João Ricardo Antunes dos Santos Soares Pereira

Licenciado em Bioquímica

Investigation of the Adhesive Properties of Bacterial Medium-Chain-Length Polyhydroxyalkanoates (mcl-PHA) for Medical Applications

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Doutora Maria Filomena Andrade de Freitas,
Senior Researcher, FCT-UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Agradecimentos

Ao longo do desenvolvimento deste trabalho foram muitos os que diretamente influenciaram o desenvolvimento do mesmo e por isso gostava de aqui deixar o meu agradecimento.

Gostaria de agradecer em primeiro lugar à minha orientadora, Filomena Freitas, por me ter recebido e apresentado o seu laboratório. Por me ter acompanhado em toda a parte do desenvolvimento deste trabalho bem como disponibilidade em esclarecimentos, conselhos e ensinamentos que me proporcionou ao longo de todo este projeto.

Queria gratificar o Professor Vítor Alves, do Instituto Superior de Agronomia, pela disponibilidade, apoio, ensinamentos, esclarecimentos e conselhos, assim como o interesse em seguir todo este trabalho.

Não posso deixar de agradecer à Professora Maria Ascensão Reis e a todo o grupo BioEng pela forma como me receberam, ajudaram e ensinaram, principalmente, na parte experimental deste projeto, com um especial apreço à Diana Araújo, Sílvia Baptista, Inês Farinha, Nádia e Sílvia Antunes por todos os conselhos, instruções, correções, paciência e comentários que me fizeram crescer tanto a nível pessoal com profissional.

E por último, mas não menos importante gostaria de agradecer aos meus colegas de mestrado, Patrícia Reis, Joana Marques, André Oliveira e Sofia Pereira, por partilharem comigo todas as frustrações, peripécias e complicações, bem como os êxitos, o companheirismo e a ajuda em ultrapassar todos os obstáculos que a realização de uma tese de mestrado acarreta.

A todos, dedico este trabalho e um muito obrigado.

Palavras-chave

Polihidroxialcanoatos de cadeia média, Glicerol, *Pseudomonas chlororaphis,* Métodos de extração alternativos, Testes de tensão, Testes de adesividade

Resumo

Os poli(hidroxialcanoatos) (PHAs) são uma classe de poliésteres biodegradáveis e biocompatíveis com possíveis aplicações em biomedicina (Sodian et al., 2000). Os PHAs são produzidos em forma de grânulos dentro das células bacterianas, como compostos de armazenamento de carbono e energia. Existem três tipos diferentes de polímeros de PHA dependendo do tamanho dos seus monómeros, nomeadamente, os PHAs de cadeia curta (scl-PHA), os PHAs de cadeia média (mcl-PHA) e os PHAs de cadeia longa (lcl-PHA). Estes polímeros são formados durante o processo de fermentação, onde as bactérias crescem em condições limitantes, com carência de um ou vários elementos (por exemplo, limitação de fósforo, azoto, micronutrientes e/ou oxigénio), mas com um excesso de uma fonte de carbono adequada (Lee, 1996; Zinn and Hany, 2005). Contudo, o uso de uma fonte de carbono eficiente para a produção de PHA representa um custo elevado no processo de fermentação (Cruz et al., 2016). Por esta razão, para reduzir substancialmente os custos de produção, é importante o uso de resíduos ou sub-produtos como fonte de carbono, como por exemplo o glicerol, gerado pela indústria do biodiesel (Muhr et al., 2013). Neste trabalho, foi estudado um grupo conhecido de bactérias acumuladoras de mcl-PHA, com o intuito de observar a acumulação de mcl-PHA usando glicerol da indústria do biodiesel como única fonte de carbono. A estirpe *Pseudomonas chlororaphis* foi selecionada para a produção de mcl-PHA a partir de glicerol, por ter atingido uma concentração final de biomassa seca de 3,28 g/L, superior às restantes estirpes testadas, e por produzir um polímero composto por monómeros de hidroxihexanoato (HHx), hidroxioctanoato (HO), hidroxidecanoato (HD) e hidroxidodecanoato (HDd).

A bactéria *Pseudomonas chlororaphis* foi estuda em ensaios em bioreactor, em três modos de cultivo diferentes, nomeadamente, *batch*, *batch* alimentado por pulsos e *batch* repetido onde foram obtidas as produtividades de 0,026, 0,023 e 0,025 g/L h, respetivamente. O polímero foi recuperado das células bacterianas por extração em Soxhlet com clorofórmio. Para o processo de purificação, foi utilizado um método modelo com redissolução do mcl-PHA em clorofórmio seguido de precipitação em etanol gelado. Contudo, o clorofórmio é um solvente perigoso e não pode ser usado em produções industriais. Sendo assim, foram estudados métodos extração alternativos usando solventes menos prejudiciais, cujos valores mais altos de pureza foram atingidos usando acetona, etil-acetato e hexano como solventes.

Os mcl-PHA têm propriedades mecânicas únicas, que foram estudadas realizando testes de tensão axial num filme de mcl-PHA produzido pela bactéria *Pseudomonas chlororaphis*. Neste ensaio, foi alcançada uma tensão de rutura entre os 2,91 e os 4,89 MPa, em quatro réplicas, juntamente com uma elongação entre os 212,39 e os 296,87% com um módulo de Young de 6 a 9 MPa.

Uma vez que o objetivo principal deste trabalho seria desenvolver um novo adesivo totalmente natural para poder ser utilizado em aplicações na área da medicina, o mcl-PHA, devido às suas propriedades adesivas, apresenta-se como o candidato perfeito para esta questão. Por esta razão, foram feitos alguns testes de adesividade, usando o mcl-PHA e pele de porco como substrato modelo. Foi realizado um teste de tensão, em três réplicas, onde foram obtidos valores tensão de separação entre os 45,02 e os 90,13 kPa. Foi feito também testes de tensão de cisalhamento, em três réplicas onde foram obtidos valores tensão de separação entre os 10,02 e os 12,87 kPa.

Keywords

Medium-chain-length poly(hydroxyalkanoates), Glycerol, *P. chlororaphis,* Alternative recovery methods, Tensile test, Adhesion tests

Abstract

Poly(hydroxyalkanoates) (PHAs) are a class of biodegradable and biocompatible polyesters with potential applications in biomedicine (Sodian et al., 2000). PHA are produced inside bacterial cells in the form of granules as carbon and energy storage compounds. There are three different PHA polymers, namely, short-chain-length PHAs (scl-PHA), medium-chain-length PHAs (mcl-PHA) and long-chain-length PHAs (lcl-PHA), depending on the size of their monomers. These polymers are formed during fermentation processes where bacteria grow with some defiant conditions (i.e. limitation in phosphorus, nitrogen, trace elements and/or oxygen), but with an excess supply of a suitable carbon source (Lee, 1996; Zinn and Hany, 2005). However, the use of effective carbon sources for production of PHA represent high costs to the fermentation process (Cruz et al., 2016). Therefore, the use of wastes or by-products as carbon sources, such as waste glycerol generated by the biodiesel industry, is important to reduce substantially the production costs of mcl-PHA (Muhr et al., 2013). In this work, a group of known mcl-PHA-accumulating bacteria, was studied for the accumulation of mcl-PHA using waste glycerol, from the biodiesel industry as the sole carbon source. *Pseudomonas chlororaphis* was found to be the most suitable candidate to produce mcl-PHA from glycerol, with a final CDW concentration of 3.28 g/L, among the tested strains, and with a polymer composed by hydroxyhexanoate (HHx), hydroxyoctanoate (HO), hydroxydecanoate (HD) and hydroxydodecanoate (HDd) monomers.

Pseudomonas chlororaphis was studied in bioreactor cultivation experiments, in three different cultivation modes, namely, batch, pulse feeding and repeated batch and productivities of 0.026, 0.023 and 0.025 g/L h, respectively, were achieved. The polymer was recovered from the bacterial cells by Soxhlet extraction with chloroform. For the purification process, a standard technique with the re-dissolution of the mcl-PHA in chloroform followed by precipitation in ice cold ethanol was used. However, chloroform is a hazard solvent and cannot be used for industrial production. Hence, alternative recovery methods were also studied, using less hazardous solvents and the highest purity values were achieved by acetone, ethyl acetate and hexane solvents.

Mcl-PHAs have unique mechanical properties that were studied by preforming an axial tensile test in a mcl-PHA film. In this assay, a tension at break values between 2.91 to 4.89 MPa were achieved in four replicates, together with an elongation at break of 212.39 to 296.87% and a Young modulus of 0.60 to 0.90 MPa.

Since the main objective in this work was to develop a new fully natural bio-based adhesive to be used in medical applications, mcl-PHA, due to his tacky behaviour, presents its self as perfect candidate for this issue. So for that purpose some adhesion tests, using mcl-PHA and porcine skin as a model substrate, were made. It was performed a tension test, in three replicates, where it was attained values

of tension at separation between 45.02 and 90.13 kPa. It was also made some shear tests, in three replicates and a tension at separation values of 10.02 to 12.87 kPa were achieved.

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Abbreviations

CDW – Cell Dry Weight (g/L)

DO – Dissolved Oxygen (%)

FCT – Faculdade de Ciências e Tecnologia

GC – Gas Chromatography

HB – HydroxyButirate

HD – HydroxyDecanoate

HDd – HydroxyDodecanoate

HHx – HydroxyHexanoate

HO – HydroxyOctanoate

HPLC – High-Performance Liquid Chromatography

HTd – HydroxyTetradecanoate

Lcl-PHA – Long Chain Lengeth Polyhydroxyalkanoate

LPS - LipoPolySaccharides

Mcl-PHA – Medium Chain Lengeth Polyhydroxyalkanoate

N – Normal unit

n. a. – Data Not Available

nm – Nanometers

OD – Optical Density

PHA – PolyHydroxyAlkanoate

PHB – Poly HydroxyButirate

PKO – Palm Kernel Oil

rpm – rotation per minute

Scl-PHA – Short Chain Lengeth Polyhydroxyalkanoate

SFAE – Substrates derived From Animal Waste

v/v – volume per volume

vvm – volume of air per volume of reactor per minute

$\mathbf{Y_{P/S}}$ – Yield of mcl-PHA on glycerol (g mcl-PHA/g glycerol)

$\mathbf{Y_{X/S}}$ – Yield of biomass on glycerol (g cell/g glycerol)

1. Introduction

1.1. Poly(Hydroxyalkanoate) (PHA)

Poly([*R*]-hydroxyalkanoate) (Fig.1.1) is a class of biodegradable and biocompatible polyesters with potential applications in biomedicine (Sodian et al., 2000). PHAs are produced by some archae and eubacteria in aerobic and anaerobic habitats. These polyesters are formed inside bacterial cells and accumulated in the form of granules, as a reserve material during fermentation processes where bacteria grow with some defiant conditions (i.e. limitation in phosphorus, nitrogen, trace elements, or, more usually, oxygen), but with an excess supply of a suitable carbon source (Lee, 1996; Zinn and Hany, 2005).

Depending on the selected bacterial species, the fermentation process, the growth conditions and the downstream processing, different types of polymers can be produced (Keshavarz et al., 2010), namely, short-chain-length PHAs (scl-PHA), with monomers of 3-5 carbon atoms, medium-chain-length PHAs (mcl-PHA), with monomers of 6-14 carbon atoms, or long-chain-length PHAs (lcl-PHA), with monomers of more than 14 carbon atoms (Ashby et al., 2008). These different types of PHA polymers have distinct properties. For example, most scl-PHAs are very rigid and brittle bio-thermoplastics, due to their high crystallinity, while mcl-PHAs are more elastic and viscous materials, characterized by low crystallinity degrees and melting temperatures (Laycock et al., 2014; Rai et al., 2011). However, they all have some characteristics in common: biodegradability, low permeation to oxygen, water and oil, and most of them are likely to be biocompatible (Jacquel et al., 2007). These properties make them suitable to a wide range of applications in different industries: like plastic industry (production of bioplastics), fine chemicals industry, medicine (e.g. implants) and fuel industry (production of biofuel) (Chen, 2009).

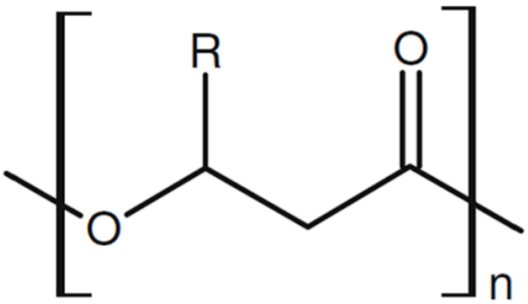


Figure 1.1 - General chemical structure of poly([R]-hydroxyalkanoate) (PHA), where R is the side chain of each monomer (it might contain functional groups) and n is the number of monomers.

1.2. Medium-Chain-Length Poly(Hidroxyalkanoate)

1.2.1. Fermentative production

The production of mcl-PHA has been already studied in various bacterial species (Hoffmann and Rehm, 2004; Lee, 1995, 1996).

Table 1.1 - Production of PHA by some bacteria.

Microorganism	Carbon Source	Type of PHA	PHA content (%CDM)	PHA productivity (g L^{-1} h^{-1})	References
Pseudomonas oleovorans NRRL B-14682	Olive oil distillate	HB	19	0.02	Cruz et al., 2016
Pseudomonas oleovorans	n-octane	3HHx-co-3HO	33	0.32	Lee et al., 1996
Pseudomonas resinovorans NRRL B-2649	Olive oil distillate	HHx-HO-HD-HDd	31	0.05	Cruz et al., 2016
Cupriavidus necator DSM 428	Olive oil distillate	HB	62	0.12	Cruz et al., 2016
Pseudomonas citronellolis NRRL B-2504	Olive oil distillate	HHx-HO-HD-HDd	10	0.01	Cruz et al., 2016
Pseudomonas putida KT2440	Glucose	mcl-PHA	32	0.006	Tan et al., 2014
Pseudomonas aeruginosa PAO1	Oil and wax product from polyethylene pyrolysis	mcl-PHA	25	-	Tan et al., 2014
Pseudomonas chlororaphis DSM 50083	Waste glycerol	HHx-HP-HO-HN-HD-HDd	29	0.14	Muhr et al., 2013

For mcl-PHA production, batch, fed-batch and continuous fed-batch fermentations have been studied. In fed-batch cultivations, the production of mcl-PHA is usually a two-step process, in which a high biomass concentration is first achieved, followed by PHA accumulation (Kim et al., 1997; Khanna et al., 2004). Batch fermentations, based on a single-step process, are quick and easy to operate. However, the production of PHA cannot be fully improved. On the other side, fed-batch fermentations are slow and sometimes difficult to operate, but high PHA concentrations can be achieved. Continuous fermentation has been studied for the production of mcl-PHA (Hartmann et

al., 2006; Prieto et al., 1999), but it has been shown that the PHA content decreased with increased growth. Since PHA is formed inside bacterial cells as a carbon and energy storage compound, the PHA content inside the cells and the carbon source concentrations during the fermentation process needs to be very well controlled in a continuous process, in order to avoid the accumulation of carbon source and polymer degradation by bacteria (Sun et al., 2007). Repeated batch fermentations joins the advantages of the batch and fed-batch processes by being a quick and easy way to reach a high concentration of PHA. The fermentation process is performed like a batch assay until a threshold point is achieved. Once that value is reached, the fermentation broth is removed and the bioreactor is replenished with fresh medium. The residual broth volume serves as inoculum for the following cycle. Usually, with the increasing number of cycles, the higher the PHA concentration (Bauer et al., 2005).

Until now, in mcl-PHA fermentation processes many different substrates were tested, like alkanes, alkanoic acids, glucose, fructose and glycerol (Tan et al., 2014; Sun et al., 2007; Lee, 1996). Alkanes and aliphatic acids (e.g. octanoic acid) are efficient carbon sources for mcl-PHA synthesis. However, some of those substrates are poorly water miscible and some other are even toxic to bacteria at relatively low concentrations (Sun et al., 2007). In order to use them as substrates for bacterial cultivation, their concentration needs to be well controlled. Sugars, like glucose and fructose, are effective carbon sources for use in mcl-PHA fermentation processes but their high price represents the majority of the expenses for the entire PHA production process (Cruz et al., 2016). Therefore, the use of wastes or by-products as carbon sources, such as waste glycerol generated by the biodiesel industry, is important to reduce substantially the production costs of PHA (Muhr et al., 2013).

For regulatory agencies to certify polymers for medical applications, very demanding specifications have to be fulfilled. The use of a waste as a carbon source in the fermentation process might be a big concern that has to be taken in account. If low grade wastes are used as substrates, more intensive downstream procedures have to be implemented to guaranty that high purity biopolymers are obtained. So, the choice of the most appropriate substrate must take into account the purity degree required for the biopolymer's final application. A balance must be made considering the cost of the substrate and that of the downstream.

