Juliana Inês Barros Silva

Bachelor's degree in Biochemistry

Production of medium-chain length polyhydroxyalkanoates (PHA) from sugar-rich extracts and hydrolysates from white wine grape pomace

Dissertation for the degree of master's in biotechnology

Supervisor: Doctor Maria Filomena Andrade de Freitas,
Assistant Professor, FCT-UNL

Co-supervisor: Doctor Alexandre Babo de Almeida Paiva,
Assistant Researcher, FCT-UNL

FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2019

LOMBADA

Production of medium-chain length polyhydroxyalkanoates from sugar-rich extracts and hydrolysates from white wine grape pomace

Juliana Silva

2019

Juliana Inês Barros Silva

Bachelor's degree in Biochemistry

Production of medium-chain length polyhydroxyalkanoates (PHA) from sugar-rich extracts and hydrolysates from white wine grape pomace

Dissertation for the degree of master's in biotechnology

Supervisor: Doctor Maria Filomena Andrade de Freitas,

Assistant Professor, FCT-UNL

Co-supervisor: Doctor Alexandre Babo de Almeida Paiva,

Assistant Researcher, FCT-UNL

September 2019

Copyright

Production of medium-chain length polyhydroxyalkanoates (PHA) from sugar-rich extracts and hydrolysates from white wine grape pomace

Ao meu avô!

x

Agradecimentos

Quero agradecer em primeiro lugar aos meus orientadores, Filomena Freitas e Alexandre Paiva por terem acreditado em mim para a realização deste projeto, por estarem sempre disponíveis, prontos a ajudar e por todos os ensinamentos transmitidos.

A todo o grupo BioEng e grupo Biocatalysis & Bioenergy por todo o apoio, disponibilidade, conselhos e ensinamentos. Um especial agradecimento ao João Pereira e Bruno Pedras, pois sem eles a vida laboratorial tinha sido bem mais complicada, obrigada pelos truques, pelos momentos de descontração, pela paciência, é graças a vocês que aprendi tanta coisa neste ano de tese.

À minha família, que me ama incondicionalmente, que me apoia em todos os meus sonhos e por estarem ao meu lado num dos maiores projetos da minha vida. Obrigada pai e mãe pela luta contante para assegurarem o meu futuro, obrigada irmã porque estares ao meu lado em qualquer momento e obrigada tio por acreditares e me fazeres acreditar nas minhas capacidades.

Finalmente quero agradecer aos meus amigos, por estarem lá quando preciso deles. Aos que estiveram no laboratório Tatiana Costa, Asiyah Esmail e Bruno Serafim, que partilhamos as dificuldades, os momentos de descontração e pela ajuda, um grande obrigada. Àquela que não esteve presente no laboratório, mas sempre na minha vida, Lia Fonseca. Para aquelas que Coimbra me deu Beatriz Gameiro, Mafalda Trovão, Rita Mendes, Inês Lopes e Tatiana Costa, obrigada por estarem ao meu lado desde o início e serem as bebés mais incríveis do mundo.

Esta é para vocês todos!

Resumo

O objetivo desta tese foi a produção de polihidroxialcanoatos (PHA), utilizando como substrato bagaço da uva. Cinco estirpes bacterianas, *Pseudomonas citronellolis* NRRL B-2504, *Pseudomonas chlororaphis* DMS 19603, *Pseudomonas resinovorans* NRRL B-2649, *Pseudomonas stutzeri* NRRL B-775 e *Burkholderia sacchari* DSM 17165, foram estudadas.

Numa primeira parte, foram realizados ensaios em *shake flask* utilizando como substrato bagaço de uva sujeito a três tipos de tratamento: extrato aquoso (1); e hidrolisados obtidos por tratamento ácido (2) ou por água quente comprimida (HCW) (3). O extrato aquoso foi utilizado para o cultivo de *Ps. chlororaphis*, *Ps. citronellolis*, *Ps. resinovorans* e *Ps. stutzeri*, enquanto os hidrolisados ácido e de HCW foram testados para o cultivo de *Ps. chlororaphis*, *Ps. citronellolis*, *Ps. resinovorans* e *B. sacchari*. Estes ensaios demonstraram que o extrato aquoso proporcionava não só um bom crescimento celular, como também acumulação de PHA, pela maioria das estirpes testadas. Os cultivos com os hidrolisados, nas condições testadas, resultaram em reduzido crescimento celular e/ou ausência de acumulação de polímero.

Numa segunda fase, foram realizados ensaios em bio-reator com as estirpes *Ps. chlororaphis*, *Ps. citronellolis* e *Ps. resinovorans* que foram identificadas como tendo maior potencial de produção de PHA. As estirpes atingiram teores de polímero na biomassa de 16.7%, 14.3% e 17.4%, respetivamente, com produtividade volumétrica de 0.04-0.08 g/(L.h). Todos os polímeros eram medium-chain length PHA (mcl-PHA), compostos principalmente por 3-hydroxydecanoato, 3-hydroxydodecanoato e/ou 3-hidroxioctanoato, e tinham peso molecular entre 1×10^5 Da e 3.1×10^5 Da. Apesar da temperatura de degradação ser semelhante, os polímeros apresentaram graus de cristalinidade diferentes, tendo o mcl-PHA produzido por *Ps. chlororaphis* o valor mais elevado (38.8%) e o da *Ps. resinovorans* o mais baixo (10.2%). Assim, o extrato do resíduo do vinho branco mostrou ser um substrato adequado para a produção de biopolímeros com propriedades físico-químicas diferentes e caraterísticas versáteis que podem ser utilizados em diferentes aplicações.

Palavras – chave: polihidroxialcanoatos, polihidroxialcanoatos de cadeia média, resíduos lignocelulósicos, bagaço da uva, hidrólise ácida, água quente comprimida

Abstract

The aim of this thesis was the production of polyhydroxyalkanoates (PHA), using grape pomace as substrate. Five bacterial strains, *Pseudomonas citronellolis* NRRL B-2504, *Pseudomonas chlororaphis* DMS 19603, *Pseudomonas resinovorans* NRRL B-2649, *Pseudomonas stutzeri* NRRL B-775 and *Burkholderia sacchari* DSM 17165, were studied.

In a first part, shake flask tests were carried out using as substrate grape pomace subjected to three types of treatment: aqueous extract (1); and hydrolysates obtained by acid treatment (2) or by compressed hot water (HCW) (3). The aqueous extract was used for the cultivation of *Ps. chlororaphis*, *Ps. citronellolis*, *Ps. resinovorans* and *Ps. stutzeri*, while acid and HCW hydrolysates were tested for *Ps. chlororaphis*, *Ps. citronellolis, Ps. resinovorans* and *B. sacchari*. These assays demonstrated that the aqueous extract provided, not only a good cell growth, but also a good accumulation of PHA by most strains tested. Cultures with the hydrolysates under the tested conditions resulted in reduced cell growth and/or absence of polymer accumulation.

In a second phase, bioreactor assays were performed with *Ps. chlororaphis*, *Ps. citronellolis* and *Ps. resinovorans* that were identified as having higher PHA production potential. The strains reached 16.7%, 14.3% and 17.4% polymer content in the biomass, respectively, with volumetric productivity values of 0.04-0.08 g/(L.h). All polymers were medium-chain length PHA (mcl-PHA), composed mainly of 3-hydroxydecanoate, 3-hydroxydodecanoate and/or 3-hydroxyoctanoate, and had molecular weight values between 1×10^5 Da and 3.1×10^5 Da. Despite the similar temperature degradation, the polymers had different degrees of crystallinity: the mcl-PHA produced by *Ps. chlororaphis* the highest value (38.8%) and that of *Ps. resinovorans* had the lowest (10.2%). Thus, the grape pomace extract proved to be a suitable substrate for the production of biopolymers with different physicochemical properties and versatile characteristics that can be used in different applications.

Keywords: polyhydroxyalkanoates, medium chain polyhydroxyalkanoates, lignocellulosic residues, grape pomace, acid hydrolysis, hot compressed water

List of Content

List of Figure

List of Table

List of Abbreviations

3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDd	3-hydroxydodecanoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HTd	3-hydroxytetradecanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
5-HMF	5-hydrixymethylfurfural
CDW	cell dry weight (g/L)
DSC	Differential Scanning Calorimetry
EGPJ	Ensiled Grass Press Juice
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
HA	Hidroxyalkanoic acid
HCW	Hot Compressed Water
HPLC	High-Performance Liquid Chromatography
LB	Luria-Bertani
LBET	Lobry de Bruyn–Alberda van Ekenstein transformation
LDPE	low-density Polyethylene
Lcl	long-medium-length
Mcl	medium – chain – length
Mn	number –average molecular weight
Mw	average molecular weight
n. a.	Data Not Available
n.d.	Data Not Detected
OD	Optical density
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-co-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PDI	Polydispersity index
PE	Polyethylene
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)

PHA	PolyHydroxyAlkanoate
PhaC	PHAsynthase
PHB	Polyhydroxybutyrate
PKO	Palm Kermel Oil
PLA	Polylactate
PP	Polypropylene
PVA	Poly(vinyl alcohol)
PVAc	Poly(vinyl acetate)
rpm	rotation per minute
Scl	short-chain-length
SEM	Scanning electron microscopy
SFAE	Substrates derived from animal waste
SWH	Subcritical water hydrolysis
T_{deg}	Degradation Temperature
TFA	Trifluoroacetic acid
TGA	Thermogravimetric Analysis
T_m	Melting Temperature
X_c	Degree of Crystallinity

1.Introduction

1.1 Polyhydroxyalkanoates (PHAs)

Plastic based material has excellent mechanical properties, light weight, stability, durability and is chemically inert, which allied to its very low production costs contributes to the dramatic growth of plastic based-products production worldwide [1]. However, these types of materials represent a huge environmental concern because they are not biodegradable, have high durability, and have low recycling rates and, thus, accumulate in land and oceans. Additionally, their production contributes to the rapid reduction of crude reserves, playing also an important role in climate change. Therefore, some strategies are being developed to work around this environmental problem, including the development of bioplastics, such as poly(lactate) (PLA) and polyhydroxyalkanoates (PHA), due to their sustainable and environment friendly characteristics [2][3].

PHA is a biodegradable, biocompatible and renewable bioplastic synthesized by different strains of gram-positive and gram-negative bacteria, thus, providing an environment-friendly alternative to petroleum-based plastics [3]. These biopolymers are polyesters of 600 to 35,000 R-hydroxyalkanoic acids (HA) (Figure 1) [4], that result from the intracellular accumulation of energy storage compounds to levels as high as 90% of the bacteria's dry cell weight (Figure 2), and are meant to be used when carbon or energy sources are imbalanced [5][6]. Under metabolic stress, triggered by a limitation of nutrients required for cell growth such as: oxygen, nitrogen or phosphorus and a high amount of a carbon source, the production of PHA increases [3] [7].
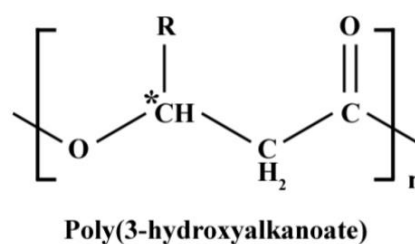
Figure 1 General chemical structure of polyhydroxyalkanoates (PHA), R is the side chain of each monomer, which determines the nomenclature and carbon number of PHA and n, the number of monomers [4].
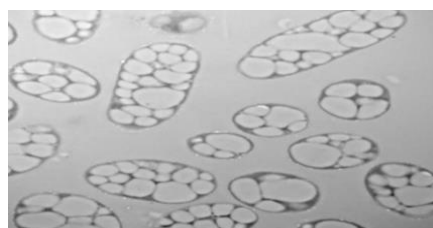
Figure 2 Microbial intracellular polyhydroxyalkanoates granules [8].

The design of PHAs and their thermal and mechanical properties can be controlled by manipulation of metabolic pathways, as well as PHA synthase structure and activity [8]. The different design options combined with their biocompatible, non-toxic, piezoelectric, hydrophobic, structurally inert, enantiomerically pure characteristics makes them a potential and competitive alternative for petrochemical-based plastics for packaging purposes, medical and therapeutic applications and agriculture and food industries [2]. However, the most relevant and appealing characteristic that distinguishes PHAs from the traditional plastics is the capacity of being biodegradable in natural environments [6][9].

