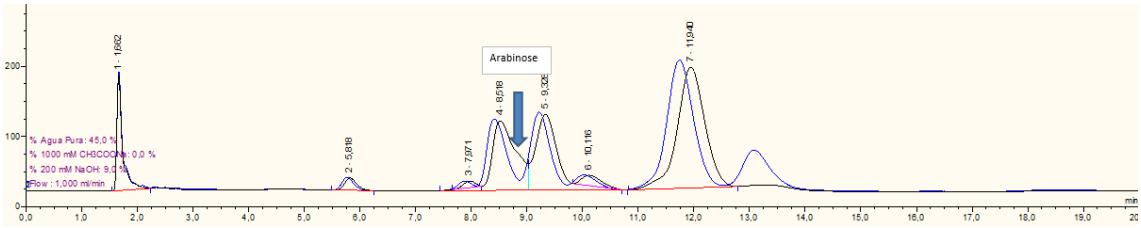

Figure A.20 - Chromatogram of the final EPS sample with arabinose as added standard sugar.

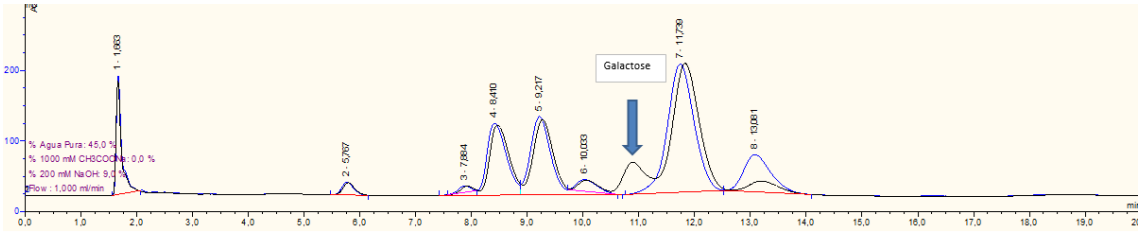

Figure A.21 - Chromatogram of the final EPS sample with galactose as added standard sugar.

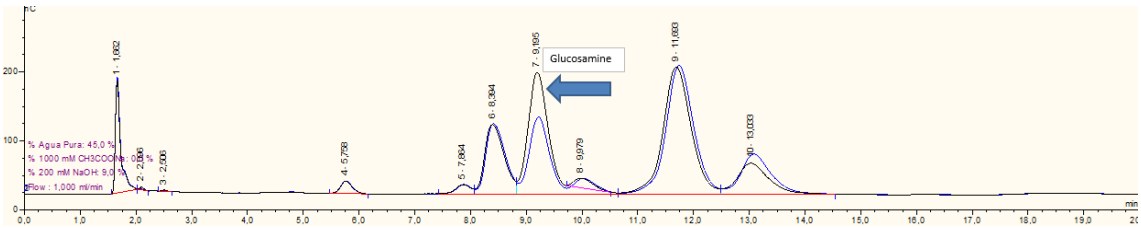

Figure A.22 - Chromatogram of the final EPS sample with glucosamine as added standard sugar.