1.2.2. Glycerol waste used as carbon source

As described above, the costs associated with fermentation feedstocks reflect the majority of the expenses of the total PHA production costs. Many low cost by-products or wastes (i.e., glycerol from biodiesel production) have been studied as fermentation substrates (Solaiman et al., 2016). With the increasing production of biodiesel and, consequently, glycerol, as a by-product, it is important to find new applications for crude glycerol (Yang et al., 2012). One potential use of crude glycerol is in feedstocks for fermentation processes. Glycerol has a greater degree of reduction than sugars, offering a unique opportunity to obtain reduced chemicals like succinate, ethanol, xylitol and

propionate (Silva et al., 2009). Some studies even revealed the use of glycerol from biodiesel production for polyhydroxybutirate (PHB) and mcl-PHA production, using *P. oleovorans* and *P. corrugata* strains (Ashby et al., 2005).

1.2.3. PHA Recovery Methods

Recovery processes of PHA from the bacterial biomass can be divided into two major categories: solvent extraction and chemical digestion of non-PHA biomass. For both processes, bacterial cells are first collected by centrifugation or filtration, being the cell-free supernatant usually discarded.

Solvent extraction consists in the selective extraction of PHA from the biomass with an organic solvent, like chloroform, methyl ether, methylene chloride (Rameshwari et al., 2014). This extraction process usually involves three steps, which are: biomass pre-treatment, solvent extraction, and polymer purification. Pre-treating dry biomass with methanol can be effective to remove some of the lipids and colouring impurities, increasing the purity of the PHA after purification (Jiang et al., 2006; Koller et al., 2013). PHA is extracted from the dried biomass with an organic solvent under stirring and, in some cases, heating or even using a Soxhlet extraction. The resulting solution is filtered or centrifuged to remove remaining cell debris. Afterwards, PHA is either precipitated with a non-solvent, or obtained by evaporation of the solvent. Usually, the precipitation and washing steps are repeated to achieve higher purity. For the extraction of PHB, the number of solvents that can be used is limited and in the majority of the cases they are toxic and dangerous, like chloroform, methylene chloride and 1.2-dichloroethane (Kunasundari et al., 2011; Fiorese et al., 2009). Therefore, other solvents, like propylene carbonate, have been studied in order to use less toxic solvents (Fiorese et al., 2009). On the other hand, for mcl-PHA extraction there is a larger number of solvents that can be used for the extraction procedure since those polymers are soluble in less hazardous solvents, like acetone, hexane, ethyl acetate (Rai et al., 2010; Jiang et al. 2006; Wampler et al 2010). For applications in the medical and pharmaceutical fields it is important to use less toxic solvents in the extraction process, since the presence of these kind impurities are not tolerated by regulatory agencies (Williams et al., 1999).

In the methods based on the chemical digestion of non-PHA biomass, the biomass typically receives a heat treatment, an enzymatic solubilisation and/or a surfactant washing, followed by a filtration or centrifugation step, being then the PHA separated from the cell debris. These steps need to be repeated several times to achieve an acceptable purity (Jiang et al., 2006; Koller et al., 2013).

Some methods can be used for disruption of the cell membrane, including the use of surfactants, the dissolution of non-PHA cell mass by acids, the use of bead mills or ultrasonication for the mechanical disruption of the cells, treatment with hypochlorite and even bursting the cells with osmotic pressure (Kunasundari et al., 2011; Rameshwari et al., 2014; Madkour et al., 2013; Koller et al., 2013).

1.2.4. Purification of PHA

The presence of considerable amounts of impurities, in the final polymer, like proteins, surfactants and endotoxins, are not tolerated by regulatory agencies for applications in the medical and pharmaceutical fields. For PHA derived from fermentation of Gram-negative bacteria, contamination with endotoxins is a serious problem (Williams et al., 1999).

For applications in the medical field, PHA of high purity is needed. This is one of the most important steps in the downstream processing. Biologically active contaminants, like proteins and lipopolysaccharides (LPS), have to be removed because they can produce some immunoreactions. LPS act as endotoxins, whereby trace quantities can have severe effects in contact with blood and trigger immunoreactions (Koller et al., 2013; Tan et al., 2014). LPS are part of the outer membrane of some bacteria. During cell lysis and product recovery, LPS are liberated from the outer membrane and can contaminate the PHA. Regulatory agencies accept only a very low content of proteins, surfactants and endotoxins. Therefore, PHA for use in the medical industry has to be carefully purified from such endotoxins (Koller et al., 2013; Tan et al., 2014; Kunasundari et al., 2011).

The standard techniques used for the purification of PHA are re-dissolution, precipitation and washing with a non-solvent, usually methanol or ethanol. However, different non-solvents, like water and ether, have been used for the precipitation and washing steps (Tan et al., 2014; Kunasundari et al., 2011). Other techniques for purification of PHA include, for example, chromatography, treatments with chemical agents and filtration (Tan et al., 2014). For mcl-PHA other, less hazardous solvents can be used in the purification process.

1.3. Material properties

The fermentation process, recovery methods and purification steps may affect the size of PHA monomers in the polymer (Keshavarz et al., 2010; Koller et al., 2013), thereby affecting the physical properties of the polymer, such as the melting point, glass transition temperature and crystallinity, affecting the final application of the polymer (Rai et al., 2011).

Interestingly, the monomers' side-chain and the distance between the ester linkages in the backbone are strongly related with the material properties of the mcl-PHA. In general, mcl-PHA are water insoluble, biodegradable, biocompatible, have low crystallinity and melting points, are very elastomeric and have a rubber-like or sometimes glue-like behaviour (Rai et al., 2011; Cruz et al., 2016). However, their properties largely depend on the functional groups of the side-chains. These functional groups can be adapted through chemical modification to improve the physical properties to different applications (Lu et al., 2009). Thereby, the field of possible applications is considerably extended. The polymer's polarity and solubility in polar solvents could be drastically changed through the insertion of hydroxyl groups in the side-chains of mcl-PHA (Eroglu et al., 2005; Lee et al., 2000; Renard et al., 2005).

1.4. Poly(Hidroxyalkanoates) in Biomedicine

To be used in biomedical industry, highly pure mcl-PHA, with a low contents of endotoxins are needed. With their physical properties, mcl-PHA can be used in many different biomedical areas, like wound management (e.g. as skin substitutes, wound dressing, sutures, adhesion barriers), vascular system (e.g. as cardiovascular patches), orthopaedic (e.g. orthopaedic pins, articular cartilage repair devices), dental (e.g. as a barrier material for guided tissue regeneration in periodontitis) and drug delivery (e.g. as a drug carrier) (Chen et al., 2005; Keshavarz et al., 2010).

The glue-like behaviour of some mcl-PHA makes them suitable candidates as alternative materials for the development of new bio-based adhesives to replace the commercial petrochemical-derived adhesives and glues used in biomedicine (Cruz et al., 2016; Solaiman et al., 2001). They can be used in wound management, as sutures and adhesives to wound closure, and drug delivery systems, like transdermal patches (Chen, 2009; Cruz et al., 2016; Keshavarz et al., 2010). Solvent-based adhesives using epoxies, urethane and cyanoacrylate are used in many areas for medical application due to their good adhesion capacity to different materials, low price and fast curing times (Cruz et al., 2016). However, most of them are non-biodegradable and their use might cause an allergic reaction when in contact with biological tissues. In addition, their production might pose environmental and public health concerns (Kim et al., 2013; Kaur, 2011). PHAs, on the other hand, can easily overcome these problems due to their biodegradable and biocompatible properties, making possible the production of a fully natural bio-adhesive that could be used for medical applications.

2. Motivation

Mcl-PHA, with a wide range of physical properties, are accessible through biosynthesis by bacteria. It was found that some bacteria can even accumulate mcl-PHA with high intracellular concentration during a fermentation process. Several of those bacteria belongs to the *Pseudomonas* Genus (Guo-Qiang Chen, 2009; Alexander Muhr et al., 2013). In this work, a group of known mcl-PHA-accumulating bacteria, namely, *Pseudomonas putida*, *P. resinovorans, P. chlororaphis, P. citronellolis* and *P. oleovorans*, was studied for the accumulation of mcl-PHA using a glycerol waste as the sole carbon source.

Pseudomonas chlororaphis was found to be the most suitable candidate to produce mcl-PHA. This strain is still understudied and there are only a few reports in literature on its ability to grow on glycerol. Therefore, this strain was studied in bioreactor cultivation experiments, in three different cultivation modes, namely, batch, pulse feeding and repeated batch. The polymer was recovered from the bacterial cells by Soxhlet extraction with chloroform. For the purification process, a standard technique with the re-dissolution of the mcl-PHA in chloroform followed by precipitation in ice cold ethanol was used.

Chloroform was used as the standard solvent for extraction of the polymers. However, this solvent is hazardous and cannot be used for industrial production. Hence, alternative recovery methods were also studied, using less hazardous solvents, like acetone (Jiang et al., 2006), hexane (Rai et al., 2010), ethyl acetate (Wampler et al., 2010), propylene carbonate (Fiorese et al., 2009), hypochlorite (Tan et al., 2014), sodium hydroxide, hydrogen peroxide (Madkour et al., 2013) and different water-based extractions using osmotic shock for disrupting the bacterial cell walls (Koller et al 2013). For medical use a good downstream processing of the polymer is needed to achieve mcl-PHAs without contaminations. With improved recovery and purification methods, it is possible to obtain a polymer with relatively low concentration in bacterial cell compounds.

The physical properties of mcl-PHAs makes them suitable candidates for several medical applications. Due to their inherent biocompatibility, biodegradability, rubber and glue-like behaviour, mcl-PHAs are being more considered as potential candidates to act like sustainable basic polymers for several applications (i.e. rubbers, smart latexes, basic materials for post-synthetic functionalization by chemical or enzymatic means, thermos-sensitive, glues and adhesives) (Chen, 2010; Zinn, 2010). Solvent-based adhesives like epoxies, urethane and cyanoacrylate are used in many areas for medical application due to their good adhesion capacity to different materials, low price and fast curing times (Cruz et al., 2016). However, most of them are non-biodegradable and their use might cause an allergic reaction when in contact with biological tissues. In addition, their production might pose environmental and public health concerns (Kim et al., 2013; Kaur, 2011). In this study, it was taken the advantage of the unique properties of mcl-PHA to be used for developing a new fully natural bio-based adhesive to be used in medical applications. This properties were assessed, using porcine skin as a model substrate.

3. Materials and Methods

3.1. Glycerol waste characterization

The glycerol waste from biodiesel (SGC Energia, SGPS, SA, Portugal) production was analysed by HPLC for glycerol quantification. Glycerol concentration was determined by high-performance liquid chromatography (HPLC) with a VARIAN Metacarb column (BioRad) coupled to an infrared (IR) detector. The analyses were performed at 50˚C, with sulphuric acid (H_2SO_4 0.01 N) as eluent at a flow rate of 0.6 mL/min. Glycerol (ReagentPlus 86-88% w/w Scharlau) standards were prepared at a concentration of 1 g/L successively diluting them to the concentrations of 0.5 g/L, 0.1 g/L, 0.05 g/L and 0.01 g/L (calibration curve in Appendix 1).

3.2. Microbial cultivation experiments

3.2.1. Screening Assay

The bacterial cultures used in this assay were *Pseudomonas oleovorans* strains NRRL B-14682 and NRRL B-14683*, Pseudomonas resinovorans* NRRL B-2649*, Pseudomonas citronellolis* NRRL B-2504*, Cupriavidus necator* DSM 428*, Pseudomonas chlororaphis* DSM 19603 and *Pseudomonas putida* KT2440. The cultures were reactivated by inoculation in Chromagar (CHROMagar™) plates (solid medium) with a sample of the cryopreserved bacteria and incubated at 30˚C for 48 h. Afterwards, isolated colonies were inoculated into 50 mL liquid Luria Bertani (LB) medium (bacto tryptone, 10.0 g/L; yeast extract, 5.0 g/L; NaCl, 10.0 g/L), pH 7.0, and incubated in an orbital shaker at 200 rpm and 30˚C, for 24 h. This culture served as pre-inocula for shake flask and bioreactor experiments.

3.2.2. Shake flask Assays

The pre-inocula (20 mL) of the cultures were used as inoculum for the 200 mL shake flask cultivations in medium E*. The medium contained the following (per liter): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g. 10 mL of a 100 mM $MgSO_4$ solution and 1 mL of a microelement solution were added. The microelement solution contained the following (per liter of 1 N HCl): $FeSO_4$- $7H_2O$, 2.78 g; MnCl $4H_2O$, 1.98 g; $CoSO_4$ $7H_2O$, 2.81 g; $CaCl_2$ $2H_2O$, 1.67 g; $CuCl_2$ $2H_2O$, 0.17 g; $ZnSO_4$ $7H_2O$, 0.29 g (Brandl et al., 1988). It was supplemented with glycerol waste as the sole carbon source at a concentration of 40 g/L. The glycerol waste was autoclaved separately for 20 minutes (at 121˚C and 1 bar) and then added to medium E*. In all shake flask experiments, the cultures were incubated in an orbital shaker at 200 rpm and 30˚C, for 72 h.

3.2.3. Bioreactors Assays

3.2.3.1. Inoculum

The bacterial culture used in these assays was *P. chlororaphis* DSM 19603. The pre-inoculum (20 mL) was transferred to a 500 mL shake flask with 200 mL medium E* supplemented with glycerol waste (concentration of 40 g/L) and incubated under the same conditions for 72 h.

3.2.3.2. Batch Fermentation

Batch cultivations were performed in a bioreactor (BioStat®B-Plus, Sartorius) with a working volume of 2 L. Medium E* with glycerol waste at a concentration of 40 g/L was used. The temperature and the pH were kept at 30 ± 0.1˚C and 7.0 ± 0.1, respectively. The pH was controlled by the automatic addition of 2 M NaOH and 2 M HCl solutions. A constant aeration rate (2 SLPM, standard liters per minute) was kept during all experiments. The dissolved oxygen concentration (DO) was controlled at 30% of air saturation by automatically adjusting the stirring speed between 300 and 800 rpm. Foam formation was automatically suppressed by addition of Antifoam A (Sigma/VWR). Samples were periodically taken from the bioreactor for quantification of cell dry weight, PHA, ammonia and glycerol waste. For quantification of the cell dry weight (CDW), cultivation broth samples (15 mL) were centrifuged, the supernatant was recovered for glycerol and ammonia quantification and the pellet was lyophilized for biomass and mcl-PHA quantification.

3.2.3.3. Pulse Feeding Fermentation

Pulse feeding cultivation were performed in a bioreactor (BioStat®B-Plus, Sartorius) with a working volume 10 L., using medium E* supplemented with glycerol waste as the sole carbon source at a concentration of 40 g/L. After 21 h of cultivation, a pulse of carbon source was fed to give a concentration of 40 g/L. In this experiment, the temperature and the pH were kept at 30 ± 0.1˚C and 7.0 ± 0.1, respectively. pH was controlled by the automatic addition of 2 M HCl and NH_4OH (25%, v/v) solutions. The NH_4OH solution also served as a nitrogen source. A constant aeration rate (2 SLPM, standard liters per minute) was kept during the experiment. The dissolved oxygen concentration (DO) was controlled at 30% of air saturation by automatically adjusting the stirring speed between 300 and 800 rpm. Foam formation was automatically suppressed by addition of Antifoam A (VWR). Samples were periodically taken from the bioreactor for quantification of cell dry weight, PHA, ammonia and glycerol waste. For quantification of the cell dry weight (CDW), cultivation broth samples (20 mL) were centrifuged, the supernatant was recovered for glycerol and ammonia quantification and the pellet was lyophilized for biomass and mcl-PHA quantification.

3.2.3.4. Repeated Batch Fermentation

Repeated batch cultivation experiments were performed in bioreactors (BioStat®B-Plus, Sartorius) with a working volume 2 L of E medium. In each cycle, glycerol waste was used as the only carbon source with an initial concentration of 40 g/L. The temperature and the pH were kept at 30 ± 0.1˚C and 7.0 ± 0.1, respectively. pH was controlled by the automatic addition of 5 M NaOH and 2 M HCl solutions. A constant aeration rate (2 SLPM, standard liters per minute) was kept during the experiment. The dissolved oxygen concentration (DO) was controlled at 30% of air saturation by automatically adjusting the stirring speed between 300 and 800 rpm. Foam formation was automatically suppressed by addition of Antifoam A (VWR). Samples were periodically taken from the bioreactor for quantification of cell dry weight, PHA, ammonia and glycerol waste. For quantification of the cell dry weight (CDW), cultivation broth samples (15 mL) were centrifuged, the supernatant was recovered for glycerol and ammonia quantification and the pellet was lyophilized for biomass and mcl-PHA quantification. The repeated batch cycles were implemented by withdrawing 1.6 L, under aseptic conditions, of cultivation broth at the end of each cycle, and supplying fresh medium E*. The broth volume (0.3 L) remaining in the bioreactor at the end of each cycle served as the inoculum for the following cycle.