1.1.1 Types of PHA

PHA is a highly diverse group of compounds, composed of monomers with different structures and molecular weight; they can be found as simple monomers or in polymer chains with different monomer combinations. The different PHAs have distinct mechanical and physical properties [5][10][11]. PHA structure is influenced by the type of synthesizing microorganism and its growth conditions, and the carbon source provided. The enzyme PHA synthase (*PhaC*) is responsible for the PHA monomer variation in the biosynthetic pathway; there are more than 150 different building blocks of PHA with different structures [5][12]. In fact, the side chain length size of the PHA polymers can be divided in short-chain-length PHA (scl-PHA), composed of monomers with 3-5 carbon units, medium-chain length PHA (mcl-PHA) with monomers with 6-14 carbon units and long-chain-length PHA (lcl-PHA), composed by more than 14 carbon units. The lcl-PHA are not well studied yet.

The scl-PHA are a group of PHAs that have been extensively studied because their physical and mechanical properties are similar to the petroleum-based plastics, such as polypropylene (PP) and polyethylene (PE), with the advantage of being biodegradable [13][14]. Scl-PHA are produced by a large array of bacteria, including *Rhodopseudomonas palustris*, *Staphylococcus epidermidis*, *Cupriavidus necator*, *Caulobacter crescentus*, among others [12][14][15]. These polymers are characterized by their high crystallinity (crystallinity index, X_c, between 55%-80%), high molecular weight, ranging from 200 000 to 3 000 000 Da, high melting temperatures (T_m) of 160-177 °C, glass transition temperature (T_g) of -4 to +15 °C, tensile strength of 15 to 40 MPa, tensile modulus of 1.1 to 5 GPa and polydispersity index of 1.9 to 2.1 [16-18]. Poly(3-hydroxybutyrate) (P(3HB)) was the first PHA reported, which showed a lack of toxicity and excellent biocompatibility with mouse tissue [18]. However, these biopolymers have poor elongation till breakage, are stiffer and have relatively high crystalline brittleness when compared to PP, which bring constrains to their commercialization. There are some alternatives

to solve these problems, such as blending P(3HB) with other degradable polymers, chemical structure design combined with processing conditions and the inclusion of plasticizers and nucleating agents to reduce the crystallization process and to improve the flexibility [12][13] [16].

Mcl-PHA are produced by several bacteria, mainly fluorescent *Pseudomonas*, namely, *Pseudomonas citronellolis, Pseudomonas resinovorans*, *Pseudomonas chlororaphis*, *Pseudomonas putida*, among others, from renewable carbon feedstocks, like carbohydrates, lipids, alcohols, organic acids [17] [19] [20]. mcl-PHA are composed of different monomers, such as 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDd), 3-hydroxytetradecanoate (3HTd).

In contrast with scl-PHA, mcl-PHAs are by far less crystalline (X_c below 40%), have low tensile strength (up 10 mPA), high elongation to break ratios, low degrees of polymerization (molecular weight usually below 100 000 Da), low melting temperatures (T_m), between 40 and 60 °C, low glass transition temperature (T_g), -50 and -25ºC [17][21][22][16]. Thus, even at temperatures far below the frosting point, these polymers do not become brittle, making them a material of interest for rubber-like materials and biomedical applications, for example in drug delivery and tissue engineering. The monomer composition influences the physical properties of PHA, so the presence of 3HO, 3HD and 3HDd in mcl-PHA makes them more flexible and elastomeric, similar to elastomers, latexes and resins [19][20].

In order to improve the performance of PHAs, various blends of PHA have been developed with characteristics increasingly similar to traditional plastics and at lower production costs. These blends consist in co-polymers which comprise more than one type of PHA, such as poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(HBHV)) with characteristics very similar to PLA. Additionally, blends of PHA with rubber, poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA) and poly(vinyl acetate) (PVAc), among others, have also been described [3].

1.1.2 Process and commercial costs

PHA commercialization has some limitations, especially because of the high cost in production and downstream processes. The production cost of PHA is three times more expensive than PP, PE and PLA [5]. These high costs result from the high energy required for sterilization and intensive aeration, slow microorganism growth, low conversion of substrates to PHA, expensive carbon sources and expensive downstream recovery costs [5][23]. The production cost of PHA has to decrease before it becomes commercially competitive.

To improve the amount of PHA accumulated by the microorganism and to expand its diversity, various biological and synthetic engineering techniques have been developed using different metabolic pathways, *e.g.* the deletion or weakening of PHA unrelated pathways, or the design of new pathways [5]. Future engineering is also focused in developing bacteria resistant to contamination, essentially extremophiles, to reduce the energy demand in sterilization and aeration [23]. Another strategy to reduce the costs of production is the use of mixed cultures. Despite achieving the lowest accumulation of PHA and only some types of PHA are produced (scl-PHA and not mcl-PHA), using mixed cultures required few control processes, no need of sterilization and have the ability of use a huge range of substrates including and there are maximum utilization of those substrates [24].

Carbon substrates are metabolized by distinct pathways in bacterial cells, mainly by fatty acid β oxidation, *de novo* fatty acid synthesis pathways and carbohydrates biosynthesis (Figure 3) [12][24]. The carbon source substrates are expensive chemicals and raw materials, which represent between 30 to 40 % of the total production cost, being the major responsible for polymer high production cost [5][24][25]. Cheaper carbon source alternatives are key to lower the costs and make PHA economically attractive. The use of inexpensive substrates, such as waste materials rich in organic matter have been tested as a promising alternative for PHA production. Waste materials include industrial, agricultural, forest residues and wastewater [12][25-26].
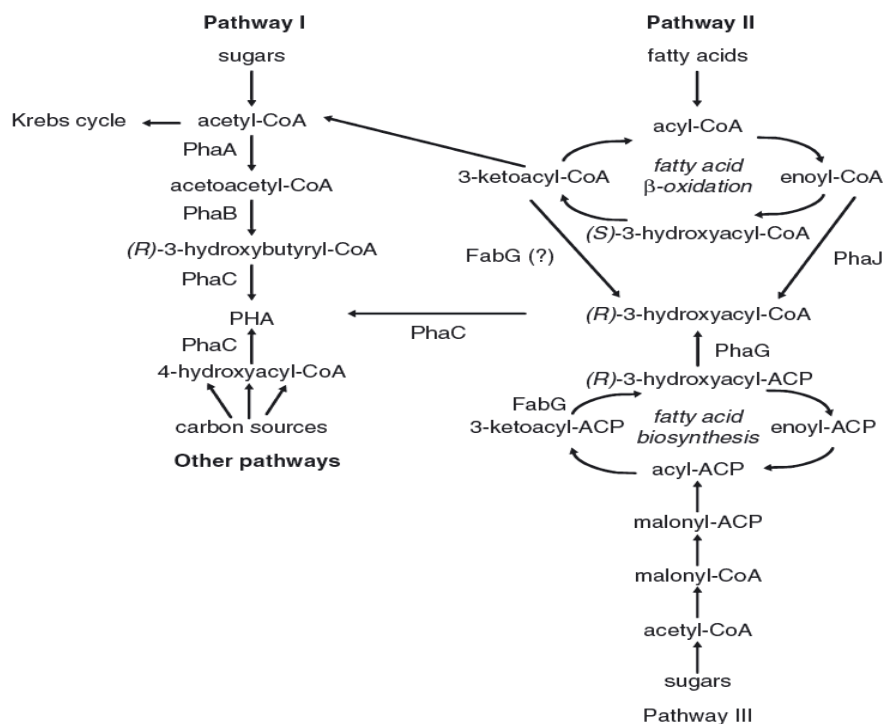
Figure 3 Metabolic pathways for PHA production [12].

The downstream process includes biomass and broth separation, as well as extraction and purification of the biopolymer, these steps also need to become cost efficient [5] [27]. To separate the cells, conventional procedures are normally used, such as centrifugation, filtration, or flocculation-centrifugation. Several techniques have been developed to recover PHA from cells. The most common method is the organic solvent extraction, which is efficient and produces PHA with a high level of purity because the polymer and the lipids are the only ones that are dissolved. The solvents most commonly used include acetone, chloroform, methylene chloride, propylene carbonate, dichloroethane. However, the need to use large amounts of solvents makes the organic solvent extraction of PHA a process that is harmful for the environment and economically unattractive [12][28]. Thus, other methods are reported such as sodium hypochlorite and surfactants treatments, chemical and enzymatic digestion, treatments with ammonia, dissolved air flotation and extraction using supercritical fluids, these alternatives have potential to substitute solvent extraction [9][12][28]. The bacteria morphology also affects the cost of the downstream processes, as it is more difficult to recover PHA from small bacteria. As a result, morphologic engineering has developed strategies to change bacterial shape and size aiming to improve the efficacy of the downstream processes [23].

1.2 Agro-industrial wastes

The increase of global urbanization and industries generates more and more agro-industrial residues, both solid and liquid. In fact, the World Bank statistics suggest that in 2025, about 2.6 billion ton of waste will be generated [29]. Agro-industrial materials consist, essentially, in stems, stalks, leaves, husks, shells, peels, lint, seed/stones, pulp cotton, groundnut, jute, vegetables, coffee, cacao, olive, tea, fruits, palm oil and crude glycerol (the main residue from biodiesel production) [30][31].

These wastes are usually incinerated or left in landfills, resulting in methane and CO_2 emission or a continued accumulation of secondary waste such as dioxins, furans, acid gases, as well as particulates, which cause environment contamination and diseases. Their non-utilization, poor valorisation and the increasing cost of waste disposal and the penalties imposed constitute a significant economical alarm [29-31]. However, with the rising concerns for the environment and pollution, a policy to effectively use waste residues has been developed, with recycling methods and the conversion of raw materials into biotechnological value-added products such as bioplastic and biofuels. These approaches would, not only have a positive impact in the environment, but also in the corresponding economies [30][31].

Agro-industrial wastes are mostly constituted by sugars, lignin, cellulose, hemicellulose, proteins, lipids and fibers. Depending on their area of application, they can be subjected to pre-

treatment strategies with biological, physical or chemical agents, followed by recovery procedures, *e.g.* to generate fermentable carbon substrates [29][31][32]. Several biotechnological approaches, using agro-industrial wastes enriched in these organic compounds has been reported, including fermentative and non-fermentative methods (table 1).

Table 1 Examples of some biotechnological strategies to add value to agro-industrial waste.

Agro-industrial Waste	Type of method used	Value added Products	Reference
Potato waste; Sugarcane bagasse; Molasse; Brewery waste;	Microorganism fermentation	Pullulan (exopolysaccharide)	[33]
Apple Pomace	Non-fermentative: Isolation and Extraction	Organic acid; enzymes; single cell proteins; ethanol; pigments	[34]
Winery wastes	Hydrolysis and Microorganism fermentation	Lactic acid; biosurfactants; xylitol; ethanol	[35]
Olive oil distillate; Biodiesel acids by-products;	Microorganism fermentation	Polyhydroxyalkanoates	[36]
Tomato Paste	Microorganism fermentation	FucoPol (exopolysaccharide)	[37]
Mango seeds	Non-fermentative: isolated by acid hydrolysis	Cellulose nanocrystals	[38]

1.3 Wineries and wine production

In 2017, the vineries occupied a total surface area of 7.5 million hectares, with a production of 69.9 of available grapes, the largest fruit crop worldwide. These grapes are used as pressed grapes in wine, musts and juices production or as unpressed grapes to consume as fresh fruit and for dried grapes production. Wine production uses about 50% of the total grapes production, a total of 32.9 million tonnes, being one of the most important agricultural activities throughout the world. The biggest producer of wine is Italy, followed by France, Spain, USA and Argentina, Portugal occupies the 11th position (Figure 4) [35][39].
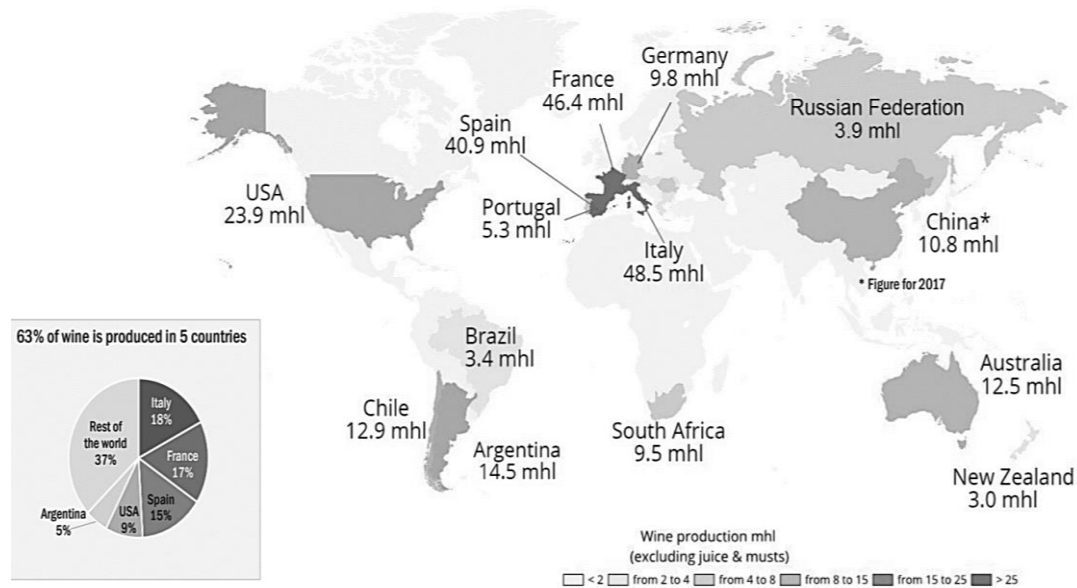
Figure 4 Countries producers of wine and volume of wine produced in millions of hectolitres (mhl), in 2018 [39].

Wine results from the total or partial alcoholic fermentation with a minimum alcohol percentage of 8.5 %, after pressing fresh grapes. Typically, the wine is still, but it can be sparkling and fortified. The still wine is made by the usual fermentation; sparkling wine has a similar process but has a final step of fermentation in the bottle, creating carbon dioxide bubbles. In fortified wines, alcohol is added during the fermentation process, which inhibits the process, consequently creating a sweeter wine as not all the sugar has been fermented and transformed into alcohol. Depending of the grapes used and the production procedures, the wine can be red, white or rosé. It can also be green or mature wine depending of the region. There are different methods to produce wine, the main difference resides in the fermentation step. To produce red wine, red grapes are used, and the grape juice is decanted after fermentation. However, in white wine production the pressed grapes are removed before the fermentation step, to produce this type of wine either red or white grapes can be used (Figure 5). Rosé wine is produced using red grapes through the white wine method, with the difference that the skins stay a short time in contact with the juice in order to give some pigment to the wine [40]. Nevertheless, it is important to note that the wine industry produces large amounts of residues, mainly grape pomace, in this way there is an opportunity to make the wine industry a more sustainable process [41][42].
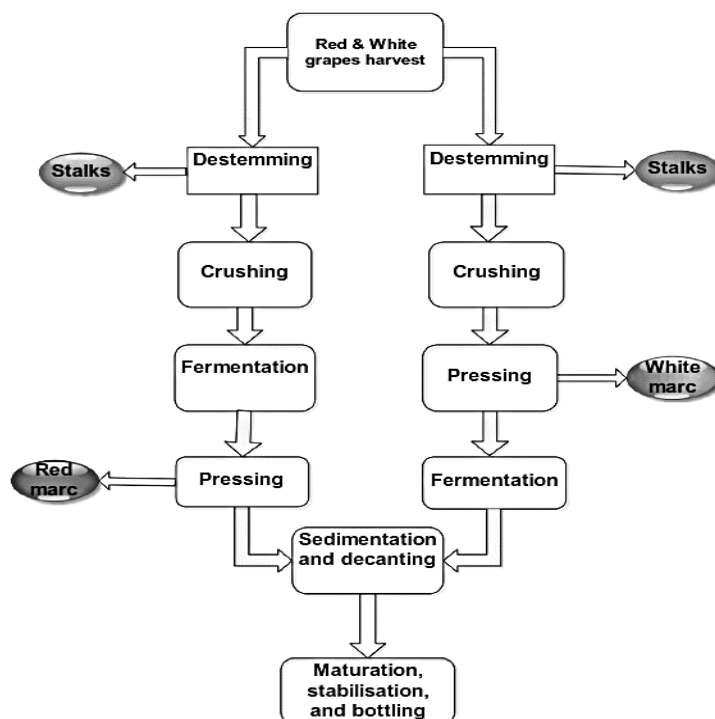
Figure 5 Red and White wine production method and step where the grape pomace (grape marc) are obtained for each method [42].

1.4 Grape Pomace

Grape pomace is the solid organic residue that remains after processing the grapes to obtain the wine. The main components are stalks, seeds, pulp and skin, which are composed of water, carbohydrates, proteins, oils, vitamins, minerals, fibers, vitamin C and phenolic compounds. Typically, large portions are discarded in landfills, used as fertilizer or processed into animal feed. Grape pomace has low pH, values ranged from 3.8 to 6.8, electrical conductivity between 1.62-6.15 dS m^{-1} and high organic matter content (669–920 g kg^{-1}), high concentrations of macronutrients, especially K (11.9–72.8 g kg^{-1}), high concentrations of polyphenols (1.2–19.0 g kg^{-1}) and heavy metals, which are incompatible with agricultural requirements. Moreover, grape pomace leaches phytotoxic agents into crops causing subsurface-flow contamination and is responsible for around 0.3% of annual greenhouse gases emission. As a result, the urgency in the development of alternatives and new approaches to the use of grape pomace is increasing [35][43].

In the last years, several studies have been performed to characterise the grape pomace in its chemical composition, functional properties, mineral content, microbiological analysis, toxic potential and biodegradability. These studies allowed the development of new biotechnological approaches to grape pomace use, rendering many products, *e.g.* purified oils, dietary fibre, phenolic compounds and bioactive compounds [44]. These products have food, cosmetic or

therapeutic applications, adding commercial value to grape pomace [45]; production of methane by anaerobic digestion [43]; production of citric acid, tartaric acid, ethanol [46]; production of bioplastics [27] and production of an antibacterial extract-based film [47]. The red and white grape pomace have also been reported to be used as substrate for the production of PHA [48][49].

In addition, grape pomace is rich in lignocellulosic material, which is a compact structure composed of hemicellulose, cellulose and lignin. Hemicellulose is composed of heterogeneous linear and branched polymer containing pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose) and/or uronic acids (α-D-glucuronic, α-D-4-O-methyl-galacturonic and a-D-galacturonic acids). Cellulose consists of a linear polymer chain of D-glucose joined by β-(1,4)-glycosidic bonds, producing a crystalline structure and is usually the mainly constituent of lignocellulose (Figure 6).
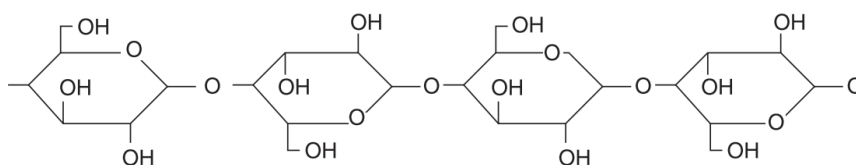
Figure 6 Structure of cellulose [31].

Lignin is an aromatic polymer of sinapyl alcohol, coniferyl alcohol and p-coumaryl alcohol synthesized from phenylpropanoid precursors and are linked together by a set of linkages to make a complicated matrix (Figure 7). Lignin is linked to hemicelluloses and cellulose forming an impermeable barrier, this structure assures strength and resistance against microbial and enzymatic attack [31][50][51].
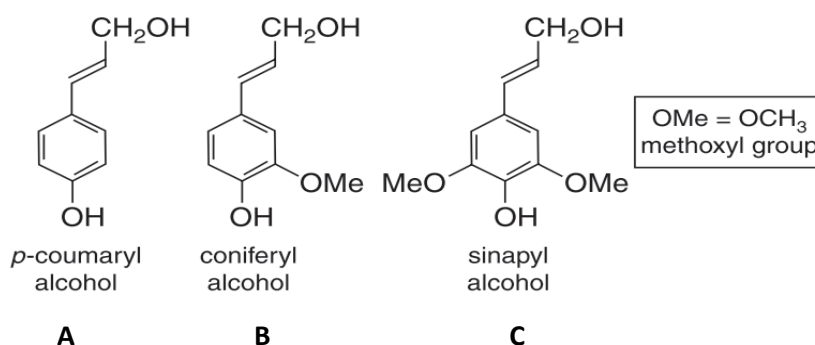
Figure 7 The constituents of lignin: p-coumaryl alcohol (A), coniferyl alcohol (B) and sinapyl alcohol (C) [31].

Lignocellulose has numerous applications in production of sustainable energy and production of fermentation product, but needs to be submitted to a pre-treatment, to break down the

polysaccharides into fermentable sugars that can then be used as substrate for microbial cultivation [29][52][53]. Thus, there are some decisive factors that influence the pre-treatment, such as specific surface area, cellulose crystallinity index, degree of polymerization, cellulose sheathing by hemicelluloses, lignin content and acetyl content [52]. The pre-treatment must be efficient and economical viable. Several lignocellulose pre-treatments are available and can be categorised as physical (milling, steam explosion, steaming treatment, hydrothermal, irradiation, freeze, extrusion), chemical (acid hydrolysis, ozonolysis, oxidative delignification, organosolv process), biological (microbiological and microaerobic treatment), enzymatic, electrical (Pulsed-Electric-Field pre-treatment) and, in some cases, a combination of these methods is use [29][50][52][54].
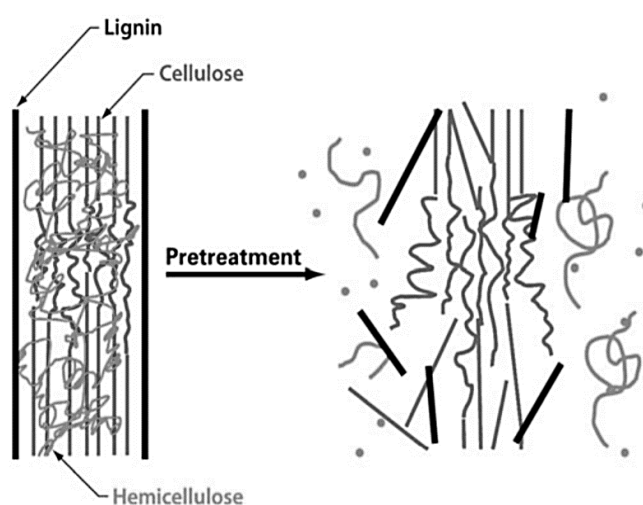
Figure 8 Representation of lignocellulosic pre-treatment [54].

1.5 Acid Hydrolysis

Acid hydrolysis uses organic or inorganic acids as catalysts, and it is one of the most commonly used methods for lignocellulosic material pre-treatment. The most applied acid is the sulphuric acid (H_2SO_4), followed by hydrochloric (HCl), nitric (HNO_3) and trifluoracetic acid (TFA). The structure of the amorphous hemicellulose is more easily hydrolysed than the crystalline cellulose. There are some parameters that can affect the efficiency of the hydrolysis, such as the acid concentration, temperature and time. This method can be divided in two general approaches, also under high acid concentration (concentrated-acid pre-treatment) and low temperatures, or under low acid concentration (dilute-acid pre-treatment) and high temperatures. Each approach has advantages and disadvantages [55][56].

Concentrated-acid pre-treatment achieves high yields of lignocellulose hydrolysis and performing at low temperatures is a clear advantage. However, the high concentration of acids

increases the corrosion of the material and high concentration of hazardous agents increases the environmental threat. Furthermore, some products derived from the degradation of hemicellulose-derived sugars (pentoses and hexoses), such as furfural and 5-hydroxymethyl furfural (5-HMF), which may not affect the enzymatic hydrolyses, can inhibit subsequent sugar fermentation and are formed during the process [52]. The inclusion of an acid recovery step is a promising way to make the process more sustainable and economic viable [55].

Dilute-acid pre-treatment can be either a step before enzymatic hydrolysis of the lignocellulosic material or the complete method of hydrolysis of the lignocellulosic material. This approach shows to be the more favourable to industrial application because it is possible to obtain high amounts of monomeric sugars from hemicellulose pre-treatment and disrupt lignin, increasing the cellulose's susceptibility to enzymatic hydrolysis. Besides that, induce less corrosion and environment problems and create low degradation products [55][56]. On the other hand, strong conditions and higher temperature is required to achieve a high yield of glucose from cellulose, resulting in a degradation of the amorphous hemicelluloses.