3.3. Analytical Techniques

3.3.1. Determination of Cell Growth

The cell growth was determined by quantification of the cell dry weight (CDW) of each sample. The cell pellet was used for the gravimetric determination of the CDW, after washing once with deionized water (resuspension in water, and centrifugation at 8000 rpm, for 15 to 20 minutes at 10˚C) the pellet was freeze-dried (ScanVac CoolSafe™, LaboGene) at -110˚C for 48 h.

3.3.2. Glycerol Quantification

The cell-free supernatant was diluted (1:50) in sulphuric acid (SIGMA-ALDRICH) (H_2SO_4 0.01 N) and filtered with Vectra Spin Micro Polysulfone filters (0.2 µm) (Whatman), at 3000 rpm for 10 minutes. Glycerol was quantified by HPLC, as described above.

3.3.3. Ammonium Quantification

Ammonium concentration was determined by colorimetry, as implemented in a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands). Ammonium chloride (Sigma) was used as standard at concentrations between of 5 and 20 mg L^{-1}.

The cell-free supernatant was diluted (1:200) in deionized water and analysed.

3.3.4. Mcl-PHA Concentration and Composition

Mcl-PHA content and composition were determined after hydrolysis of dried cell samples (5 to 10 mg) in 2 mL 20% (v/v) sulphuric acid (SIGMA-ALDRICH) in methanol (Fisher Chemical) and 2 mL of benzoic acid in chloroform (1 g/L) (SIGMA-ALDRICH), on oil bath at 100˚C, for 4 h. Then, 1 mL of water was added and the organic phase was recovered and analysed by GC (430-GC, Bruker) with a Restek column of 60m, 0.53 mmID, 1 µM df, Crossbond, Stabilwax. The injection volume was 2.0 µL, with a running time of 32 min, a constant pressure of 14.50 psi and helium as carrier gas. The heating ramp was 0 to 3 min a rate of 20˚C/min until 100˚C, 3 to 21 min a rate of 3˚C/min until 155˚C and 21 to 32 min a rate of 20˚C/min until 220˚C. Mcl-PHA (VersaMer™ PHAs, PolyFerm Canada) standards were prepared at 2 g/L and then diluted to give concentrations in the range 0.1 and 1.75 g/L.

3.3.5. Production Calculus

The yields of biomass on substrate (Yx/s, g.g^{-1}) and mcl-PHA on substrate (Yp/s, g.g-1) were determined using the following equations:

$$Yx/s = \frac{\mathrm{xf}-\mathrm{xi}}{Sf-Si} \quad \text{Eq. 3.1}$$

$$Yp/s = \frac{pf-pi}{sf-si} \quad \text{Eq. 3.2}$$

where xf and xi are the final and initial biomass concentration, Sf and Si are the final and initial substrate, pf and si are the final and initial mcl-PHA produced, respectively.

The mcl-PHA volumetric productivity (rP, g L^{-1}h^{-1}) was determined by the following equation:

$$rP = \frac{dP}{dt} \quad \text{Eq. 3.3}$$

where P is the mcl-PHA (g L-1) produced at time t (hours).

3.4. Polymer extraction and characterization

After centrifugation (8000 rpm for 15 minutes at 4˚C) of the cultivation broth recovered from the bioreactor experiments, the pellets were lyophilized and subjected to a Soxhlet (250 mL) extraction with chloroform (CARLO ERBA Reagents S.A.S.) at 80˚C for 24 - 48 h. The cellular debris were removed by filtration with syringe filters with a pore size of 0.45 µm (GxF, GHPmembrane, PALL) and the mcl-PHA was precipitated in ice-cold ethanol (CARLO ERBA Reagents S.A.S.) (chloroform/ethanol 1:10). The precipitate was then recovered in a pre-weighted flask and left at room temperature, in a fume hood, for solvent evaporation. The polymer's composition was determined by GC, as described above.

3.4.1. Polymer extraction method tests

Different extraction methods were tested, using either solvents or osmotic shock treatments.

3.4.1.1. Solvent Testing

In the solvent extraction tests, 1 g of biomass was mixed with 30 mL of each solvent: chloroform (CARLO ERBA Reagents S.A.S.), acetone (LABCHEM), hexane (JOSÉ MANUEL GOMES DOS SANTOS, LDA), ethyl acetate (CARLO ERBA Reagents S.A.S.), propylene carbonate (Fluka), hypochlorite (Auchan), sodium hydroxide (1 M) (eka) and hydrogen peroxide (Auchan). The samples were placed in an orbital shaker, at 200 rpm and 30˚C, overnight. The samples were centrifuged and the pellets or supernatants were collected depending on the solvent used. For chloroform, acetone, hexane, ethyl acetate and propylene carbonate solvents, the supernatant containing the solubilized polymer was recovered, filtered, precipitated in ice cold ethanol (solvent/ethanol 1:10) and dried at room temperature. For hypochlorite, sodium hydroxide and hydrogen peroxide, the pellets containing the polymer were separated from the aqueous soluble cell components, and sequentially washed with deionized water (30 mL) and ethanol (30 mL) and then freeze dried. The polymer's composition and purity were determined by GC, as described above.

3.4.1.2. Osmotic Shock Testing

In these experiments, 3 different conditions of osmotic shocks were tested: hyper-osmotic shock with saturated solutions of sodium chloride or sucrose, and hypo-osmotic shock with deionized water. Another kind of hypo-osmotic shock treatment was also tested, by freezing the samples at -80˚C and thawing them after 24h. For each test, a 0.5 g biomass sample was mixed with 20 mL of the appropriate solution. The samples were then placed at an orbital shaker, at 200 rpm and 30˚C, for 1h. Then, the samples were centrifuged (8000 rpm, for 10 minutes at 10˚C), the pellets were ressuspended in deionized water and placed, once again, in the shaker under the same conditions. The samples were observed under the optical microscope prior and after each treatment to check for cell disruption.

3.4.2. Mcl-PHA films

3.4.2.1. Film preparation by Solvent Evaporation

Mcl-PHA films were prepared by dissolving 0.85 g of polymer in 20 mL of chloroform (CARLO ERBA Reagents S.A.S.) and transferring the solution to a glass petri dish that was placed in the fume hood for evaporation of the solvent.

3.4.2.2. Mechanical properties

The mcl-PHA film was cut into rectangular-shaped pieces with 3 samples of 3 x 1.5 cm^2 and one sample of 2.5 x 1.5 cm^2. The film samples were firmly attached to metal clamps of the testing holders. The elongation was measured in axial tension test at room temperature using a texturometer (TA.XT.plus Texture analyser, Stable Micro Systems) and a 5 Kg load cell. The film strained at a constant velocity of 0.5 mm/s until break.

The section area (A, m^2) was determined by using the following equation:

$$A = \delta * L \qquad \text{Eq. 3.4}$$

Where δ is the thickness (m) and L is the wideness (m) of the mcl-PHA.

The tension applied to the mcl-PHA biofilm (Ƭ, Pa) was determined by using the following equation:

$$Ƭ = \frac{F}{A} \qquad \text{Eq. 3.5}$$

Where F is the force applied to the mcl-PHA (N) and A is the section area (m^2).

The deformation (Ɛ, -) was determined by using the following equation:

$$Ɛ = \frac{(l - li)}{li} \qquad \text{Eq. 3.6}$$

where l is the actual length (mm) and li is the initial length (mm)

The stiffness of the mcl-PHA was represented by the young modulus. It was determined by measuring the slope of a line tangent to the initial tension-deformation curve, in the linear part.

3.5. Adhesive Tests

3.5.1. Tension Tests

Porcine skin (purchased at a local butcher) was used as a soft tissue model. The porcine skin was cut into 2.5 x 2.5 cm^2 square-shaped pieces and their dermis side was firmly attached to metal testing holders (figure 3.1).

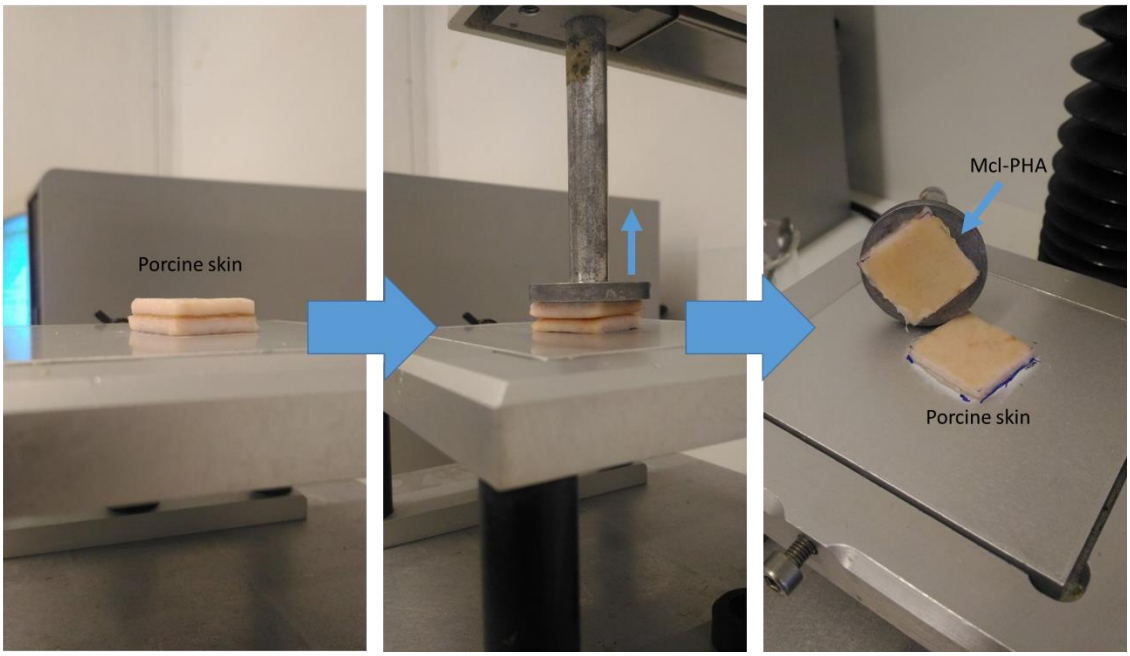


Figure 3.1 - Schematic assembly for the tension tests.

The mcl-PHA was melted at 45˚C and spread uniformly (100 – 190 mg) on the epidermis side of one porcine skin piece and immediately pressed against the epidermis side of other porcine skin piece by applying a 2.58 N load for 30 seconds, at room temperature. After 20 min, the bonding strength was measured in tension mode at room temperature using a texturometer (TA.XT.plus Texture analyser, Stable Micro Systems) and a 5 Kg load cell. The two parts of the joint were strained at a constant velocity of 0.5 m/s until separation was achieved.

3.5.2. Shear Tests

Porcine skin (purchased at a local butcher) was used as a soft tissue model. The porcine skin was cut into 6 x 2.5 cm^2 rectangular-shaped pieces. Mcl-PHA melted at 45˚C (210 – 280 mg) were spread uniformly, in the contact area (2.5 x 2.5 cm^2), on the epidermis side of one porcine skin piece which were immediately attached to the epidermis side of other porcine skin piece by applying a 2.56 N load on the pieces for 30 seconds, at room temperature. After 20 min, the skin was then firmly attached to metal clamps of the testing holders and the bonding strength was measured in shear mode at room temperature using a texturometer (TA.XT.plus Texture analyser, Stable Micro

Systems) and a 30 Kg load cell. The two parts of the joint were strained at a constant velocity of 0.5 mm/s until separation was achieved (figure 3.2).

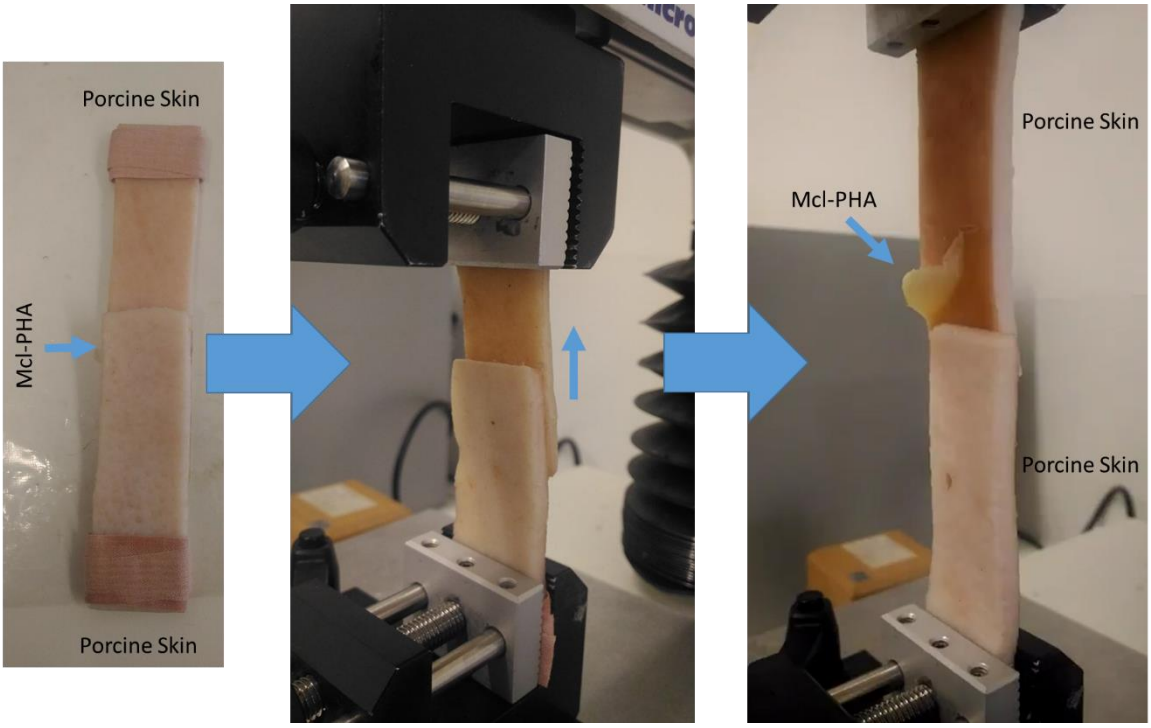


Figure 3.2 - Schematic assembly for the shear tests

3.5.3. Adhesive Tests Calculus

The bonding strength was calculated on the all samples and was defined as the maximum force felted at separation divided by the bonding area (Eq. 3.5).

3.5.4. *In vivo* peeling tests

In this experiment a human arm was used as soft tissue model. The mcl-PHA was melted at 45˚C and spread uniformly through a rectangular-shaped plastic sheet (2 x 6 cm^2), and left at room temperature to dry. After the polymer dried, the mcl-PHA was separated from the plastic sheet and applied to the skin (figure 3.3).