Recent studies report organic acids such maleic, succinic, oxalic and fumaric acids as alternatives to inorganic acids, avoiding equipment corrosions and sparing the energy needed for acid recovery [56]. Other pre-treatment method considered to be a green alternative is fractionation of the lignocellulosic material using Hot Compressed Water (HCW), since it does not use hazardous chemicals, only requires simple equipment, does not cause equipment corrosion and it is not deleterious to the environment [57].

1.6 Hot Compressed Water

Hot Compressed Water (HCW) is a hydrolysis method that uses water as "green" solvent at temperatures between the boiling point (100 °C) and the critical temperature (374 °C) and under a pressure between 3.5 and 20 MPa, which maintain the water in liquid state during the process, called subcritical state of water. This technology is also known as subcritical water hydrolysis, superheated water or pressurised hot water [58][59].

In the subcritical state of the water, the ionic product of the water (Kw), *i.e.* the product of the H^+ and OH^- ions concentrations, changes drastically with temperature, ranging between Kw=10^{-14} at 25 °C to kw=10^{-11} at 300 °C. Consequently, water behaves as an acid or base catalyst [60]. The water polarity declines significantly with increasing temperature, because of breakdown of the hydrogen bond. The solubility of the hydrophobic molecules increases, while the solubility of the ionic molecules decreases, reaching values similar to the organic solvent-water mixtures ones. The increase in temperature, also decreases the viscosity and surface tension, improving the mass transfer rates [61].

HCW is environmentally friendly because it only uses water as solvent, not needing any chemical and hazardous reagents. Since it only uses water, the raw material does not need to be dehydrated and recovered, a process that usually has high energy costs. Furthermore, being a short reaction time process, imposes less equipment corrosion concerns and produces less waste and less degradation products. HCW shows to be energy-efficient and an economical procedure [57][62].

The ability to extract/hydrolyse several compounds using HCW has been extensively researched. For example, this technology was shown to be effective in the extraction of polysaccharides from soy hulls [59], it is also capable of extracting different monosaccharides that can be used as sources of renewable biofuel and bioactive compounds from different lignocellulosic material [58][61][62].

1.7 Motivation

The consumption of petroleum-derived plastics worldwide is increasing drastically due to its highly versatile qualities, namely to its: light weight, stability, durability and chemical inertness. They are used in a huge range of applications in domestic, medical and industrial fields [3]. However, traditional plastic is not biodegradable and its continuous accumulation represents a very serious pollution problem due to the persistence of these materials in the environment [12]. Some alternatives have been developed such as the production of microbial PHA. PHA is synthetized by many bacteria has a carbon and energy reserve. The biodegradability, biocompatibility and characteristics similar to the petroleum-based plastics such as polypropylene (PP) and low-density polyethylene (LDPE) makes it a promise alternative to the conventional plastics [8]. To encourage the use of PHA in a way that this residue can reach its potential has an alternative to conventional plastic, it is essential that its production is economically viable, one way to significantly reduce production cost is to use inexpensive substrates, such as agro-industrial waste [25].

This work had as main objective the production of mcl-PHA using grape pomace, a waste from the white wine production. This residue is rich in free sugars, such as fructose and glucose (about 40%) and structural sugars (about 16%) [57], because the grape pomace from white wine production does not suffer fermentation, making it a potential microbial carbon source.

The main goal was pursued following two different approaches. The first one was the production of mcl-PHA by *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649, *Ps. citronellolis* NRRL B-2504 and *Ps. stutzeri* NRRL B-775 using the free sugars present in the grape pomace extract as carbon source. These bacteria are known to produce mcl-PHA from various substrates, including sugar-rich feedstocks [36][63][64].

In the second approach, grape pomace, already stripped from the free sugars, was hydrolysed by two different treatments: dilute-acid hydrolysis and HCW. Then, both hydrolysates were used as feedstocks to produce mcl-PHA by *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649, *Ps. citronellolis* NRRL B-2504 and P(3HB) by *B. sacchari* DSM 17165.

The bottom-line goal of this work was to valorise wine grape pomace by its conversion into value-added bioplastics, mcl-PHA and P(3HB), that are strong candidates to substitute traditional plastics.

2.Material and Methods

2.1 Grape Pomace Aqueous Extract

2.1.1 Grape Pomace Aqueous Extract Preparation

Grape pomace was provided by a Portuguese wine producer, from Herdade do Esporão, Alentejo. The water content was provided by vinery and had about 40 wt.% of water. The wet grape pomace was milled to the smallest particle size possible, using a blender machine (Tristar BL-4427), and diluted with deionized water to obtain a total solids concentration of 30 g/L, considering that the residue contained 40 wt.% of water. The solution was autoclaved (20 min, 121 °C, 1 bar), to potentiate the extraction of free sugars. Then, the autoclaved solution was centrifuged twice at 9000xg and 4 °C, during 25 min. The pellets containing most of the insoluble material of the residue were dried in an oven overnight, at 60 °C, and stored to be used latter. To remove the small particles in suspension, the supernatant was filtered with paper coffee filters. The grape pomace extracts obtained were used as sole feedstock for shake flask and bioreactors assays.

2.1.2 Grape Pomace Aqueous Extract Characterization

The free sugars present in the grape pomace extract were quantified by High Performed Liquid Chromatography (HPLC) as described below. The pH value was measured (pH1100L, VWR pHenomeralTM).

2.2 Grape Pomace Hydrolysates

2.2.1 Hot Compressed Water

The Hot Compressed Water extraction was performed to obtain sugar-rich extracts from the structural sugars present in the grape pomace pellets obtained by centrifugation as described in Grape Pomace Extract Preparation.

2.2.1.1 Semi-continuous Hot Compressed Water

A TOC7-20-G REACTOR (HiP High-Pressure Equipment Company, USA), 51 cm long, with 5 cm external diameter and 2.6 cm internal diameter stainless steel tube, was used to perform the semi-continuous HCW process (Figure 1). The reactor was filled with the dry grape pomace residue pellets (≈60 g), mixed with glass beads (≈200 g) to fill up the free volume. The material was kept between pour discs, to avoid clogging. The reactor was then placed in an electrical oven with temperature control. Distilled water was pumped through the reactor using a preparative pump 1800 (KNAUER 40). Before entering the reactor, the water was heated to achieve the desired temperature (190 °C). The temperature and pressure of the system were controlled by a thermocouple monitor and Back Pressure Regulator (BPR; Tescom Europe®, 26-1000), respectively. The valves and fittings used were from HIP and SWAGELOK. The water

exiting the reactor was collected for analysis, to determine which conditions yielded a higher amount of sugars. To start the experiment, the pump was switched on at the selected flow rate (between 5 and 10 mL/min) and the BPR was set to the intended pressure (100 bar). When the pressure reached, the heating was turned on. Two temperatures where tested, 190 °C and 250 °C, obtaining two different fractions of grape pomace hydrolysate. The first sample was collected when the temperature of the outlet stream reached 190 °C and was kept at constant temperature for 30 minutes. The second sample was taken at a temperature between 190 °C and 250 °C and at constant final temperature for 30 minutes. The samples were stored in Schott flasks, at 4 °C. The quantity of sugar in each sample was analysed by HPLC, as described below.
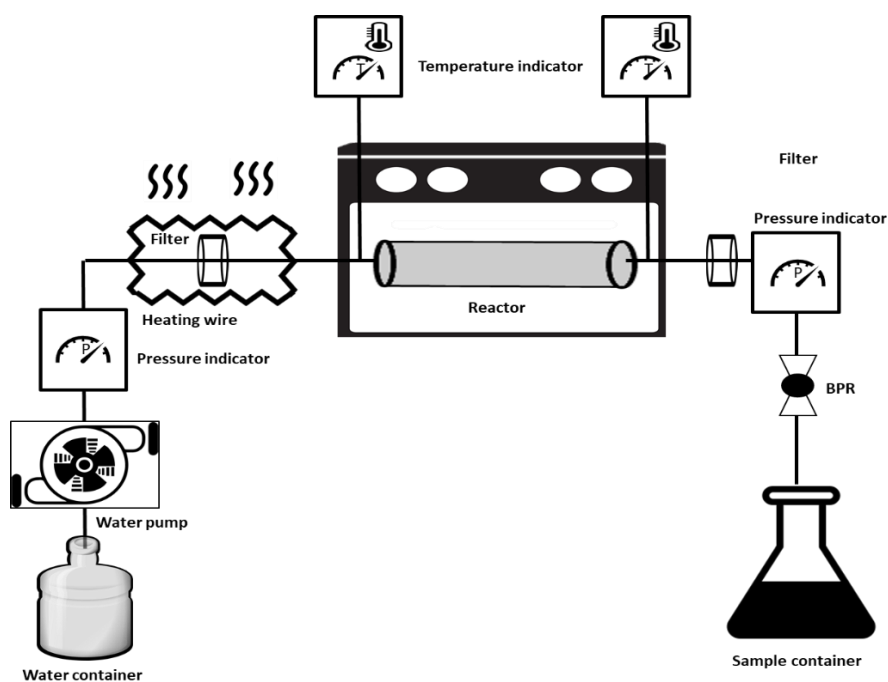
Figure 9 Schematic of the Semi-Continuous Hot Compressed Water experimental set-up.

2.2.1.2 Batch Hot Compressed Water

The batch process was performed in a reactor (Parr Instrument Company) with 1200 mL capacity. The reactor was filled with the dry grape pomace residue pellets (≈80 g) and deionized water (800 mL) and was correctly assembled in the high-pressure system (4540 High Pressure Reactors). The pressure was selected to 50 bar using nitrogen to maintain the pressure inside the reactor. The electrical heater was placed and the temperature was controlled (Parr 4848 Reator Controller) to test two different temperatures 190 °C and 250 °C. The agitation was turned on and remained constant throughout the experiment. All the valves and equipment were from Parr Instruments Company. Samples of the liquid fraction (10mL) were taken when the desired temperature was reached inside the reactor, after which samples were taken at constant temperature in time intervals of 10 and 20 minutes. When the experiment was over,

the pressure was relieved, and the electrical heater disassembled. When the equipment reached the room temperature the broth was collected to be treated and used in bacterial cultivation experiments.
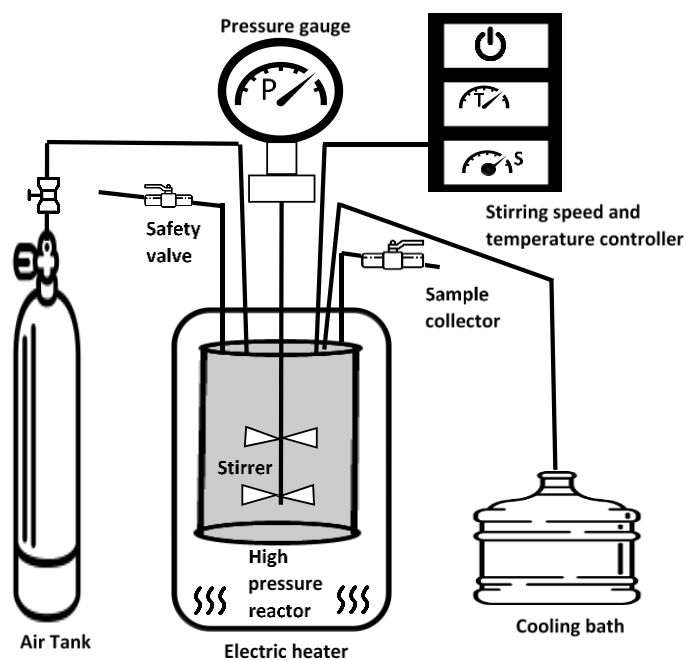
Figure 10 Schematic of the Batch Hot Compressed Water experimental set-up.

2.2.1.3 Grape Pomace HCW extract Preparation and Characterization

The extracts obtained by the different HCW procedures were characterized by HPLC to quantify their sugar content and composition. After the analyses, the batch HCW at 190 ºC treatment was selected to obtain extract in quantities required for the cultivation experiments. The HCW extract collected was centrifuged at 9000xg and 4 ◦C, during 30 min and filtered with paper coffee filters. The filtered solution was concentrated 5 time, using Rotavapor (Rotavapor R-210). The pH of the concentrated HCW extract was measured (pH1100L, VWR pHenomeralᴛᴍ) (≈ 4.5) and neutralized by the addition of NaOH (pH=7). The grape pomace HCW extract was used as sole feedstock for shake flask assays.

2.2 Dilute Acid Hydrolysis

A dilute acid hydrolysis was also perfomed to obtain simple sugars from the structural sugars present in the grape pomace residue (after extraction in the autoclave, as decribed above).

2.2.1 Grape Pomace acid hidrolysate Preparation and Characterization

The dried grape pomace residue pellets (500 g) were mixed in 2 L deionized water containing 3% (v/v) sulfuric acid (H_2SO_4, 95-97 % Sigma Aldrich). The solution was autoclaved at 121 °C, for 20 min. The solution was centrifuged at 8000xg and 4 °C, for 30 min. The supernatant was collected and filtered with paper filters, to remove some solids that remained in suspension. The

pH was measured (pH1100L, VWR pHenomeral™) (≈ 0.5) and NaOH pellets were added to obtain a neutral solution (pH= 7). The solution was used to perform the shake flask experiments.

2.3 Screening Assays

2.3.1 Grape Pomace Extract

2.3.1.1 Microorganism and pre-inoculum

Four bacterial cultures from the Genus *Pseudomonas*, namely, *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649, *Ps. citronellolis* NRRL B-2504 and *Ps. stutzeri* NRRL B-775 were used in this assay. All the bacterial strains were cryopreserved in liquid Luria Bertani (LB) medium with glycerol (20% v/v) at -80 °C. To reactivate the cultures, a sample of each cryovial was cultivated in Chromagar (CHROMagar™Orientation) plate and inoculated at 30 °C, during 48 h.

Thereafter, an isolated colony of each culture was inoculated into 20 mL liquid LB medium (10.0 g/L bacto tryptone (Bechon, Dickinson and Company); 5.0 g/L yeast extract (Panreac AppliChem); 10.0 g/L NaCl (Panreac AppliChem)) (pH=7) in 50 mL baffled shake flask and incubated in an orbital shaker (New Brunswick Scientific), at 200 rpm and 30 °C, for 24 h. These cultures were used as pre-inoculum for shake flask and bioreactor assays.

2.3.1.2 Shake Flask assay

The pre-inoculum (20 mL) of each culture was used as inoculum into 500 mL shake flasks containing 150 mL grape pomace as the sole feedstock, and 20 mL Medium E* concentrated 10x, with the following composition (per litter): $(NH_4)_2HPO_4$, 33 g; K_2HPO_4 58 g; and KH_2PO_4, 37 g, 100 mL of a 100 mM $MgSO_4$ solution and 100 mL of a microelements solution. The microelements solution contained (per litter) $FeSO_4$- $7H_2O$, 27.8 g; $MnCl_2$ $4H_2O$, 1.98 g; $CoSO_4$ $7H_2O$, 2.8 g; $CaCl_2$ $2H_2O$, 1.67 g; $CuCl_2$ $2H_2O$, 0.17 g; $ZnSO_4.7H_2O$, 0.29 g. The cultures were incubated in an orbital shaker at 200 rpm and 30 °C, for 72 h. During the shake flask experiment, two daily sample (10 mL) were collected for OD_{600nm} measurement, cell dry weight, ammonium, sugars and mcl-PHA quantification and Nile Blue staining. All the manipulation was done in a laminar flow chamber (Heraeus SB48) and all the solutions and material used were previously autoclaved (20 min, 121 °C, 1 bar), in order to prevent contamination of the cultures.

2.3.2 Grape Pomace Hydrolysates

2.3.2.1 Microorganisms and pre-inoculum

Three mcl-PHA producing bacteria, *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649, *Ps. citronellolis* NRRL B-2504 and one producing PHB, *Burkholderia sacchari* DSM 17165, were used in this assay. To reactivate the cryopreserved cultures, a sample of each cryovial was cultivated in Chromagar plates and incubated at 30 °C, during 48h. An isolated colony of each culture were inoculated into 15 mL liquid LB medium (10.0 g/L bacto tryptone; 5.0 g/L yeast

extract; 10.0 g/L NaCl) (pH=7) in 50 mL baffled shake flask and incubated in an orbital shaker (New Brunswick Scientific) at 200 rpm and 30 °C, for 24h. These cultures were used as pre-inoculum for shake flask assay.

2.3.2.2 HCW Hydrolysate Shake Flask Experiments

The pre-inoculum (15 mL) of each culture were used as inoculum into 200 mL shake flasks cultivations with the medium E* (15 mL) and grape pomace hydrolysate from batch compressed water hydrolysis at 190 °C (150 mL) as the sole feedstock. All the conditions and complementary solutions were the same as previously described. During the shake flask experiments, two daily samples (8 mL) were collected for OD_{600nm} determination, cell dry weight, ammonium, sugars and mcl-PHA quantification and Nile Blue staining.

2.3.2.3 Acid Hydrolysate Shake Flask Experiments

The pre-inoculum (15 mL) of each culture was used as inoculum into 200 mL shake flasks cultivations with the medium E* (15 mL) and grape pomace hydrolysate obtained by acid hydrolysis with H_2SO_4 (150 mL) as the sole feedstock. All the conditions and complementary solutions were the same as previous reported. During the shake flask experiment, two sample (8 mL) were collected daily for OD_{600nm} determination, cell dry weight, ammonium, sugars and mcl-PHA quantification and Nile Blue staining. During the shake flask experiment, two sample (8 mL) were collected daily for OD_{600nm} determination, cell dry weight, ammonium, sugars and mcl-PHA quantification and Nile Blue staining.

2.4 Bioreactor assays

2.4.1 Batch assay

2.4.1.1 Bacterial strain and inoculum

The strain used in batch assay was *Ps. chlororaphis* DMS 19603. The inoculum was prepared by transferring a cryovial, previously prepared, to a 500 mL sake flask with 200 mL of LB medium. The shake flask was incubated in an orbital shaker (New Brunswick Scientific) at 200 rpm and 30 °C, for 24 h.

2.4.1.2 Bioreactor operation

The batch cultivation of *Ps. chlororaphis* DMS 19603 was performed in 2L BioStat®B-Plus bioreactor (Sartorius, Germany). The cultivation medium was composed of 1.6 L grape pomace (prepared as described above), supplemented with 200 mL medium E* (prepared as described above). A 10% (v/v) inoculum (200 mL) was used.

The pH was controlled at 7.0 ±0.1 by the automatic addition of 2 M NaOH or 2 M HCl, and the temperature was controlled at 30.0 ± 0.1 ºC. A constant air flow rate (1 SLPM, standard litre per minute) was kept during all the experiments. The dissolved oxygen concentration (DO) was

controlled at 30% of the air saturation by automatically adjusting the stirring speed between 300 and 800 rpm. Foam formation was automatically suppressed by addition of Antifoam A (Sigma-Aldrich). During the assays, samples (18 mL) were periodically taken from the bioreactor for OD_{600nm} determination and quantification of the cell dry weight, ammonia, sugars concentration and mcl-PHA accumulation. The batch operation was performed during 24 h.

2.4.2 Fed-batch assays

2.4.2.1 Bacterial strain and inoculum

The strains used in the fed-batch assays were *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649 and *Ps. citronellolis* NRRL B2504. The inoculum was prepared by transferring a cryovial, previously prepared, to a 500 mL sake flask with 200 mL of LB medium. The shake flask was incubated in an orbital shaker (New Brunswick Scientific) at 200 rpm and 30 ºC, for 24 h.

2.4.2.2 Bioreactor operation

The fed-batch cultivations of *Ps. chlororaphis* DMS 19603, *Ps. resinovorans* NRRL B-2649 and *Ps. citronellolis* NRRL B-2504 were performed in 2 L BioStat®B-Plus bioreactors. The cultivation medium was composed of 1.6 L grape pomace as the sole carbon source, supplemented with 200 mL medium E* (prepared as described above). A 10% (v/v) inoculum (20 mL) was used in all assays. After 6 h of cultivation, *Ps. chlororaphis* DMS 19603 and *Ps. citronellolis* NRRL B-2504 bioreactors were fed with concentrated grape pomace extract (10x), at a constant feed-rate of 50 mL/h. For *Ps. resinovorans* NRRL B-2649, the assay, the fed-batch phase was initiated after 20 h of cultivation.

The pH was controlled at 7.0 ±0.1 by the automatic addition pf 2 M NaOH or 2 M HCl, at 30.0 ± 0.1 ºC. A constant air flow rate (1 SLPM, standard litre per minute) was kept during all the experiments. The dissolved oxygen concentration (DO) was controlled at 30% of the air saturation by automatic adjustment of the stirring speed between 300 and 800 rpm. Foam formation was automatically suppressed by addition of Antifoam A (Sigma-Aldrich). During the assay, samples (18mL) were periodically taken from the bioreactor for OD_{600nm} determination and quantification of the cell dry weight, ammonia, sugars concentration, mcl-PHA and EPS. The assays took 30 h for *Ps. chlororaphis* DSM 19603 and *Ps. citronellolis* NRRL B-2504 cultivations, and 54 h for *Ps. resinovorans* NRRL B-2649 cultivation.

2.5 Biopolymer extraction

At the end of the bioreactor cultivation assays, the broth was centrifuged (9000xg, during 20 min, at 10 ◦C) and the cell pellets were washed by suspending them twice in deionized water and centrifuged under the same conditions. Afterwards, the cell pellet was lyophilized (ScanVAc CoolSafe TM, LaboGene) and the polymer was extracted by Soxhlet extraction with chloroform

(Sigma-Aldrich, HPLC grade) as solvent (7g of biomass for 250 mL of chloroform) at 80˚C during 48 h. The cellular debris was 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), under vigorous stirring. The precipitate was then recovered in a pre-weighted flask and left at room temperature, in a fume hood, for solvent evaporation.

2.6 Analytical Techniques

2.6.1 Cell growth

Cell growth was evaluated during the experiments by measuring the optical density, at 600 nm (OD_{600nm}) (VWR V-1200 spectrophotometer, Portugal), of the broth samples with the necessary dilution in deionized water. Deionized water was used as zero reference. Two replicas were measured.

2.6.2 Biomass quantification

The cell dry weight (CDW) of each sample was determined by gravimetry. The samples were centrifuged (10000xg, during 15 min, at 4 ºC) and the cell pellets were washed, suspended once in deionized water and centrifuged. The pellets were freeze-dried (ScanVac CoolSafeTM, LaboGene) at -110 °C, for 48 h. The cell pellets were weighted to obtain the CDW. Two replicas were measured.

2.6.3 Nile Blue Staining

Nile Blue (0.1% v/v) was added to an Eppendorf tube with 0.5 mL of each broth sample, covered with aluminium foil and placed in an oven at 100 °C, for 5 min. Then, slides where prepared to be observed under the microscope (Olympus BX51 epifluorescence) under contrast and fluorescent light, both with a magnification of 100x.

2.6.4 Quantification of sugars

Sugar concentration from the assay using grape pomace aqueous extract was determined by High Performance Liquid Chromatography (HPLC), using a VWR Hitachi Organizer (Pump L-2130, Auto sampler L-2200, Column Oven L-2350) and Detector (Merck Differencial Refractometer RI-71). The column used was Aminex HPX-87H 300x7.8mm and Biorad 125-0129 30x4.6mm at 30°C, 0.5mL/min, during 18 minutes with an injection volume of 99 µL. The cell free supernatants were diluted (1:50) in eluent, H2SO4 0.01 (SIGMA-ALDRICH) and filtered with Vectra Spin Micro Polyssulfone filter (0.2 µm) at 3000 rpm, for 15 min. Standards were prepared using a sugar mix of fructose and D-(+)glucose (Sigma-Aldrich, 99%), with a concentration between 0.0625 and 1 g/L.

Sugar concentration from the assay using grape pomace hydrolysate (obtain by acid hydrolysis

and HCW) was determined by HPLC using Dianex ICS3000 and Detector PAD (pulsed Amperometric Detection). The column used for Xylose and Mannose analysis was Carbopac SA10 250x40 mm, using 1mM NaOH as eluent, at 40 °C, 1.2 mL/min, with an injection volume of 10 μL. To analyse the other sugars and uranic acids the column used was CarboPac PA10 250x40 mm and AminoTrap 50x40 mm, the eluents was 200mM NaOH (9-100%) and 1M CH_3COONa (0-17%), at 25 °C, 1mL/min, with an injection volume of 5 μL. The cell free supernatants were diluted (1:50) with deionized water and filtered with Whatman Nylon Filters (0.2 μm) to vials. Standards were prepared using a mix of D-(-)-fucose (98 %, Scharlau), D(-)Arabinose (99 %, Sigma), D-(+)-glucose (99 %, Fluka), D-(+)-Galactose (99 %, Fluka), D-(-)-Fructose (98.5 %, Scharlau), L-rhamnose monohydrate (99 %, Fluka), D-(+)-Galacturonic acid (97 %, Fluka) and D-glucuronic acid (98 %, Alfa Aesan) and a mix D-(+)-mannose (99 %, Fluka) and D-(+)-Xylose (99 %, Sigma-Aldrich), both prepared with deionized water in concentrations between 5 ppm and 100 ppm.