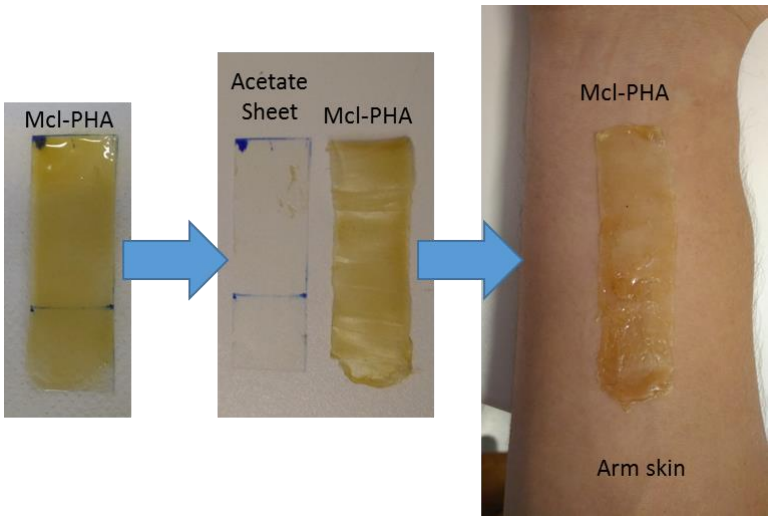


Figure 3.3 – Schematic assembly for the peeling test

After the polymer adhere to the skin, it was made a peeling assay. The mcl-PHA was separated from the skin by, simply, pulling the polymer until separation was achieved as showed in figure 3.4,

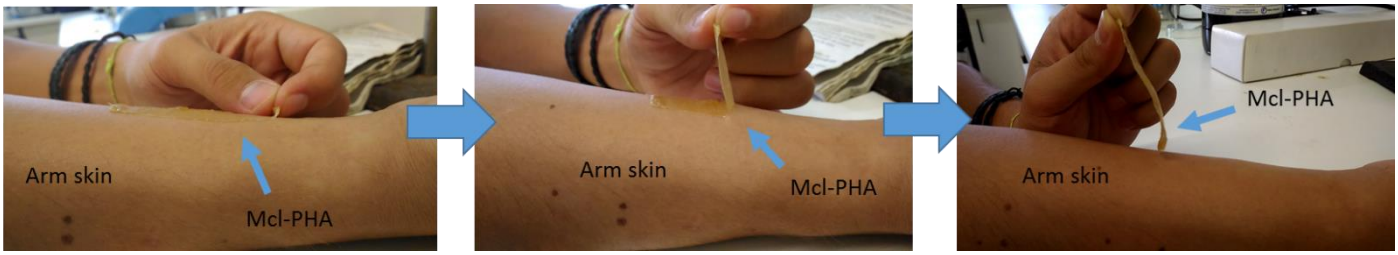


Figure 3.4 - Peeling test assay

4. Results and Discussion

4.1. Screening Assay

The screening assay was performed to see which bacteria would grow better by using only glycerol waste (a biodiesel by-product) as a carbon source. The ability of each strain to accumulate PHA, especially mcl-PHA, was also evaluated. With the increasing biodiesel production all over the world, it is important to find new applications for its cheap wastes or by-products. One of the possible applications is to use these by-products as substrates for mcl-PHA production, reducing drastically the production costs of those polymers. In this assay, group of well-known PHA accumulating bacteria, namely *Pseudomonas putida*, *P. resinovorans, P. chlororaphis, P. citronellolis, P. oleovorans* and *C. necator*, were studied for the ability of mcl-PHA accumulation, using glycerol waste as the sole carbon source. *C. necator* is a well-known producer of PHB, *P. putida* is a recognizable producer of mcl-PHA (Tan et al., 2014) and *P. resinovorans, P. citronellolis and P. oleovorans* have also been described to synthesize mcl-PHA from wastes including biodiesel co-products. (Morais et al., 2014). *P. chlororaphis,* on the other hand, a little is known about this strain and its ability to produce mcl-PHA in glycerol, until now it has been described to synthesize mcl-PHA from wastes (Muhr et al., 2013). These assays were carried out using shake flasks with 200 mL of Medium E* supplemented with glycerol waste as the sole carbon source (40 g/L). All assays took 72 h.

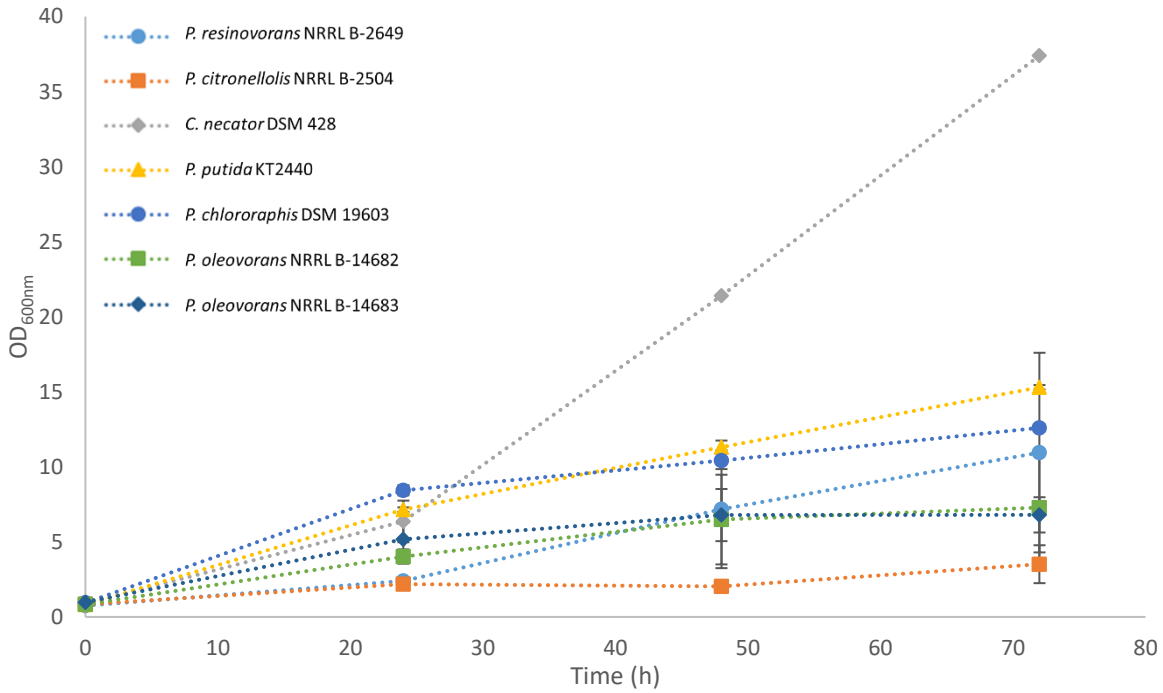


Figure 4.1 - Growth profile of the different strains of bacteria in raw glycerol (a biodiesel by-product).

By looking at the growth profile of the screening assay (Figure 4.1), it is possible to see that all cultures were able to grow on glycerol by-product, but the one with the highest growth was *C. necator* DSM 428 that reached an OD of 37.4 within 72 h of cultivation. Among the tested mcl-PHA producers, *P. putida* KT2440 *and P. chlororaphis* DSM 19603 had the highest cell growth, as shown by the OD attained (15.3 and 12.6, respectively). These results were confirmed by the gravimetric quantification of

the CDW (table 4.1), in which the highest value was obtained for *C. necator* (9.69 g/L) and for the mcl-PHA producers, *P. putida* (4.44 g/L) and *P. chlororaphis* (3.82 g/L).

To verify the production of PHA inside the bacterial cells, Nile blue staining was made for samples collected from the shake flasks at 24 and 72 hours after the inoculation. The bacteria were observed under the microscope in phase contrast and using fluorescence, in order to visualize the bacteria and the PHA inside the cells, respectively. By looking at figure 4.2, it is possible to see that in all strains the concentration of bacterial cells increased between 24 and 72 hours, which reflects the bacterial growth. Looking at the fluorescence images (Figure 4.2), it is also visible that PHA was produced and accumulated by all bacterial strains. In figure 4.2 it remains clear that the strains of bacteria with higher cell growth and PHA accumulation were *C. necator* DSM 428*, P. putida* KT2440 *and P. chlororaphis* DSM 19603.

Table 4.1 - Overall growth and PHA production, and composition of the polymer obtained in the screening assay, after 72 h batch shake flask cultivations on waste glycerol as the sole carbon source.

Bacteria Strain	Biomass (g/L)	Type of PHA	Composition
***P. resinovorans* NRRL B-2649**	1.68	mcl-PHA	HD-HDd
***P. citronellolis* NRRL B-2504**	1.21	mcl-PHA	HD-HDd
***C. necator* DSM 428**	9.69	PHB	HB
***P. putida* KT2440**	4.44	mcl-PHA	HHx-HO-HD-HDd
***P. oleovorans* NRRL B-14683**	2.01	PHB	HB
***P. oleovorans* NRRL B-14682**	1.60	PHB	HB
***P. chlororaphis* DSM 19603**	3.82	mcl-PHA	HHx-HO-HD-HDd

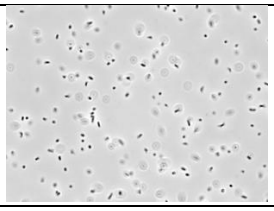
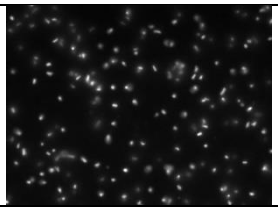
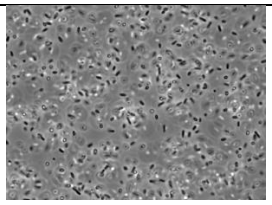
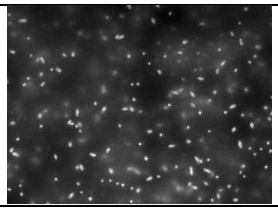
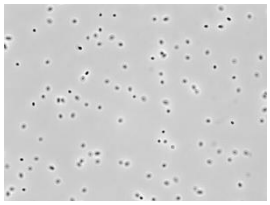
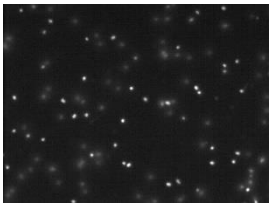
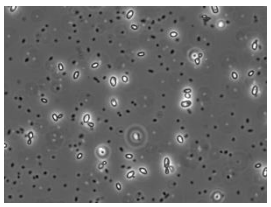
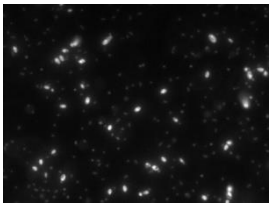
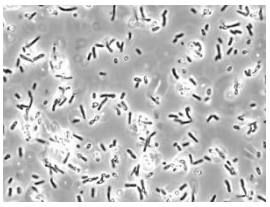
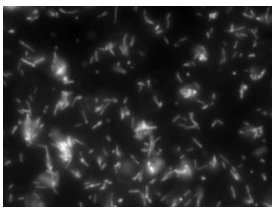
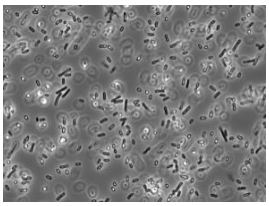
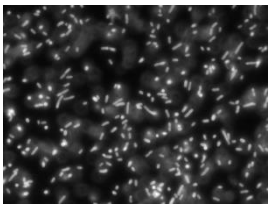
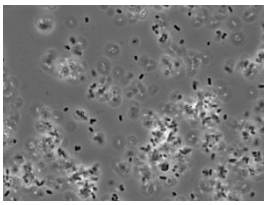
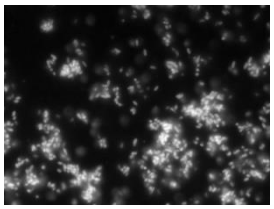
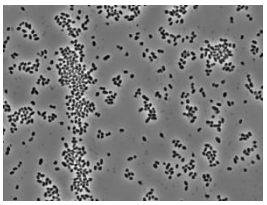
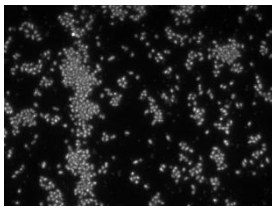
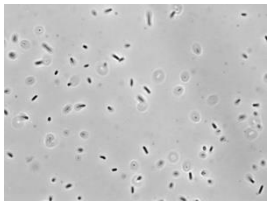
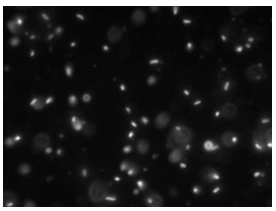
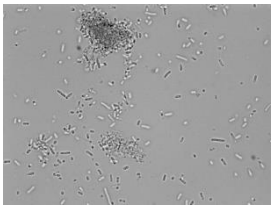
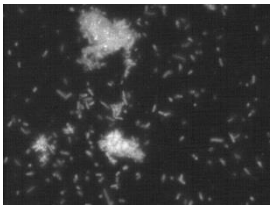
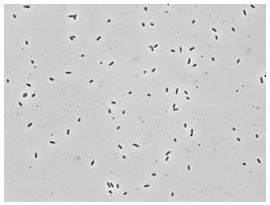
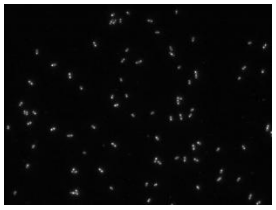
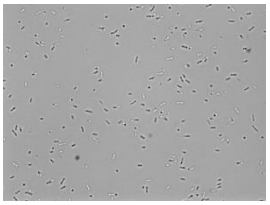
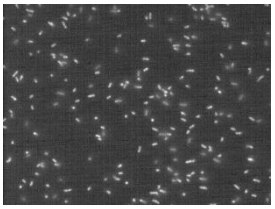
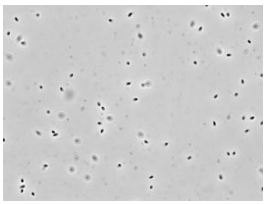
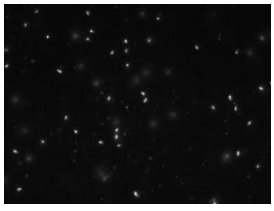
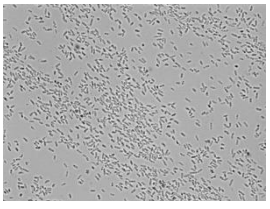
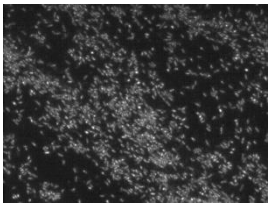
Bacteria Strain	24 h after inoculation		72 h after inoculation	
	Phase contrast	Flourescence	Phase contrast	Flourescence
P. resinovorans **NRRL B-2649**				
P. citronellolis **NRRL B-2504**				
C. necator **DSM 428**				
P. putida **KT2440**				
P. oleovorans **NRRL B-14683**				
P. oleovorans **NRRL B-14682**				
P. chlororaphis DSM 19603				

Figure 4.2 - Visualization of the bacterial cells under the optical microscope (100x) for samples collected from shake flasks screening assay, at 24 and 72 hours after inoculation, under phase contrast and with fluorescence after Nile blue staining.

C. necator is a well-known PHB-producer able to grow on glycerol (Tan et al., 2014, Mothes et al., 2007) and was used in this study as a control. So, as expected, it only accumulate the hydroxybutyrate homopolymer (table 4.1). Both tested *P. oleovorans* strains also produced PHB (table 4.1), which is in accordance with literature reports (Ashby et al., 2004, Yang et al., 2012). The other tested *Pseudomonas* strains synthesized mcl-PHA (table 4.1). The co-polymers produced by *P. resinovorans* and *P. citronellolis* were composed of HD and HDd monomers. On the other hand, *P. putida* KT2440 *and P. chlororaphis* DSM 19603 (table 4.1) produced polymers of HHx, HO, HD and HDd monomers. According with the literature for *P. resinovorans* and *P. citronellolis,* using oil substrates, it is possible to achieve mcl-PHA polymers with HHx, HO, HD and HDd monomers (Cruz et al., 2015), which are slightly different than the ones achieved in this assay. For *P. chlororaphis* it has only been showed that is possible to achieve a mcl-PHA polymer with a composition similar to the one attained in this experiment, by using different carbon sources (Muhr et al., 2013, Yun et al., 2003). It has also been reported that *P. putida,* in this substrate, produces mcl-PHA with the composition of HHx, HO, HD, HDd and HTd (Poblete-Castro et al., 2014), similar to the one attained in the screening assay. *P. putida* has been widely studied over the past few years, using different substrates for the production of mcl-PHA. However, yet much less is known about *P. chlororaphis.* So far, this strain has been studied for the production of mcl-PHA using substrates derived from animal waste (SFAE) and Palm Kernel Oil (PKO) as carbon sources (Muhr et al., 2013, Yun et al., 2003). So, in order to study and learn more about *P. chlororaphis* and its ability to produce mcl-PHA from glycerol, this strain was selected to proceed with the bioreactor assays for mcl-PHA production.

4.2. Mcl-PHA Production by *P. chlororaphis*

To characterize the production of mcl-PHA by *P. chlororaphis* under controlled bioreactor conditions, three experiments were performed, under different cultivation modes: batch, pulse feeding and repeated batch.

4.2.1. Batch Fermentation

A batch fermentation was performed in a 2 L bioreactor with Medium E* and glycerol waste as carbon source for 28 hours. After a short lag phase (2 h), *P. chlororaphis* entered a growth phase that lasted around 17 hours (figure 4.3), after which the concentration of ammonium has become very low (0.08 g/L), limiting the growth of the bacteria. A CDW of 4.62 g/L was attained at 19 h of cultivation, when growth limiting conditions occurred. Afterwards, the CDW still increased to 5.57 g/L, which was mainly due to the intracellular polymer accumulation. During this assay it is possible to see that as the cell dry weight (CDW) and mcl-PHA concentrations increased, while glycerol concentration decreased. This means that glycerol was consumed for bacterial growth and mcl-PHA accumulation. mcl-PHA production was only quantified starting at 19 h of cultivation. At that time, the culture had accumulated 0.28 g/L of polymer (figure 4.3). At the end of the run the polymer content in the biomass was 13%, giving a final polymer concentration of 0.72 g/L (table 4.2).