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

2.6.6 PHA characterization

2.6.6.1 Composition

The PHA content in the biomass and composition were determined by Gas Chromatography with flame ionization detector (GC-FID) , (430-GC, Bruker) with a Restek column of 60 m, 0.53 mmID, 1 μM df, Crossbond, Stabilwax. In this procedure were achieved the mcl-PHA composition of dried cell samples as well of purified polymers extract with chloroform. The dried cell (10 mg) and the purified polymer (2 mg) were hydrolysed with 2 mL 20% (v/v) sulphuric acid (SIGMA-ALDRICH, HPLC grade) in methanol (Fisher Chemical) solution and 2 mL of benzoic acid in chloroform (1 g/L) (SIGMA-ALDRICH). The hydrolysis was performed on a dry bath at 100˚C, for 4 h. Afterwards, 1 ml of deionized water were added and the organic phase was recovered in vials to be analysed. 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 at a rate of 20˚C/min until 100 ˚C, 3 to 21 min at a rate of 3˚C/min until 155˚C and 21 to 32 min at a rate of 20˚C/min until 220˚C.

The PHA standard was prepared at 2 g/L and diluted into different concentrations ranging from 0.1 to 1.75 g/L with benzoic acid in chloroform (1 g/L) and 2 mL 20% (v/v) sulphuric acid (SIGMA-

ALDRICH, HPLC grade) in methanol (Fisher Chemical) solution. The composition of the mcl-PHA standard was 2.1 wt% 3-hydroxyhexanoate (3HHx), 15.5 wt% 3-hydroxyoctanoate (3HO), 63 wt% 3-hydroxydecanoate (3HD), 10.7 wt% 3-21hydroxydodecanoate (3HDd) and 8.7 wt% 3-hydroxytetradecanoate (3HTd) previously analysed and certificated by GC-MS. The PHA standards used was P(3HB-co-3HV) (Sigma-Aldrich, 88 mol% 3HB, 12 mol% 3HV) with concentrations ranging from 0.4 to 8.0 g/L.

2.6.6.2 Molecular Mass Distribution

A sample (15 mg) of each polymer was dissolved in 3 mL of chloroform, for 18 h at room temperature. Then, the solution was filtered with a glass fibber filter 47 mm (PALL) and analysed by a Size Exclusion Chromatography (SEC) System (Waters Millenium) with support SEC: PLgel 5 μm Guard; Polymer Laboratories; 50×7.5 mm, PLgel 5 μm 104 Å; Polymer Laboratories; 300×7.5 mm, PLgel 5 μm 500 Å; Polymer Laboratories; 300×7.5 mm. Using a temperature of equilibration of 30˚C, with a flow rate of 1 mL/min, degasing and chloroform as the mobile phase. Samples were stored at 4 ˚C before injecting 100 μL in the SEC circuit. A RI detector (Waters 2410) was used for polymer detection using a sensitivity of 512 and a collection period of 25 min. The relative molecular weight (Mw) of the polymers were determined adopting monodisperse polystyrene standards with Mw ranging between 800 Da to 504 kDa. SEC Water software relying upon the universal calibration method was used to calculate the relative Mw of mcl-PHA.

2.6.6.3 Thermal Properties

Differential Scanning Calorimetry (DSC) analysis was performed using a differential scanning calorimeter DSC 131 (Setaram, France). The samples were placed in aluminium crucibles and analysed at temperatures ranging between -90 and 120 °C, with heating and cooling speeds of 10 °C/min. Thermogravimetric Analysis (TGA) was performed using a thermogravimetric equipment Labsys EVO (Setaram, France). Samples were placed in aluminium crucibles and analysed at temperatures ranging between 25 and 500 °C, with heating and cooling speeds of 10 °C/min. The melting temperature (T_m, ˚C) was determined at the minimum of the exothermic peak. The degree of crystallinity (X_c, %) was calculated by comparing the area of the melting peak (ΔHm, J/g) with the melting enthalpy of 100% crystalline P(3HB) (ΔHm100%). The heat of melting of an infinite crystal of P(3HB) was estimated as 146 J/g.

$$Xc = \frac{\Delta Hf}{\Delta Hf,100} \times 100 \qquad (1)$$

2.6.7.1 Polysaccharide quantification

Polysaccharide presence in the grape pomace extract and cell-free supernatant was evaluated first by dialyse and after by gravimetry. Samples (5mL) were dialyzed with a 12000 MWCO

membrane (ZelluTrans Carl Roth Cellulose Membrane SO farblos) against deionized water (5 L) with a constant stirring. Sodium azide (1 mg) was added to prevent contamination. The water was renewed 4 times a day until the conductivity value was below 10 µS/m. Afterwards the dialyzed samples were freeze dried (Scanvac, CoolSafe) during 48 h. To quantify the polysaccharide, the dried samples were weight. Three replicas of each sample were measured.

2.6.7.2 Polysaccharide determination

The identification and quantification of the constituent monosaccharides were performed by High Performance Liquid Chromatography (HPLC). Polysaccharide samples (5 mg) were dissolved in deionized water (5 mL) and hydrolysed with trifluoroacetic acid (TFA) (0.1 mL TFA 99 %) in a dry bath at 120 °C, during 2 h. After the hydrolysis the samples (1mL) were filtered with Whatman Nylon Filters (0.2 µm) to vials. The column used for Xylose and Mannose analysis was Carbopac SA10 250x40 mm, using 1mM NaOH as eluent, at 40 °C, 1.2 mL/min, with an injection volume of 10 µL. To analyse the other sugars and uranic acids the column used was CarboPac PA10 250x40 mm and AminoTrap 50x40 mm, the eluents was 200 mM NaOH (9-100%) and 1M CH_3COONa (0-17%), at 25 °C, 1 mL/min, with an injection volume of 5 µL. Standards were prepared using a mix of D-(-)-fucose (98 %, Scharlau), D(-)Arabinose (99 %, Sigma), D-(+)-glucose (99 %, Fluka), D-(+)-Galactose (99 %, Fluka), D-(-)-Fructose (98.5 % Scharlau), L-rhamnose monohydrate (99 %, Fluka), D-(+)-Galacturonic acid (97 % Fluka) and D-glucuronic acid (98 %, Alfa Aesan) and a mix D-(+)-mannose (99 % Fluka) and D-(+)-Xylose (99 % Sigma-Aldrich), both prepared with deionized water (5 mL) and hydrolysed with trifluoroacetic acid (TFA) (0.1 mL TFA 99 %) in a dry bath during 2 h. Then the hydrolysed standards were diluted in concentrations between 5 ppm and 100 ppm with deionized water.

2.7 Calculations

The active biomass yield on substrate basis $(Y_{X/S})$ and polymer production yield on substrate basis $(Y_{P/S})$ was calculated by equation 2 and 3, respectively:

$$YX/S = \frac{xf - xi}{Sf - Si} \tag{2}$$

$$YP/S = \frac{Pf - Pi}{Sf - Si} \tag{3}$$

where xf and xi are the final and initial active biomass, Sf and Si are the final and initial concentration of sugars and Pf and Pi are the final and initial PHA produced.

The volumetric productivity (r_p, g/L.h) was determined by equation 4:

$$rP = \frac{dP}{dt} \tag{4}$$

where P is the final PHA produced (g/L) at t time (hours).

3. Results and discussion

3.1 White wine grape pomace

The grape pomace was constituted by the solid residue that remained after pressing the grapes to obtain white wine. It was composed of skins, seeds, stalks and pulp (Figure 11).

Figure 11 White wine grape pomace.

The grape pomace contains around 40 wt% of soluble carbohydrates and 16 wt% structural carbohydrates [57]. In order to value the residue to its maximum potential, strategies to use both types of carbohydrates were implemented. The soluble sugars were extracted with deionized water, autoclaved, centrifugated and filtered (Figure 12). The supernatant was grape pomace extract with the soluble sugars, namely: glucose and fructose, at a concentration of 12.34±0.46 and 10.77±0.39 g/L, respectively (Table 2). This extract was used as feedstock for microbial growth and mcl-PHA production in shake flask and bioreactor experiments.

Figure 12 Aqueous extract of grape pomace used for microbial growth in shake flask and bioreactor experiments.

Table 2 Soluble sugar constituents of grape pomace aqueous extract.

Glucose (g/L)	12.34 ±0.46
Fructose (g/L)	10.77 ±0.39

The polysaccharides present in the grape pomace extract were quantified after its dialysis obtaining 2.75 ±0.25 g/L (Table 3). The main constituent monosaccharides were galacturonic acid and arabinose, at concentrations of 0.13 g/L and 0.12 g/L, respectively, followed by galactose, 0.06 g/L. There was also rhamnose, glucose, xylose and mannose, all at a concentration of 0.02 g/L each.

The pellet obtained from the centrifugation of the grape pomace was constituted mostly of structural sugars (Figure 13). The pellet was dried overnight and hydrolysed to obtain monosaccharides. Two approaches were tested: HCW and acid hydrolysis. The grape pomace hydrolysate was used as feedstock for microbial growth and production of PHB and mcl-PHA.

Figure 13 Dried pellet obtain from the centrifugation of grape pomace with deionized water.

Batch HCW and semi-continuous HCW were both evaluated at two different temperatures, 190 °C and 250 °C. All the fractions obtained were centrifuged and filtered. The monosaccharides present in the grape pomace HCW hydrolysate were analysed in terms of sugar composition (Table 3). Semi-continuous and batch HCW hydrolysis are completely different processes, with different heating rates and different residence times, since in the batch hydrolysis, after dissolving, there is still reaction and in the semi-continuous process the water with the hydrolysate sugars were continuous collected, so in Batch hydrolysis is more probably that the monosaccharide degradation occurs. However, at the same temperature, the semi-continuous HCW and the batch HCW did not differ significantly. Glucose, xylose and arabinose were the most abundant monosaccharides obtained from hemicellulose in both approaches, batch and semi-continuous, at 190 °C (Table 3). Fructose was also one of most abundant monosaccharides. However, fructose is not part of the lignocellulosic structure. The high levels of fructose detected

could result from some soluble fructose that remained in the pellet after the centrifugation to obtain the grape pomace extract or could be due to the Lobry de Bruyn– Alberda van Ekenstein transformation (LBET), which consists in the conversion of D-glucose into D-fructose, with the reverse reaction being much slower. This normally occurs under room temperature and at high pH, but the increased ionic product of HCW at the temperatures reached in the experiments could have triggered this reaction even at neutral pH [57]. Fucose, rhamnose, galactose, mannose, acid galacturonic and acid glucuronic were also detected, but in lower concentrations (Table 3).

The increase in temperature, generally led to an increase in the extent of hydrolysis, which is consistent with the increase in the ionic product of water that, thus, became a stronger catalyst for the hydrolysis of biomass [57]. However, when the temperature used was 250 °C, both HCW semi-continuous and batch, almost all the monosaccharides are not available (Table 3). These results may indicate that although the lignocellulosic structure was hydrolysed in monosaccharides, these simple sugars where degraded due the high temperatures.

Table 3 Sugar composition of the hydrolysed polysaccharide extract, obtained by batch HCW and semi-continuous HCW, both at 190 °C and 250 °C and by acid hydrolysis 3 %(v/v) H_2SO_4.

Monosaccharides (g/L)	Semi-continuous HCW (190 °C)	Semi-continuous HCW (250 °C)	Batch HCW (190 °C)	Batch HCW (250 °C)	Acid Hydrolysis 3 %(v/v) H_2SO_4
Fucose	0.02	n.a.	0.02	n.a.	0.08
Rhamnose	0.07	n.a.	0.06	n.a.	0.14
Arabinose	0.07	0.02	0.29	0.11	0.82
Glucosamine	n.a.	n.a.	n.a.	n.a.	n.a.
Galactose	0.03	n.a.	0.13	n.a.	0.59
Glucose	0.37	n.a.	0.29	n.a.	3.21
Mannose	0.02	n.a.	n.d.	n.d.	n.d.
Xylose	0.11	n.a.	n.d.	n.d.	n.d.
Fructose	0.80	n.a.	0.21	0.01	2.22
Ribose	n.a.	n.a.	0.01	n.a.	0.02
Galacturonic acid	0.02	n.a.	0.01	0.01	0.47
Glucuronic acid	0.01	n.a.	0.01	0.02	n.a.

n.a. – data available; n.d.- not detected

Although the semi-continuous HCW at 190 °C was the technique were the concentration of monosaccharides obtained was higher (1.52 g/L), the technique chosen for microbial growth

and polymer accumulation in shake flask experiments was the batch HCW at 190 °C (Figure 14) due to the simpler equipment utilization and the much lower time spent in the batch procedure.

Figure 14 Hydrolysate obtained from batch HCW at 190 °C used for microbial growth and polymer accumulation.

For the acid hydrolysis, the pellet was mixed with deionized water and 3% (v/v) H_2SO_4 was added to the solution and was autoclaved. Then the solution was centrifuged and filtered (Figure 15). The pH obtained was 0.5 and NaOH was added to obtain a pH=7.
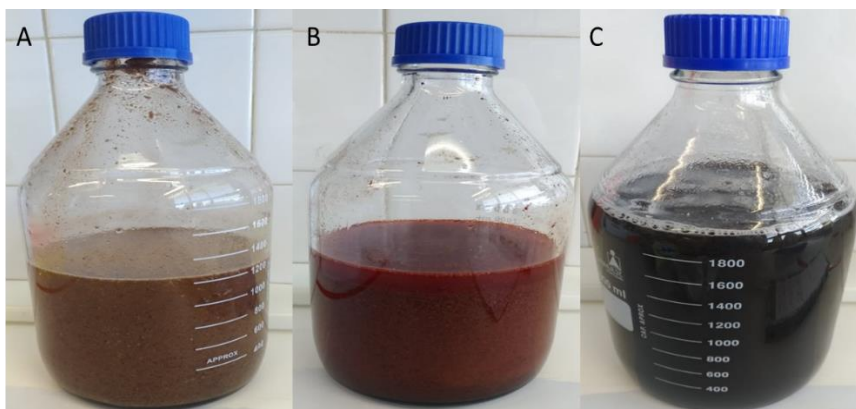
Figure 15 A-Dried pellet mixed with deionized water and H_2SO_4 B- mixture A autoclaved; C- hydrolysed solution with the pH neutralized.

The monosaccharides obtained by acid hydrolysis were the same as by HCW at 190 °C, except for glucuronic acid, which was not detected (Table 3). The sugars present at the highest concentration were glucose and fructose, 3.21 g/L and 2.22 g/L respectively. The presence of fructose may be explained as mentioned above for the HCW hydrolysate. The sugars arabinose (0.82 g/L), galactose (0.59 g/L) and galacturonic acid (0.47) had also considerable high concentrations (Table 3). Fucose, rhamnose and ribose are also present in trace concentrations (Table 2). Additionally, mannose/xylose was detected but it was not possible to quantify these

sugars. The total sugar concentration, considering all the detected monosaccharides, was 7.59 g/L. The hydrolysed solution (Figure 15C) was used as feedstock for microbial growth and polymer accumulation in shake flask experiments.

3.2 Screening assay

3.2.1 Grape pomace aqueous extract

First, shake flask experiments were used to evaluate the ability of different bacterial strains from the Genus *Pseudomonas* (Figure 16) to use the soluble carbohydrates (glucose and fructose) of grape pomace aqueous extract for cell growth and PHA accumulation, specifically mcl-PHA.

This screening assay studied a well-known group of mcl-PHA producing bacteria, namely, *Ps. citronellolis* NRRL B-2504, *Ps. chlororaphis* DMS 19603, *Ps. stutzeri* B-2504 and *Ps. resinovorans* NRRL B-2649 and aimed to identify which one(s) would show higher cell growth and more efficient polymer accumulation when using grape pomace aqueous extract as sole feedstock, thus, which one(s) would provide the highest added-value to the residue. The experiments were performed over 52 h (Figure 16).
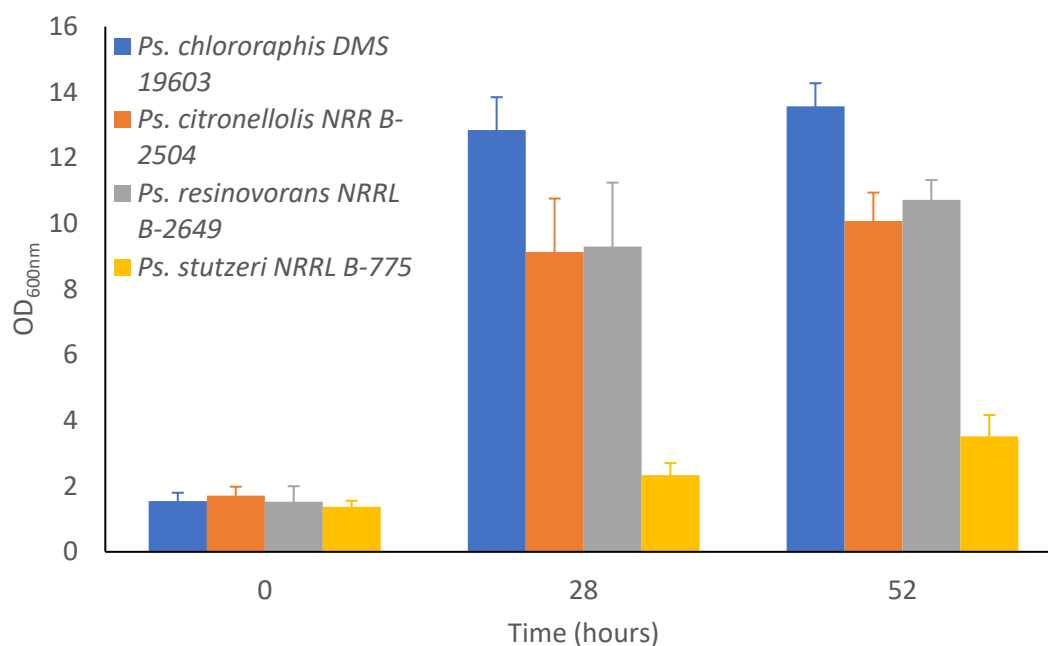
Figure 16 Cellular growth profile of the different bacterial strains using grape pomace as sole feedstock.

Through the analysis of the screening assay results presented in Figure 16, it is possible to observe that there was an increase in the optical density (OD) at 600nm over time for *Ps. chlororaphis* DMS 19603, *Ps. citronellolis* NRRL B-2504 and *Ps. resinovorans* NRRL B-2649 that reached OD values of 13.57 ±0.7, 10.08 ±0.89 and 10.73 ±0.60, respectively, within 52 h of cultivation. This shows that these bacterial strains are able to grow using grape pomace aqueous

extract as sole carbon source. On the contrary, *Ps. stutzeri* NRRL B-775 reached an OD of only 3.52 ±0.65 at the end of 52h of cultivation showing a very slow cellular growth rate. In conclusion, *Ps. stutzeri* was the only bacterial strain, among the ones tested, that was not able to efficiently grow with grape pomace as sole feedstock, and for that matter the only one that could clearly be eliminated from the following experiments.

The gravimetry quantification of CDW (Table 4) confirmed these results. *Ps. chlororaphis* DMS 19603 had the highest CDW value (3.63 g/L), followed by *Ps. resinovorans* NRRL B-2649 (3.82 g/L), *Ps. citronellolis* NRR B-2504 (2.83 g/L) and, at the end of the list, *Ps. stutzeri* B-2504 (0.39 g/L).

Table 4 Cell dry weight produce, sugar consumption and PHA accumulated by the different bacterial strains tested in screening assay using grape pomace as sole carbon source.

Bacterial strain	CDW (g/L)	Sugar (g/L)	PHA (%)	PHA (g/L)
Ps. chlororaphis DMS 19603	3.63 ±0.60	13.22 ±1.53	9.10	0.33
Ps. citronellolis NRR B-2504	2.83 ±1.03	11.70 ±1.07	2.32	0.07
Ps. resinovorans NRRL B-2649	3.92 ±0.40	9.86 ±2.43	3.27	0.13
Ps. stutzeri B-2504	0.39 ±0.65	5.05 ±0.74	1.53	0.01

Table 4 also shows the total sugar consumed during the experiments by each bacterial strain. With the exception of *Ps. stutzeri* NRRL B75, all other bacteria strains tested where are able to consume both soluble sugars, glucose and fructose, of the extract. *Ps. stutzeri* NRRL B75 consumed only 5.05 g/L of sugar, which is in agreement with its reduced cellular growth (Figure 16) and the low CDW production (Table 4). During the 52 hours of the experiments, *Ps. chlororaphis* DMS 19603, *Ps. citronellolis* NRRL B-2504, *Ps. resinovorans* NRRL B-2649 consumed 13.22 g/L, 11.70 g/L and 9.86 g/L of sugars, respectively. Thus, these three strains consumed the majority of the available sugars in grape pomace with *Ps. chlororaphis* DMS 19603 exhibiting the highest levels of sugar consumption and cell growth. Although *Ps. citronellolis* NRRL B-2504 consumed more sugar than *Ps. resinovorans* NRRL B-2649, it reached a lower final CDW. However, none of these bacteria consumed to exhaustion the sugars present in the grape pomace extract.

To confirm the production of PHA by the bacteria in the shake flask experiences, Nile Blue staining was performed to detect PHA accumulation inside bacterial cells samples collected 28

and 52 hours after the inoculation. The cells were observed in a microscope with phase contrast and using fluorescence, to observe the microorganisms and the granules of mcl-PHA accumulated inside, respectively. Figure 17 shows an increase of bacteria cells of *Ps. chlororaphis* DMS 19603, *Ps. citronellolis* NRRL B-2504 and *Ps. resinovorans* NRRL B-2649 strains at the end of the experiences, which agrees with the cellular growth of these strains shown in Table 4.

The intensification in fluorescence in these strains' samples along the assay, also reflects the increase in the accumulation of mcl-PHA within the cells. *Ps. chlororaphis* DMS 19603 demonstrated to be the bacterium with the highest concentration of mcl-PHA, 0.33 g/L (Table 5), being 9.10 % of the total CDW produced. *Ps. resinovorans* NRRL B-2649 and *Ps. citronellolis* NRR B-2504 bacterial strains also accumulated mcl-PHA inside the cells, at concentrations of 0.13 g/L and 0.07 g/L, respectively. The intensification of fluorescence (Figure 17) is in accordance with the mcl-PHA quantification.

Ps. stutzeri NRRL B-775 did not have a notable fluorescence and only accumulated 0.01 g/L of mcl-PHA inside the cells, this can be justified by the fact that this bacterium exhibited a poor cellular growth.