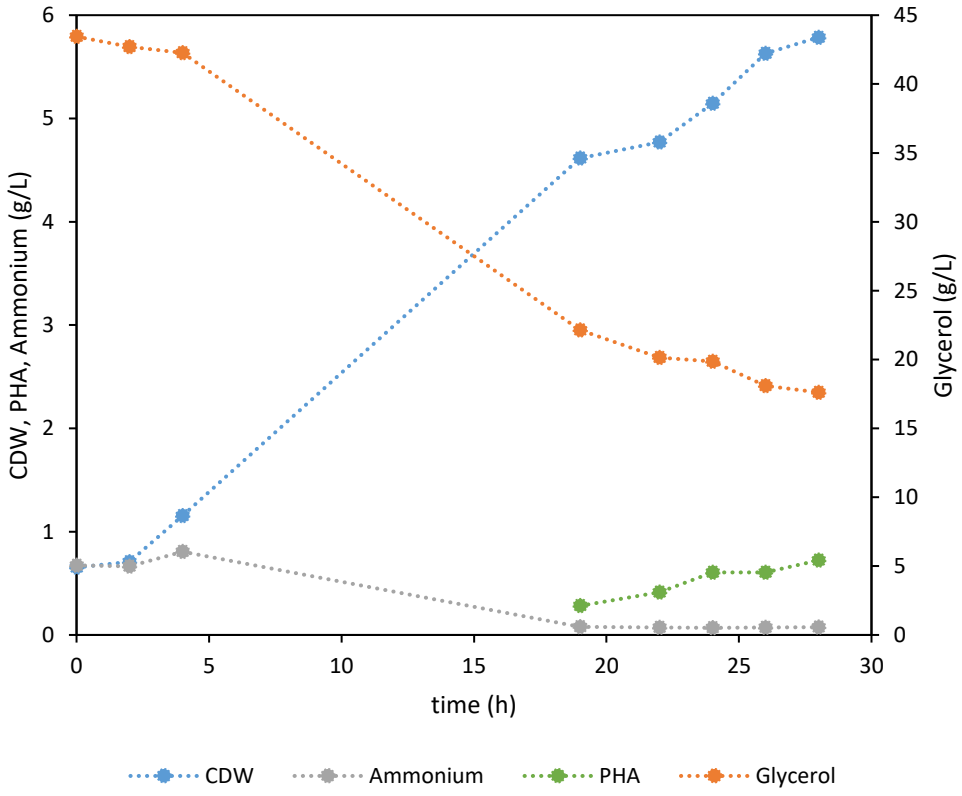


Figure 4.3 - Cultivation profile of the batch bioreactor fermentation of *P. chlororaphis* DSM 19603 using glycerol by-product as sole carbon source.

By looking at figure 4.3, it is possible to see that, in the first 19 hours the bacterial growth was responsible for the major consumption of glycerol (21.31 g/L). Starting at 19 hours the glycerol was being consumed mostly for PHA accumulation since the bacterial growth was limited by the absence of ammonia. For PHA accumulation, 4.53 g/L of glycerol were consumed making a total of 25.84 g/L throughout the whole assay. A yield of 0.20 and 0.028 for $Y_{X/S}$ and $Y_{P/S}$ were achieved, respectively.

At the end of the assay, a CDW of 5.57 g/L was achieved, with a polymer content of 13% (table 4.2). The final mcl-PHA concentration was 0.72 g/L, corresponding to a volumetric productivity (r_p) of 0.026 g L^{-1} h^{-1} (table 4.2).

According with the literature (table 4.2), in other conditions, using SFAE as a carbon source, the values of CDW, yields and productivity are a higher than the ones achieved in this assay, however it is visible that the PHA content is within the range of values described in the literature. The composition of the polymer attained in this assay is a little bit different than the ones that have been reported, yet the polymer was obtained under different conditions and with different carbon sources. Although, it is visible that for both assays the polymer is manly composed by HO and HD, having minor concentrations of HHx and HDd monomers.

In summary *P. chlororaphis* could use glycerol for bacterial growth, although the production wasn't very high, there was some mcl-PHA accumulation (as described in the literature for SFEA substrate), with the composition different to the one found in the literature for this culture.

4.2.2. Pulse Feeding Fermentation

A pulse feeding fed-batch strategy was performed. This assay was carried out in a 10 L bioreactor with the same conditions as the batch assay. However, after 23 hours of cultivation, a pulse of glycerol, about 40 g/L, was given and the assay proceeded until 41 hours. After a lag phase of 6 hours, *P. chlororaphis* entered an exponential growth phase that lasted around 15 hours. The initial glycerol (39.24 g/L) was totally consumed in the growth phase (23 h). In this experiment, ammonium hydroxide was used for pH control throughout the run, aiming at providing non-limiting conditions of nitrogen to promote a higher growth of the culture than in the previous batch experiment. Consequently, the glycerol that was added to the medium, afterwards, was consumed not only for bacterial growth but also for PHA synthesis.

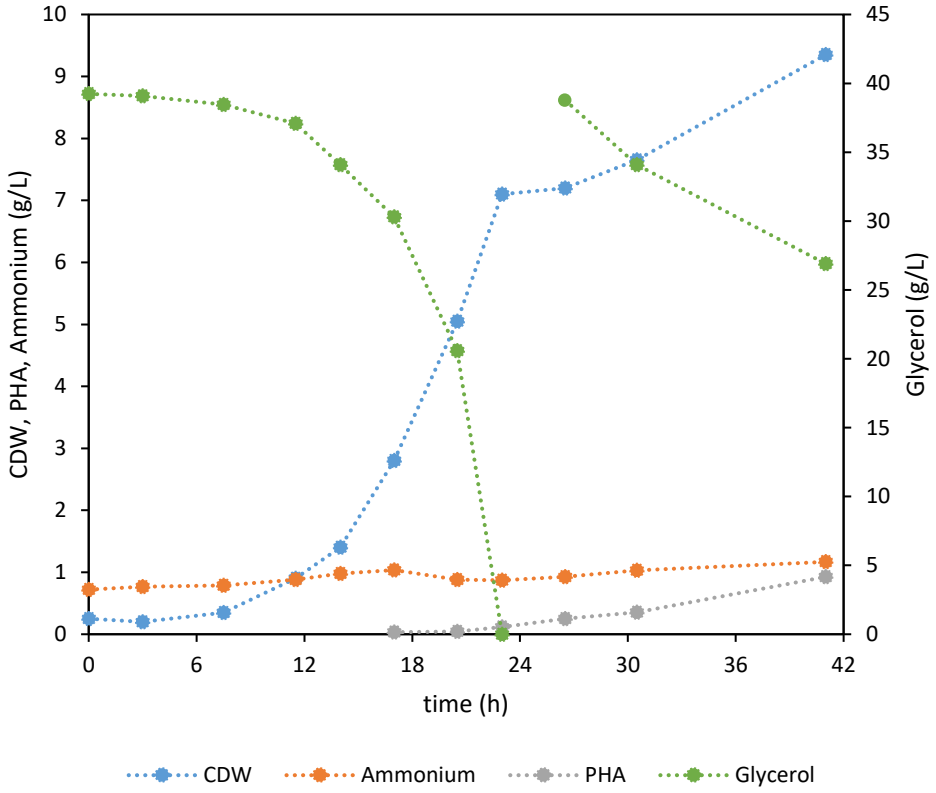


Figure 4.4 - Pulse feeding fermentation of *P. chlororaphis* DSM 19603 in a 10 L biorector.

As expected, at the end of the assay, a CDW concentration of 9.35 g/L was achieved. This value is considerably higher than that attained in the batch bioreactor cultivation (5.57 g/L). The biomass had a polymer content of 10% (table 4.2), this value is lower than the one achieved in the batch fermentation, which means that more bacterial cells were obtained but their PHA content was lower. The CDW concentration was much higher than the one achieved with the batch fermentation (table 4.2). Yet in this assay, 51.1 g/L of glycerol were consumed throughout the whole experiment. The yield values were higher than the ones attained in the previous batch fermentation ($Y_{X/S}$ – 0.30 and $Y_{P/S}$ – 0.030). This increase of $Y_{X/S}$ is a result of not having limiting conditions for the cell growth, this means that more carbon was used for cell growth than in the previous batch assay, which was the main objective of this experiment. A final PHA production of 0.93 g/L was reached, with a productivity (r_p) of 0.023 g L^{-1} h^{-1} (table 4.2). This last value was lower than the one attained in the batch fermentation, possibly because of the over stimulation of the cell growth, meaning that the bacterial cells were consuming glycerol for growth instead of polymer accumulation, resulting in lower mcl-PHA content and productivity values.

It has been reported in the literature that by using other carbon source (SFAE), with the same cultivation mode it is possible to achieve higher CDW, yield and productivity values (table 4.2), than the ones attained in this assay. However, the composition of the polymer attained in this fermentation is close to the one achieved in the previous batch experiment, meaning that this polymer is, still, a little bit different than the ones that have been reported (table 4.2). Although, it remains clear that for all assays the polymer is manly composed by HO and HD, having minor concentrations of HHx and HDd monomers.

In short, in this experiment it was possible to, considerably, increase the biomass production, without affecting the production and the composition of the final mcl-PHA polymer.

4.2.3. Repeated Batch Fermentation

To evaluate the ability of the culture to produce mcl-PHA in a repeated batch strategy, 3 consecutive cycles were performed with *P. chlororaphis*. This assay was carried out in a 2 L bioreactor with the same conditions as the batch assay, namely, each cycle was initiated with a glycerol concentration of 35 to 37 g/L and the pH was controlled with NaOH. After approximately 22 hours, 1.6 L of the culture broth was withdrawn from the bioreactor and it was replenished with fresh medium E* supplemented with glycerol. The remaining broth volume (0.3 L) served as inoculum to the following cycle. This fermentation strategy was never been attempted in a *P. chlororaphis* culture for production of mcl-PHA before, since that according with the literature the only approaches for this issue, was in shake flasks and pulse fed-batch fermentations (Muhr et al., 2013, Yun et al., 2003).

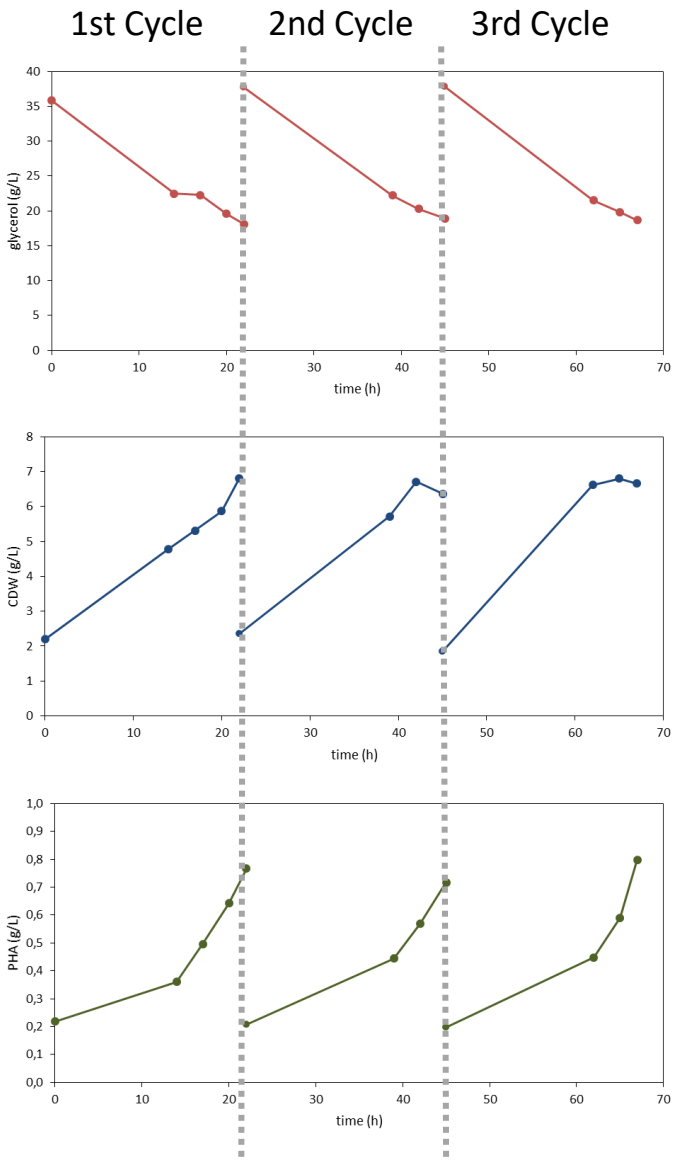


Figure 4.5 - Repeated batch fermentation of *P. chlororaphis* DSM 19603 in a 10 L biorector.

Through the observation of figure 4.5 it appears that all 3 cycles were very similar with each other, which is concomitant with table 4.2, showing the reproducibility of this assay. In all cycles as the glycerol concentration lowers, the CDW and PHA concentrations increased, which means that glycerol has been consumed for bacterial growth and PHA accumulation. In the first cycle it was consumed 17.67 g/L of glycerol to achieve a CDW of 6.82 g/L with a final concentration of mcl-PHA of 0.77 (12%). In the second cycle it was consumed 18.78 g/L of glycerol to attain a CDW of 6.37 g/L with a final concentration of mcl-PHA of 0.72 (11%). In the third cycle it was consumed 19.11 g/L of glycerol to obtain a CDW of 6.67 g/L with a final concentration of mcl-PHA of 0.80 (12%) (table 4.2). In this experiment, the results attained in all cycles are very similar with the batch experiment, with a slightly increase of CDW and, consequently, the $Y_{X/S}$ value. Meaning that the CDW and yields values are lower than the ones attained in the previous pulse feeding fermentation, for the same reasons as described above. Once again, similar to what happens in the batch assay, the productivity and the mcl-PHA content values were higher than the pulse feeding experiment.

All cycles lasted approximately the same (22 hours), in order to avoid PHA degradation by the bacteria. Similar volumetric productivities (0.022 to 0.027 g/L h) and PHA content (11 to 12%) were attained in all cycles but the highest volumetric productivity (0.027 g/L h) and PHA content (12%) was achieved by the third cycle (table 4.2). This suggests that prolonging the experiment by performing more cycles might further improve the productivity. Additionally, the length of the cycles could also be extended so higher PHA production is reached in each cycle.

Similar to what happens with the other two fermentation strategies the composition of the final polymer is similar with the one attained in the previous assay, although it has slightly difference with the composition observed in the literature. However, once again, it remains that for all this examples the polymer is manly composed by HO and HD, having minor concentrations of HHx and HDd monomers.

After this assay it is clear that it is possible to keep a *P. chlororaphis* culture to produce mcl-PHA for consecutive cycles. This cycles are reproducible and could be optimized to attain higher mcl-PHA production.

In table 4.2 it remains clear that in all the types of fermentation using *P. chlororaphis* and glycerol as sole carbon source, the composition of the mcl-PHA recovered in this assays was approximately the same. This shows that type of fermentation does not have a significant impact on the composition of the final mcl-PHA obtained. However the composition of the mcl-PHA obtained in the shake flask assay is a little bit different than the others this might be because in a bioreactor a more controlled environment can be achieved.

Table 4.2 - Yields, productivity, mcl-PHA content and composition at the end of all fermentation processes

Experiment	CDW (g/L)	mcl-PHA content (%)	mcl-PHA (g/L)	Productivity r_p (g/L h)	Yx/s (g/g)	Yp/s (g/g)	mcl-PHA Composition				References
							HHx	HO	HD	HDd	
Batch Fermentation	5.57	13	0.72	0.026	0.20	0.028	5.2	22.6	61.7	10.5	This study
Pulse Feeding Fermentation	9.35	10	0.93	0.023	0.30	0.030	6.1	26.9	54.1	13.0	This study
Repeated batch Fermentation											This study
Cycle 1	6.82	12	0.77	0.025	0.26	0.031	5.5	23.5	60.3	10.7	
Cycle 2	6.37	11	0.72	0.022	0.21	0.027	5.8	24.3	59.2	10.6	
Cycle 3	6.67	12	0.80	0.027	0.25	0.031	5.9	24.6	58.8	10.7	
Batch – Shake Flask	3.82	n. a.	n. a.	n. a.	n. a.	n. a.	6.8	34.3	45.0	13.8	This study
Pulse Feeding Fermentation with SFAE	30	11.9	n. a.	0.071	0.62	0.075	15.5	50.6	26.1	5.0	Muhr et al., 2013
	41.3	10.0	n. a.	0.094	0.73	0.07	14.6	45.7	27.3	6.7	
	41.2	15.2	n. a.	0.138	0.66	0.101	10.2	47.9	31.4	6.2	
Batch Fermentation with PKO	3.3	45	1.49	n. a.	0.67	n. a.	4.7	34.7	32.5	1.4	Yun et al., 2003

n. a. – data not available;
SFAE - substrates derived from animal waste
PKO - Palm Kernel Oil

4.3. Recovery Methods

The most commonly used solvents for PHA recovery are chlorinated hydrocarbons, such as chloroform. However, their use for extraction and purification of biopolymers from biomass are forbidden nowadays, since they are highly aggressive to the environment and to human health. On the other hand, for applications in the medical and pharmaceutical fields it is important to use less toxic solvents in the extraction process, since the presence of these kind of impurities are not tolerated by regulatory agencies. To try not to use chloroform in the recovery methods alternative recovery methods were studied using less hazardous solvents: acetone, hexane, ethyl acetate, propylene carbonate, hypochlorite, sodium hydroxide, hydrogen peroxide and different water-based extractions using osmotic shock for disrupting the bacterial cell wall.

These experiments were performed using biomass produced in a bioreactor cultivation (different from the ones presented), using glycerol by-product as sole carbon source. The biomass has a polymer content of 22%. The efficiency of each extraction methods was determined by comparison of each recovery method with the Soxhlet extraction at 80˚C, using chloroform as solvent.

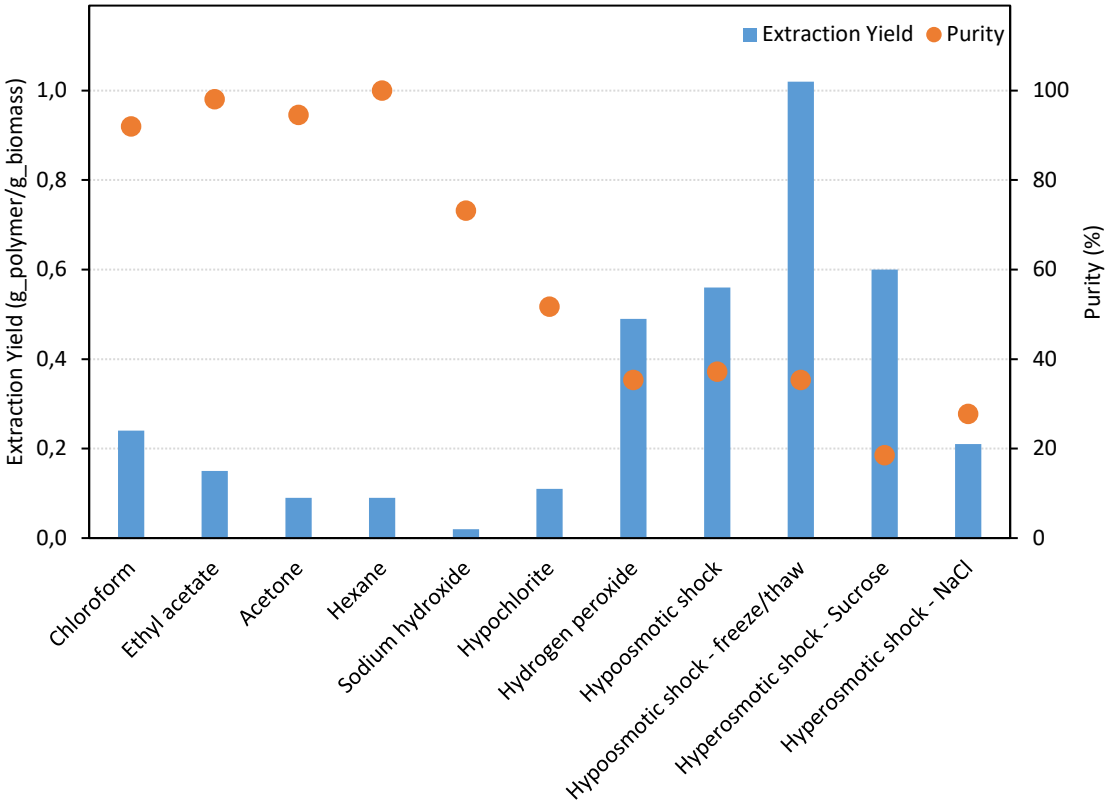


Figure 4.6 - Extraction yield and mcl-PHA purity for different recovery methods tested.

By looking at figure 4.6 it is easy to understand that recovery process was successful using chloroform, ethyl acetate, acetone and hexane since it resulted on a polymer with high purity levels (above 90%). However, the efficiency of the extraction was different: with chloroform was attained more polymer (although it was a less pure polymer than the one attained with the ethyl acetate, acetone, hexane and Soxhlet extraction, this might be because of the conditions used in this assay). It is also visible that the content of at the end of this test is above 0.22 (which is the content of the cellular mcl-PHA) this means that this chloroform sample at the end of the extraction had some impurities. With ethyl acetate, considering the conditions used in this assay, seems to be the one who had better result having a high purity polymer with a high yield. With acetone and hexane, although the polymer had high purity values, only a small portion of mcl-PHA was extracted (in this conditions not all of the polymer was extracted from the biomass) this was observed in the ethyl acetate sample too but in a minor percentage.

It is possible to see that for chloroform extractions the results attained are concomitant with the literature for PHB polymers, having high yield and purity values, even in different conditions. However, for HO extraction the yield was very low, but even though, this result was the best according with the experiments reported by Rai et al., 2011. For acetone, the results attained are, once again, according with the letter for PHB polymers. Although, for recovery of HO polymers the yield was again very low, but not too different from the chloroform results attained in the same conditions (Rai et al., 2011).

For the extraction using ethyl acetate the results attained were completely different from the ones described in the literature having high purity and yield values, however the polymer, the bacteria

and the conditions were a little bit different from the ones used in this assay. Despite all of that, ethyl acetate showed some interestingly results for mcl-PHA extraction from *P. chlororaphis*. Similar to what happen with ethyl acetate, hexane results showed to be very different from the literature, once again, the conditions were not the same as used in this assay, but they still are interesting and promising results for mcl-PHA extraction from *P. chlororaphis*.

To increase this yields and purity values it might be a good idea, performing a Soxhlet extraction using each one of these solvents (chloroform, acetone, ethyl acetate and hexane).

Table 4.3 - Different PHA recovery methods reported

Extraction Method	Solvents	Strain	PHA Monomers	Results		Reference
Solvent Extraction	Chloroform	*Bacillus cereus SPV*	HB	Yield: 31%	Purity: 92%	Valappil et al., 2007
	Chloroform	*C. necator DSM 545*	HB	Yield: 96%	Purity: 95%	Fiorese et al., 2009
	Propylene carbonate			Yield: 95%	Purity: 84%	
	Acetone	*P. putida GPo1*	HO	Yield: 94%	Purity: n. a.	ELbahloul et al., 2009
	Ethyl acetate	*P. putida KT2440*	HO	Yield: 12 %	Purity: n. a.	Wampfler et al., 2010
	Acetone	*P. mendocina*	HO	Yield: 21.38% ± 1.2%	Purity: n. a.	Rai et al., 2011
	Chloroform			Yield: 23.04% ± 2%	Purity: n. a.	
	Hexane			Yield: 8.83% ± 2%	Purity: n. a.	
Cell Disruption	Hypochlorite	*C. necator*	HB	Yield: n. a.	Purity: 86%	Hahn et al., 1995
		Recombinant E. coli	HB	Yield: n. a.	Purity: 93%	
	Hypochlorite-Chlorofom	*C. necator*	HB	Yield: 91%	Purity: 97%	Madkour et al., 2013
	Chelate-Hydrogen Peroxide	*C. necator*	HB-co-HV	Yield: n. a.	Purity: 99.5%	Madkour et al., 2013

n. a. – Data Not Available

In this conditions, sodium hydroxide wasn't a very efficient extraction method since the yield was very low (0.02), however it had a reasonable purity (73%) which means that in some other kind of applications this method could be used. Hypochlorite had a yield similar to the acetone or hexan, but the polymer attained with this recovery method was less pure, this means that some cell components (non-PHA mass) remained in the sample after the extraction. According with the literature a very pure PHB polymer can achieved using hypochlorite in the recovery process, although using different conditions. The use of sodium hydroxide and hypochlorite solvents, in this conditions, appear to be a not very efficient extraction method.

So far, there was no recovery method using hydrogen peroxide as a solo solvent for the extraction of mcl-PHA. With this solvent a high yield extraction was achieved however the polymer attained with this method was very impure, which means that there was not an efficient separation of the polymer from the biomass, resulting in a sample with a high non-PHA material.

Aiming to evaluate osmotic shock as a strategy to rupture the cells of *P. chlororaphis*, different treatments were performed. Hypo-osmotic shock was done by subjecting the cells to a hypotonic medium, namely, deionized water. This treatment could cause for bacterial cells to swell up and disrupt, exposing the inside of the cell (Koller et al 2013). Hyper-osmotic shock was performed by subjecting the cells to two different hypertonic media, namely, saturated solutions of NaCl or sucrose. This two solutions were selected to promote a salt shock and a sugar shock, respectively and in both of this treatments the bacterial cells tend to shrink. When re-suspended in a solution with low concentration NaCl or sucrose, the cell incorporates high amounts of water (by osmosis). This sudden changes causes the cell to burst because of the osmotic pressure, exposing the inside of the cells (Lang et al., 2005). In figure 4.7 it is possible the results of these extraction methods and it is visible that the bacterial cell are not intact. When compared with figure 4.2 (in the screening assay) it seems that, in phase contrast, the bacterial cells are bigger and have a different shape than the ones observed in figure 4.7.

The cells exposed to deionized water seemed to be disrupt, since it is visible in figure 4.7 that bacterial cells are not intact. Once again, when compared with figure 4.2 it seems that, in phase contrast, the bacterial cells are bigger and have a different shape than the ones observed in figure 4.7. For this same reason, it seems that for all samples treated by osmotic shock there was disruption of the cell wall, however the separation of the polymer from the cell components wasn't efficient resulting in a low purity polymer.

The freeze/thaw test improved the cell wall rupture when compared with deionized water in figure 4.7 it becomes clear that the water crystals disrupted the cell wall when the sample was freezed, making it possible for the polymer to aggregate outside the cell when the sample was thawed.

For almost all samples treated by osmotic shock, the recovery process wasn't very efficient since, although it had very high extraction yields (0.21 to 1.02%), there were low purity values (18 to 37%), which means that the polymers contained many impurities. So far there's been reported that it is possible to extract mcl-PHA from the biomass using osmotic shock treatment (Koller et al., 2013), although no comparable data were found. The osmotic shock treatment methods were not very good to

recover mcl-PHA as a solo method, but they might be suitable as a pre-treatment for mcl-PHA extraction when coupled with other extraction methods. This type of extractions only use water and could be coupled with other non-hazardous solvents decreasing the recovery costs and making the extraction less hazardous for the environment and to workers.

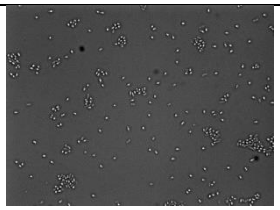
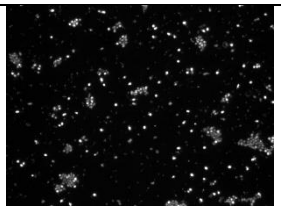
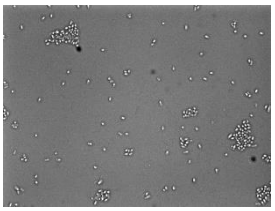
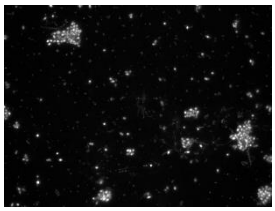
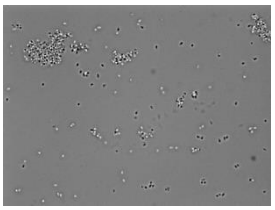
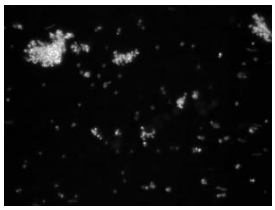
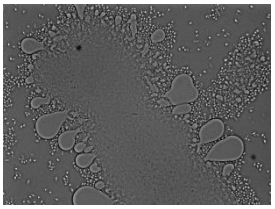
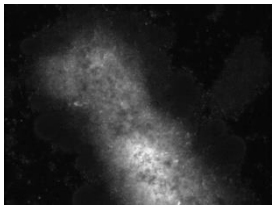
Recovery method	Phase contrast	Fluorescence
Hypoosmotic shock		
Hyperosmotic shock – Sucrose		
Hyperosmotic shock – NaCl		
Hypoosmotic shock – Freeze/Thaw		

Figure 4.7 - Visualization of the bacterial cells under the optical microscope (100x) for samples collected after osmotic shock treatment, under phase contrast and with fluorescence after Nile blue staining

4.4. Mcl-PHA Film Mechanical Tests

P. chlororaphis mcl-PHA films were prepared by solvent casting. They were elastic and translucid (figure 4.8).

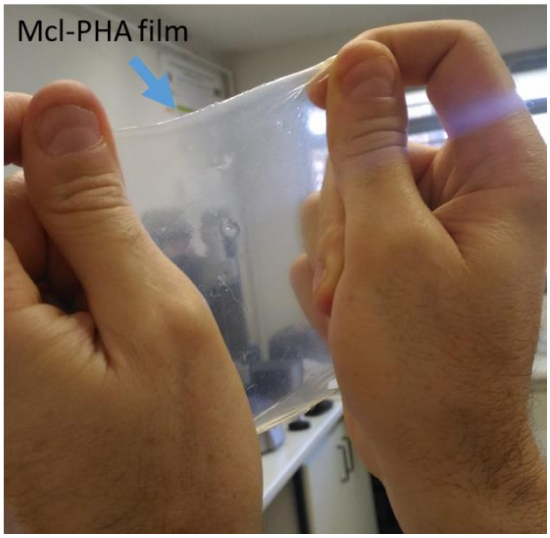


Figure 4.8 – Mcl-PHA film prepared by solvent casting.

To measure the mechanical properties of mcl-PHA films, a tensile test was carried out. For this assay, the mcl-PHA film was cut into rectangular-shaped pieces and an axial tension was applied, at room temperature, using a texturometer, with a 5 kg load cell. The film was strained at a constant velocity until break (figure 4.9).

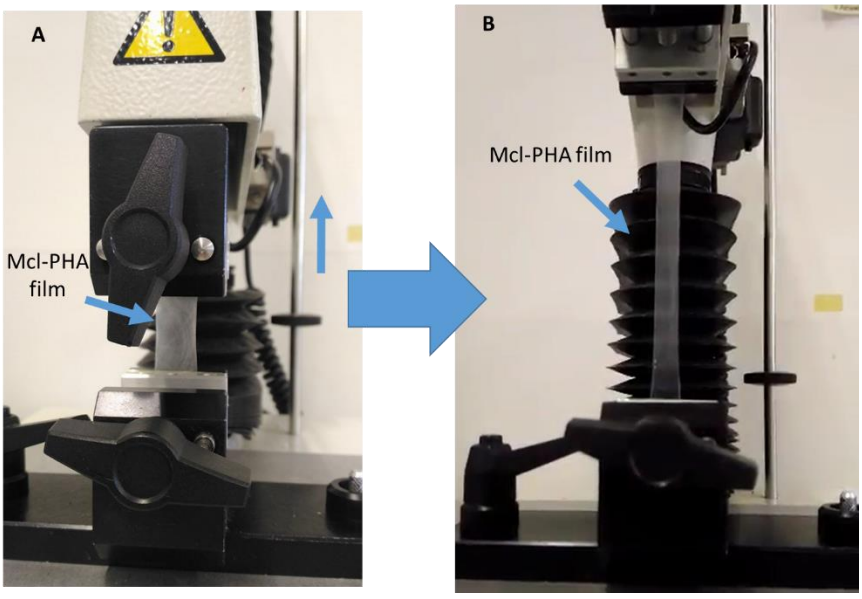


Figure 4.9 - Mcl-PHA film elongation before (A) and after (B) mechanical test.

Through the observation of the stress and strain curve (appendix 2) and table 4.4 it is possible to see that *P. chlororaphis* mcl-PHA films have elongated more than twice their original length (3 cm), and had a high percentage of plastic deformation without breaking. Comparing *P. chlororaphis* mcl-PHA with the other PHA, namely, PHB, P(HB-co-HHx), PHA-L and PHA-LE, it is clear that the latter form films much more resistant to deformation, as shown by their higher Young Modulus and lower elongation at break. When mcl-PHA is compared with Tussicol films (or Tisseel is a synthetic fibrin sealant from Baxter Healthcare commercially used in surgery) it is possible to see that it has a similar elongation at break and a higher tensile strength at break, which means that *P. chlororaphis* mcl-PHA is more resistant than Tissucol. This suggests that *P. chlororaphis* mcl-PHA might have properties suitable for the replacement of the synthetic adhesive, Tissucol, in commercial applications.

The Young Modulus is a physical parameter representing the resistance to deformation of a material (Ashby et al., 2000). It was determined by measuring the slope of a line tangent to the linear initial region of the tension-deformation curve. From table 4.4, it is possible to conclude that PHA-L, PHA-LE and Glubran2 films, having higher young modulus values (767.80 MPa, 510.60 MPa and 283.90 MPa, respectively) and lower values of elongation at break, are more likely to be stiffer. On the other hand, mcl-PHAs and Tissucol have low young modulus values (8.00 MPa and 0.04 MPa, respectively) and higher elongation at break values, which means they are less stiff and more flexible, more prone to plastic deformations.

These results show that *P. chlororaphis* mcl-PHA forms flexible, elastic and resistant films, which seems to suggest that this biodegradable and biocompatible biopolymer has potential for use as a material for wound management. Since mcl-PHA is a hydrophobic polymer, it will not dissolve when in contact with water, making it resistant to this kind of environments.

Table 4.4 - Tensile properties of PHA

Film	Thickness (mm)	Tensile strength at break (MPa)	Elongation at Break (%)	Young modulus (MPa)	Reference
mcl-PHA*	0.16 ± 0.03	3.94 ± 0.69	272.65 ± 26.57	8.00 ± 8.17	This Study
mcl-PHA*[2]	0.16	2.91	212.39	6.00	This Study
PHB	0.3	27.80	15.00	n. a.	Zhao et al., 2008
P(HB-co-HHx)	0.3	14.20	212.00	n. a.	Zhao et al., 2008
PHA-L*[3]	0.1	25.00 ± 1.70	4.40 ± 0.50	767.80 ± 78.70	Ashby et al., 2000
PHA-LE*[3]	0.1	20.70 ± 1.50	29.20 ± 3.90	510.60 ± 21.50	Ashby et al., 2000
Glubran2*[4]	n. a.	20.44 ± 2.83	13.21 ± 4.32	283.90 ± 30.50	Kull et al., 2009
Tissucol*[5]	n. a.	0.14 ± 0.02	363.10 ± 30.58	0.04 ± 0.01	Kull et al., 2009

n. a. - data not available

*mcl-PHA with 30 mm of initial length (Average of 3 Samples). Tensile-Deformation and Young modulus curves available in appendix 2 and 3, respectively

*[2]mcl-PHA with 25 mm of initial length. Tensile-Deformation and Young modulus curves available in appendix 2 and 3, respectively

*[3]PHA-L – mcl-PHA from linseed oil composed by HHx, HO, HD, HDd and HTd; PHA-LE – Epoxidized mcl-PHA from linseed oil composed by HHx, HO, HD, HDd and HTd. Measurements were made after the film aged for 100 days.

**[4]Glubran2 is synthetic surgical glue composed of modified n-butyl-2-cyanoacrylate approved for internal and external use in Europe.

**[5]Tissucol or Tisseel is a synthetic fibrin sealant from Baxter Healthcare commercially used in surgery

4.5. Adhesion evaluation

4.5.1. Tension Tests

To measure the adhesion properties of the mcl-PHA, tension tests were made using porcine skin as the model substrate. In this assay, pieces of porcine skin were cut into square-shaped pieces and their dermis side was attached to metal testing holders. The mcl-PHA was melted and spread uniformly on the epidermis side of one porcine skin piece and immediately pressed against the epidermis side of other porcine skin piece. The bonding strength was then, measured in tension mode at room temperature using a texturometer with a 5 kg load cell. The two parts of the joint were strained axially at a constant velocity until separation was achieved (figure 4.10).

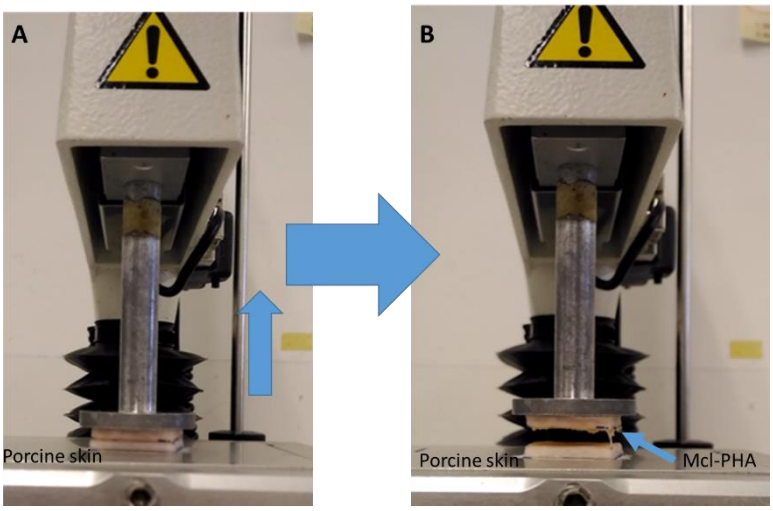


Figure 4.10 - Adhesive test in Porcine Skin before (A) and after (B) tension test.

Looking at table 4.5 it is easy to see that on tension tests in porcine skin, *P. chlororaphis* mcl-PHA performed better than all other adhesives tested with the exception of cyanoacrylate. The tension needed for skin separation with mcl-PHA was 61.13 kPa, while those with Glubran2 and Tissucol (which are commercially used glues in surgery) were only 21.00 kPa and 0.70 kPa respectively. Only cyanoacrylate conferred a higher tension at separation, however this is a synthetic adhesive present in many products for use in medicine for wound closure. Mcl-PHA is a raw polymer without any kind of treatment and already shows higher tension at separation values comparing with other natural adhesives. Hence, this natural material seems to be a promising candidate for the development of novel skin adhesives formulations to be used, for example, for wound closures in surgical applications.

Table 4.5 - Tension tests results made in porcine skin

Material	Tension at Separation (kPa)	Contact Area (10^{-4} m^2)	Reference
mcl-PHA*	61.13 ± 20.55	6.25	This Study
Gelatin - Alginate*[2]	5.50	4.00	Shefy-Peleg et al., 2014
Gelatin - Alginate - EDC*[3]	10.00	4.00	Shefy-Peleg et al., 2014
Mussel Extract*[4]	40.00	3.43	Ninan et al., 2003
Fibrin*[5]	40.00	3.06	Ninan et al., 2003
Cyanoacrylate*[6]	990.00	2.92	Ninan et al., 2003
Glubran2*[7]	21.00 ± 60	n. a.	Kull et al., 2009
Tissucol*[7]	0.70 ± 0.6	n. a.	Kull et al., 2009

n. a. - data not available
*The mcl-PHA tension tests results were determined by an average of 3 samples, using 100-190 mg of melted mcl-PHA (tension tests curve available in appendix 4)
*[2]Gelatin concentration of 200 mg/mL and Alginate concentration of 60 mg/mL.;
*[3]Gelatin - concentration of 200 mg/mL, Alginate concentration of 40 mg/mL and EDC concentration of 20 mg/mL; EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimidehydrochloride.
*[4]Average values of 10 samples after 3h of curing time, using 300 mg of the adhesive material
*[5]Average values of 8 samples after 3h of curing time, using 202 mg of the adhesive material
*[6]Average values of 10 samples after 3h of curing time, using 200 mg of the adhesive material
*[7]It was used 60 µL of glue amount and it was applied a 2 N load for 10 minutes

4.5.2. Shear Tests

To measure the adhesion properties of the mcl-PHA a shear test was also carried out. In this assay porcine skin was cut into rectangular-shaped pieces. Mcl-PHA was melted and spread, in the contact area, on the epidermis side of one porcine skin piece which were immediately attached to the epidermis side of other porcine skin. The skin was then attached to metal clamps of the testing holders and the bonding strength was measured in shear mode at room temperature using a texturometer with a 30 Kg load cell. The two parts of the joint were strained axially at a constant velocity until separation was achieved (figure 4.11).

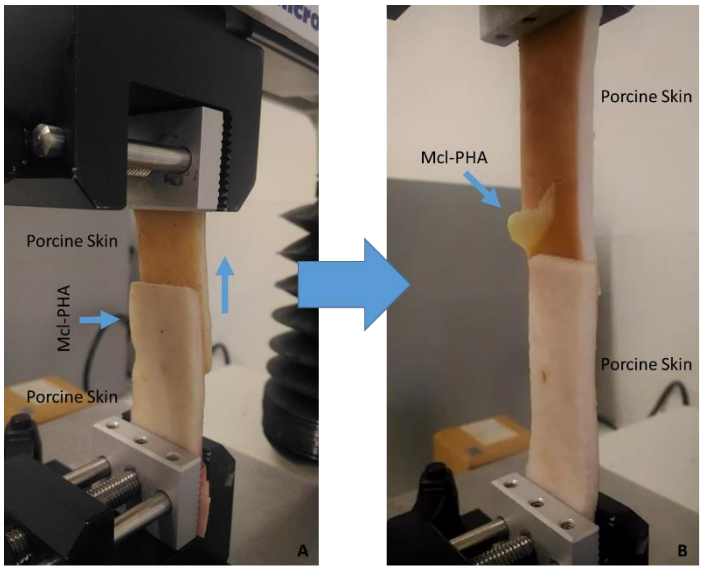


Figure 4.11 - Adhesive test in Porcine Skin before (A) and after (B) shear test.

Table 4.6 - Shear tests results made in porcine skin

Material	Tension at Separation (kPa)	Contact Area (10^{-4} m^2)	Reference
mcl-PHA*	12.72 ± 2.14	6.25	This Study
Glubran2*[2]	32.60 ± 89.00	n. a.	Kull et al., 2009
Tissucol*[2]	2.20 ± 1.30	n. a.	Kull et al., 2009
Tissucol*[3]	17.60 ± 2.10	6.25	Sierra et al., 1992
Gelatin-mTG*[4]	12.00 – 23.00	0.90 ± 0.04	McDermott et al., 2004
Chitosan	3.00	n. a.	McDermott et al., 2004
Cyanoacrylate	68.00	n. a.	McDermott et al., 2004
Fibrin-Gelatin based sealent*[5]	Up to 27.00 ± 8.00	6.25	Sierra et al., 1992

n. a. - data not available
*The mcl-PHA shear tests results were determined by an average of 3 samples, using 210-280 mg of melted mcl-PHA (shear tests curve available in appendix 4)
*[2]Was used 60 µL of glue amount and it was applied a 2 N load for 10 minutes
*[3]0.125 mL of the component was spread on the test surface
*[4]Gelatin-mTG (microbial transglutaminase), different concentrations of gelatin were studied, it was used 0.15 mL of glue amount and it was applied a 47.5 ± 0.3 gm lead weight for 120 minutes
*[5]Different concentrations of fibrin with the different gelation times were tested, 0.125 mL of the component was spread on the test surface

According to table 4.6, it can be seen that regarding the shear tests in porcine skin, the mcl-PHA has a similar performance when comparing to some natural adhesives tested and reported in the literature. The tension at separation value of the mcl-PHA (12.72 kPa) was lower than Glubran2 (32.60 kPa) and fibrin-gelatin based sealent (up to 27.00 kPa). However the mcl-PHA values are within the range of that reported for gelatin-mTG and Tissucol (12.00-23.00 kPa and 2.20-17.60 kPa, respectively), this last being commercially used in surgery. Mcl-PHA had higher values, by far, than chitosan (3.00 kPa). Once again, cyanoacrylate had the highest tension at separation.

With this assay mcl-PHA showed to be a promising candidate to act like an adhesive to be used for medical applications. Once again, it seems to be a promising candidate for the development of novel skin adhesives to be used, for example, for wound closures in surgical applications.

4.5.3. Peeling Tests

In this experiment a human arm was used as soft tissue model. The mcl-PHA was melted and spread uniformly through a rectangular-shaped plastic sheet and left at room temperature to solidify. After, the mcl-PHA was separated from the plastic sheet and applied to the skin (figure 4.12 A).

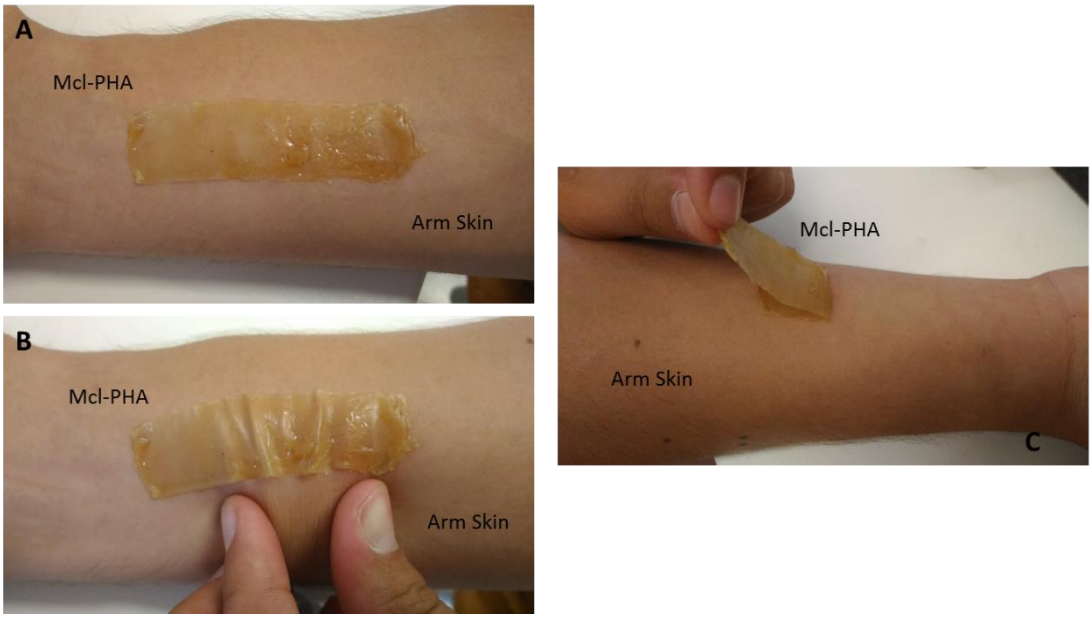


Figure 4.12 – Mcl-PHA peel tests in vivo in arm skin. (A) Mcl-PHA adhered in arm skin; (B) Mcl-PHA adhered in a wrinkled skin; (C) Mcl-PHA being peeled out from the skin

In this assay it was not possible to evaluate quantitatively the adhesion *in vivo,* because the available equipment did not permit it. So the polymer was then applied to a human arm skin (figure 4.12 A) and pulled out to check the adhesion. The mcl-PHA had a good adhesion to the skin and that is visible in figure 4.12 B, where it is noticeable that the polymer adheres to every wrinkle in the skin when the skin was wrinkled. In figure 4.12 C, it is clear that when the polymer was peeled out from the skin, there was no harm done to the skin and there was no evidence of residue of mcl-PHA in the skin.

Comparing with a commercial synthetic adhesive for wounds, like a bandage, the force used to take off the bandages is higher, however due to this strong adhesion in many cases the use of this bandages ends up harming the skin. The mcl-PHA provides a good and painless adhesion, making possible the production of a fully natural, biodegradable, biocompatible, bio-based adhesive.

In the previous assays (tension and shear tests) the final application was to use the mcl-PHA as a wound closure material (i.e. to be used in a surgery). With the peel tests it is clear that the main application changes a bit, since this test evaluates the capability of the mcl-PHA work as a wound dressing material (i.e. to be used in bandages) instead of a wound closure material. Thereby increasing the number of final applications that this polymer might have.

5. Conclusions and Future Work

In this work, it stays clear that *P. chlororaphis* could use glycerol for bacterial growth and mcl-PHA production in batch mode. Although the production wasn't very high, the mcl-PHA accumulation was similar to that described in the literature for SFEA substrate. The polymer was composed by HHx, HO, HD and HDd monomers. By performing a pulse feeding fermentation, it was possible to considerably increase the biomass production, without affecting the production and the composition of the final mcl-PHA polymer. After a repeated batch fermentation, it was possible to keep a *P. chlororaphis* culture to produce mcl-PHA in consecutive and reproducible cycles. These results were already a great step towards the optimization of the production of mcl-PHA in *P. chlororaphis.* However, the bioprocess can still be considerably improved to reach maximum optimization of the mcl-PHA production. For instance, other bioreactor conditions need to be tested to see how long do the assays has to last and when is the right moment to give a pulse, or to start a feeding, or even, to change a base, these are all important issues that have to be taken in account to maximize the polymer production.

In this work, alternative mcl-PHA recovery methods were also studied, using less hazardous solvents, since the use of chloroform is not permitted at industrial scale. In those experiments, the solvents that revealed to be better at recover mcl-PHA from the biomass were acetone, ethyl acetate and hexane. These methods should be further studied for the full optimization of the recovery process. Performing Soxhlet extractions using each one of these solvents (acetone, ethyl acetate and hexane) might reveal some interesting results in terms of yield and polymer purity.

It was also studied some recovery methods using water (Hypotonic, Hypertonic and Freezed/Thaw extractions). This type of recovery methods couldn't separate the mcl-PHA polymer from the biomass as a solo method. Nevertheless, they showed some interesting results and might be coupled with other recovery methods, using non-hazardous solvents, decreasing the recovery costs and making the extraction less hazardous for the environment and to workers.

In this thesis, it also remains clear that mcl-PHA forms flexible, elastic, resistant translucid films that have potential for use as a material for wound management. Since the mcl-PHAs are hydrophobic polymers, they will not dissolve when in contact with water, making them resistant to such kind of environments.

The tension and shear tests revealed that mcl-PHA seems to be a promising candidate for the development of novel skin adhesives to be used, for example, for wound closures in surgical applications.

The peel tests showed that the main application of the mcl-PHA could be changed, instead of wound closure adhesive mcl-PHA could also be used as a wound dressing material (i.e. to be used in bandages). Increasing the number of applications that this polymer might have.

At the end of this thesis countless number of tests remain to be done, for fully explore all the possible applications of the mcl-PHAs. Toxicity tests, anti-bacterial tests, polymer modification (during and post

the fermentation process) these are just some examples that increase exponentially the mcl-PHA application area, showing the diversity of these polymer.

6. References

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7. Appendices

Appendix 1 – Glycerol calibration curve

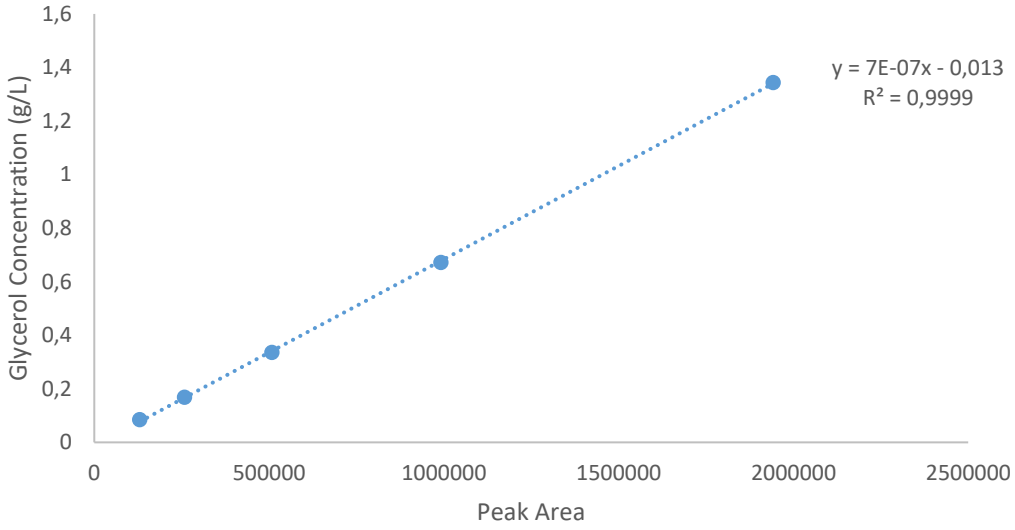

Figure 7.1 - Glycerol clibration curve

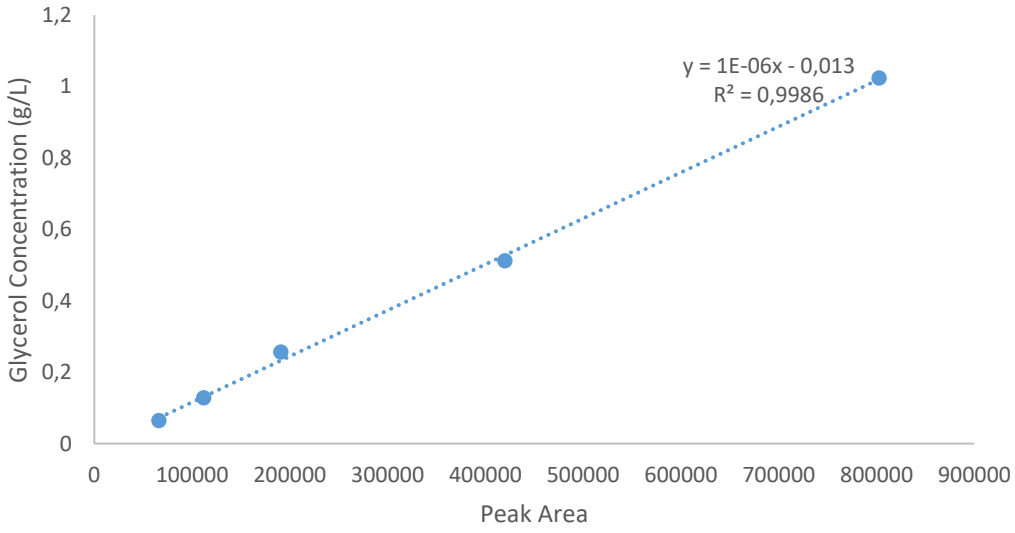

Figure 7.2 - Glycerol clibration curve

Appendix 2 – Tensile-deformation curve of the mcl-PHA film

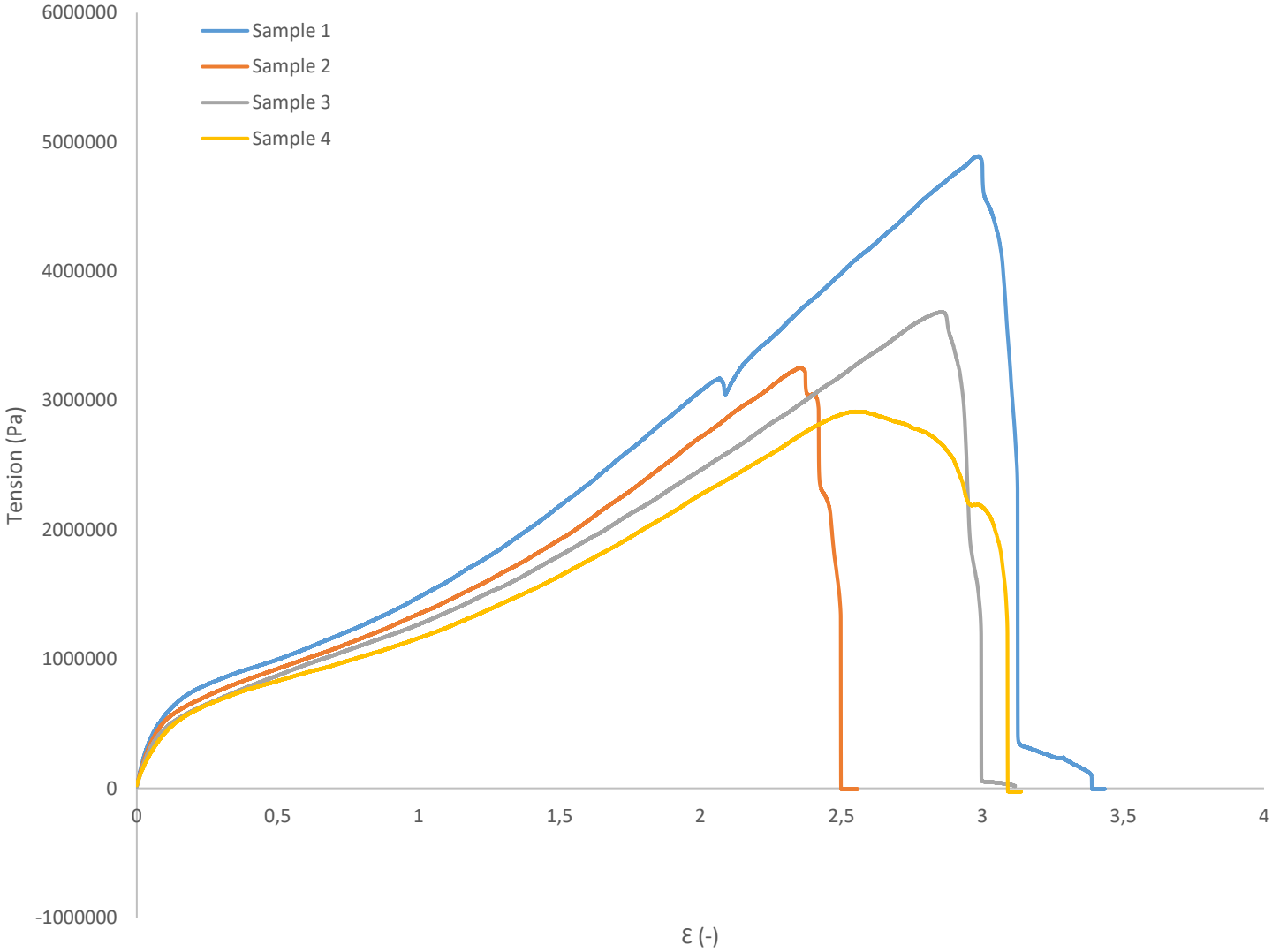

Figure 7.1 - Tensile-Deformation curve of mcl-PHA films

Appendix 3 – Young modulus of the mcl-PHA film

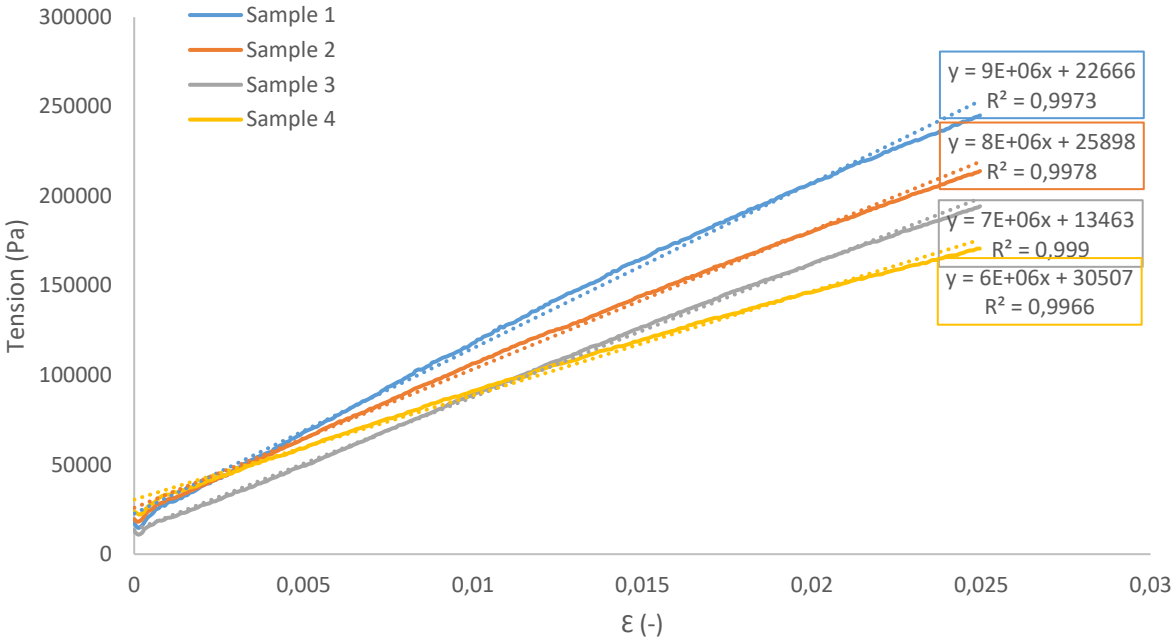

Figure 7.2 - Tensile-Deformation curve

Appendix 4 – Adhesive tests in porcine skin

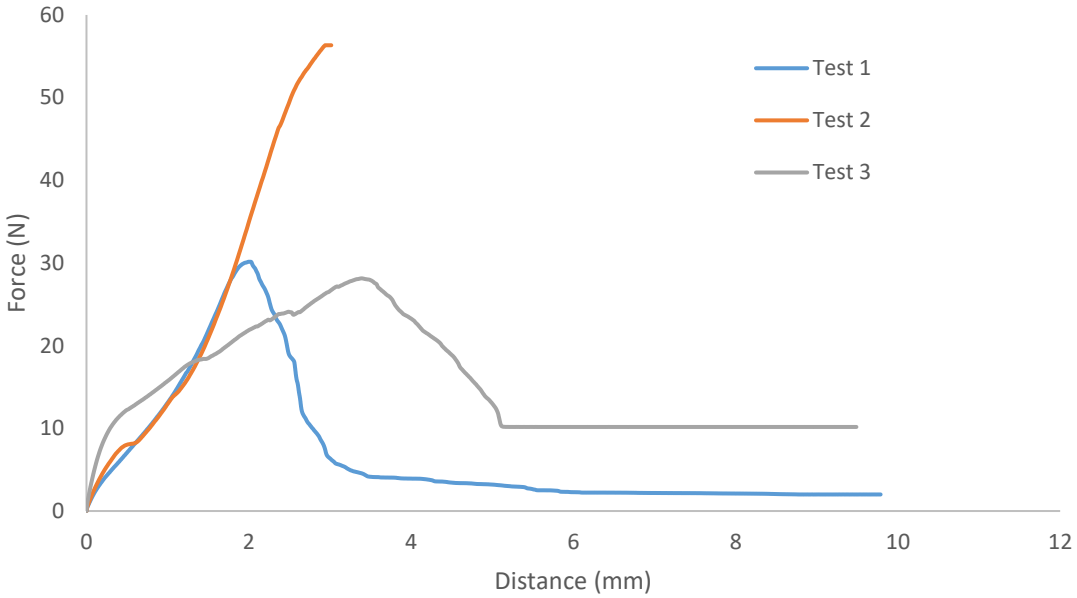

Figure 7.3 - Tension strength tests in porcine skin

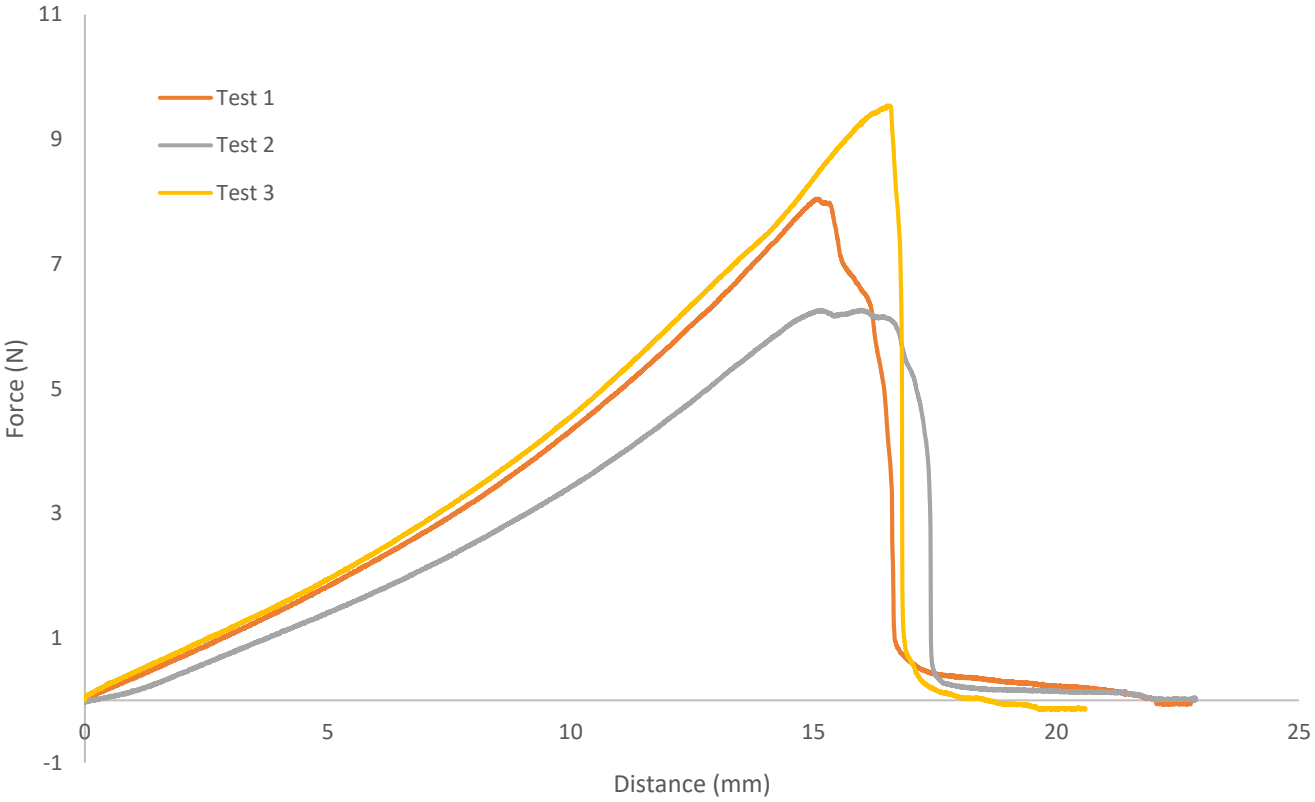

Figure 7.4 - Shear strength tests in porcine skin