The bacteria *Ps. chlororaphis* DMS 19603, *Ps. citronellolis* NRRL B-2504 and *Ps. resinovorans* NRRL B-2649 have been reported as mcl-PHA producers, through the use of a variable number of carbon sources, resulting in mcl-PHA with different amounts of constituent monomers. The production of mcl-PHA by *Ps. chlororaphis* has been studied using biodiesel waste [63], substrate derived from animal waste [16] and palm kernel oil [65] as feedstocks; *Ps. citronellolis* has been also tested with different feedstocks, including fruit waste (*e.g.* apple) [64], saturated biodiesel fractions, fatty acids by-products, olive oil distillate [36] and tallow fatty acids [16]. *Ps. resinovorans* has been tested using olive oil deodorizer distillate, biodiesel fatty acids-by product [36], used cooking oil, octanoate, fruit pomace (*e.g.* cherries, apricots and grapes) and waste frying oil [66]. In order to study and understand the ability of these bacteria strains to produce mcl-PHA using grape pomace, these microorganisms were selected to proceed to mcl-PHA production in bioreactor assays.
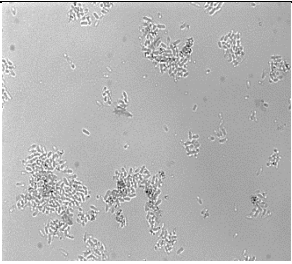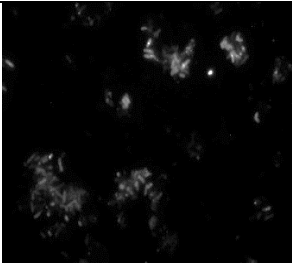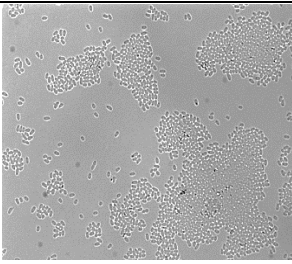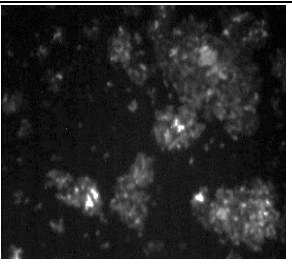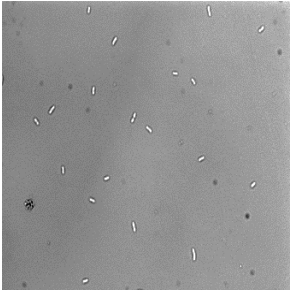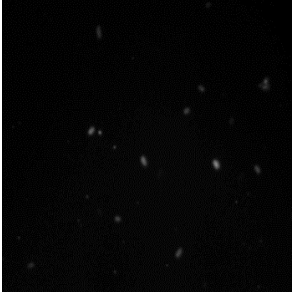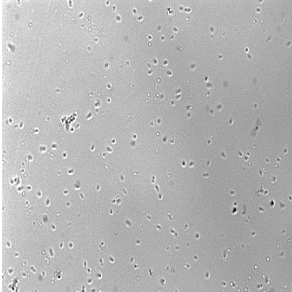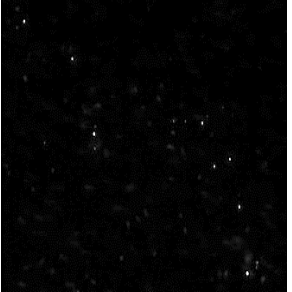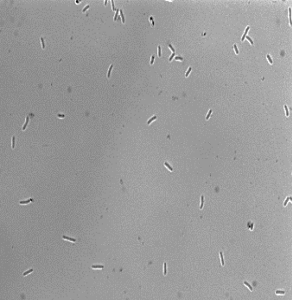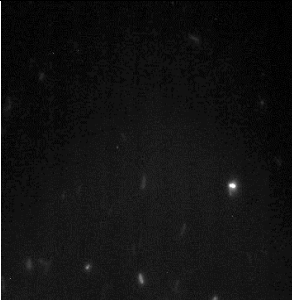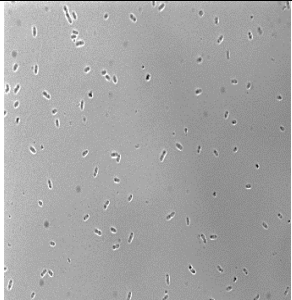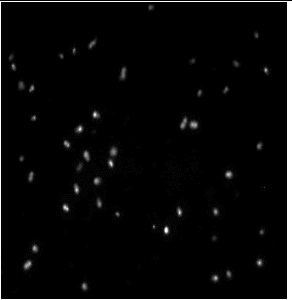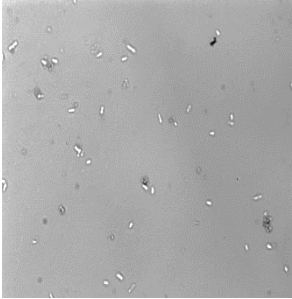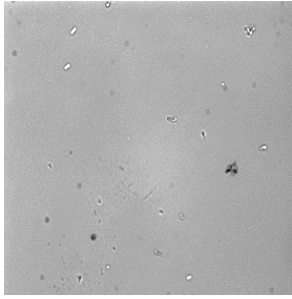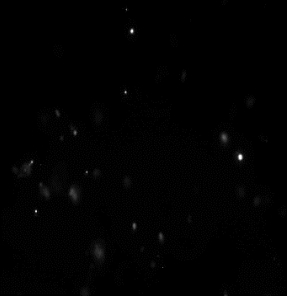
Bacterial strain	28 hours after incubation		52 hours after incubation	
	Phase contrast	Fluorescence	Phase contrast	Fluorescence
Ps. chlororaphis DMS 19603				
Ps. citronellolis NRR B-2504				
Ps. resinovorans NRRL B-2649				
Ps. stutzeri NRRL B-775				

Figure 17 Visualization of the different bacterial strain cells under the microscope (100x) for sample of the broth cultivation collected after cultivation at 28 and 52 hours after the inoculation under phase contrast and fluorescence after Nile Blue.

3.2.2 Grape Pomace Hydrolysate

To test if different bacteria were able to use hydrolysate of grape pomace, obtained by acid and HCW hydrolysis, for cell growth and PHA accumulation, namely mcl-PHA and PHB, shake flask experiments were performed.

These screening assays studied a well-known group of mcl-PHA producing bacteria, namely, *Ps. citronellolis* NRRL B-2504, *Ps. chlororaphis* DMS 19603 and *Ps. resinovorans* NRRL B-2649 and a PHB producer *B. sacchari* DSM 17165, because the hydrolysates contain xylose and this bacteria is recognised by produce PHB using xylose as carbon source[27] [67], aiming to identify which one(s) exhibit a higher cell growth and the most efficient polymer accumulation when using the hydrolysate of grape pomace as sole feedstock. The assays were performed over 52 h of cultivation.

Table 5 Cell dry weight produced, sugar consumption and mcl-PHA produced by the different bacteria strains tested in screening assay using grape pomace hydrolysate, obtained by acid and HCW hydrolysis, as sole carbon source.

Bacterial strains	Hydrolysate	DO	CDW (g/L)	Sugars (g/L)	PHA	
					(%)	(g/L)
Ps. chlororaphis	Acid	0.3	0.03	2.47	2.28	0.02
DMS 19603	HCW	2.1	0.16	1.28	0.005	0.004
Ps. citronellolis	Acid	6.1	3.68	3.29	1.33	0.06
NRR B-2504	HCW	2.7	0.21	0.43	0.004	0.002
Ps. resinovorans	Acid	15.2	5.06	3.74	2.63	0.14
NRRL B-2649	HCW	1.8	0.25	1.30	0.006	0.003
B. sacchari	Acid	10.3	3.62	1.81	n.a.	n.a.
DMS 17165	HCW	1.9	0.15	0.31	n.a.	n.a.

n.a.-not available

Table 5 shows that *Ps. citronellolis* NRRL B-2504, *Ps. resinovorans* NRRL B-2649 and *B. sacchari* DSM 17165 had the ability to grow using the grape pomace acid hydrolysate, reaching OD values of 6.1, 15.2 and 10.3, respectively, within 52 h of cultivation, however the OD of *Ps. chlororaphis* DMS 19603 observed, demonstrate that this strain could not use grape pomace acid hydrolysate as feedstock to grow. These results are confirmed by gravimetric quantification of CDW. *Ps. resinovorans* was the culture that had the highest cell growth, with CDW of 5.06 g/L as shown by the cellular growth profile. The CDW reached by *Ps. citronellolis* NRRL B-2504 and *B. sacchari* DSM 17165 were similar, 3.68 g/L and 3.62 g/L, respectively (Table 5). However, when these strains are cultivated in grape pomace HCW hydrolysate, any of them reaching to a significantly

value of OD or CDW (Table 5). It demonstrates that probably the HCW hydrolysis process formed compounds that inhibit bacterial growth.

All the tested bacteria strains are able to use the sugars present in the broth using acid hydrolysate (Table 5). Within 52 h of cultivation, *Ps. resinovorans* NRRL B-2649 was the strain that consumed the highest amount of sugars, 3.74 g/L, which justifies the highest cellular growth observed and consequently the highest mcl-PHA accumulated in cells (Table 5). This strain and *Ps. citronellolis* NRRL B-2504 consumed almost all the sugars present in the substrate, namely arabinose, glucose, xylose/mannose and fructose (Figure 18). *Ps. chlororaphis* DMS 19603 had a sugar consumption of 2.47g/L, preferentially glucose and fructose and did not consume xylose/mannose. However, there is a practically null cell growth but produce mcl-PHA, 0.02 g/L, equivalent to 2.28% (Table 5). This strain showed that it could not grow on this substrate but can produce PHA with it. So, other carbon source could be used by this strain to grow and the acid hydrolysate could be used then to accumulate PHA. Over the 52 h of cultivation, *B. sacchari* DMS 17165 consumed only 1.81 g/L of sugars (Figure 18). This bacterium is known to consume sugars as xylose to grow and to produce PHB. However, the consumption of xylose/mannose from grape pomace hydrolysate was very low, consuming preferentially other sugars. This strain did not accumulate polymer inside the cells. *Ps. resinovorans* NRRL B-2649 shows to be the most capable bacteria to use the acid hydrolysate to growth and produce mcl-PHA.
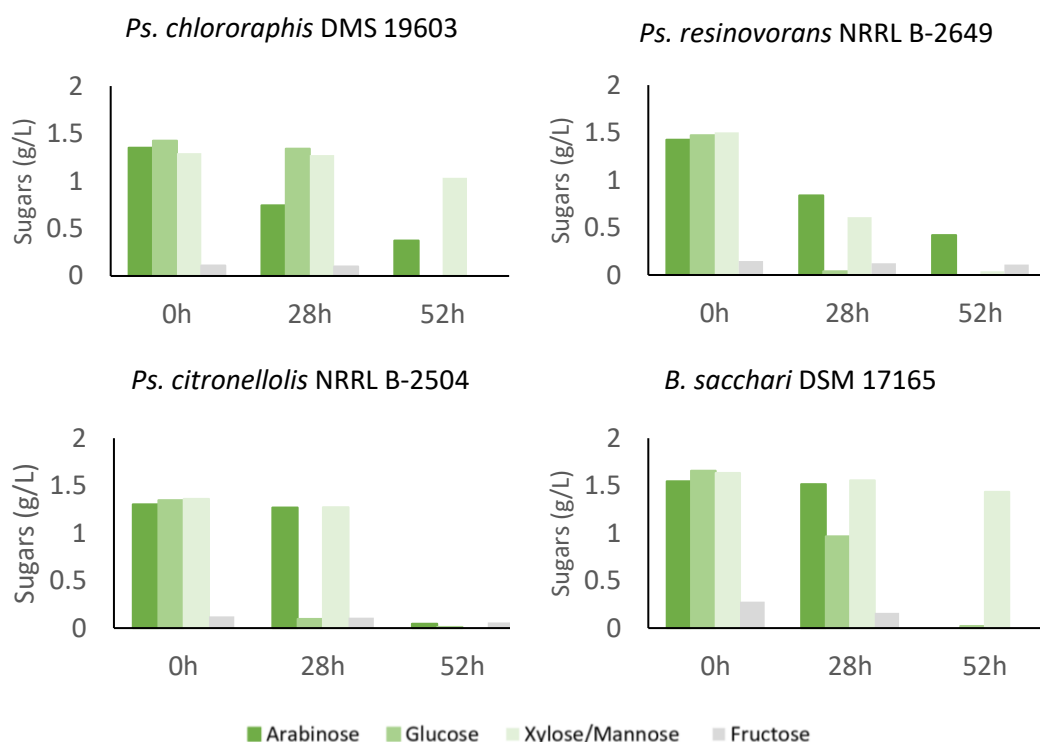
Figure 18 Sugar profiles, namely arabinose, glucose, xylose/mannose and fructose in the supernatant of each shake flask assay with different bacteria strains, with grape pomace acid hydrolysate as the sole carbon source.

The *Ps. chlororaphis* DMS 19603 and *Ps. resinovorans* NRRL B-2949 were the strains that consume more sugars from HCW hydrolysate, consuming 1.28 g/L and 1.30 g/L, respectively (Table 5). *Ps. chlororaphis* DMS 19603 consume all the glucose and fructose, but do not consume any arabinose and only some vestigial xylose/mannose (Figure 19). *Ps. resinovorans* NRRL B-2949 consume all the glucose, but none of the other sugars. In the other hand, *Ps. citronellolis* NRRL B-2504 only consume 0.43 g/L of sugars, mainly xylose/mannose (Figure 19). *B. sacchari* DMS 17165 had the lower consumption of sugars, 0.31 g/L, consuming all the arabinose and fructose, but did not consume the glucose or xylose/mannose, as expected.

All the strained tested using HCW hydrolysate as carbon source accumulated only vestigial amounts of PHA. During this hydrolysis, compounds that inhibit the microbial growth and consequently the polymer production should be generated.
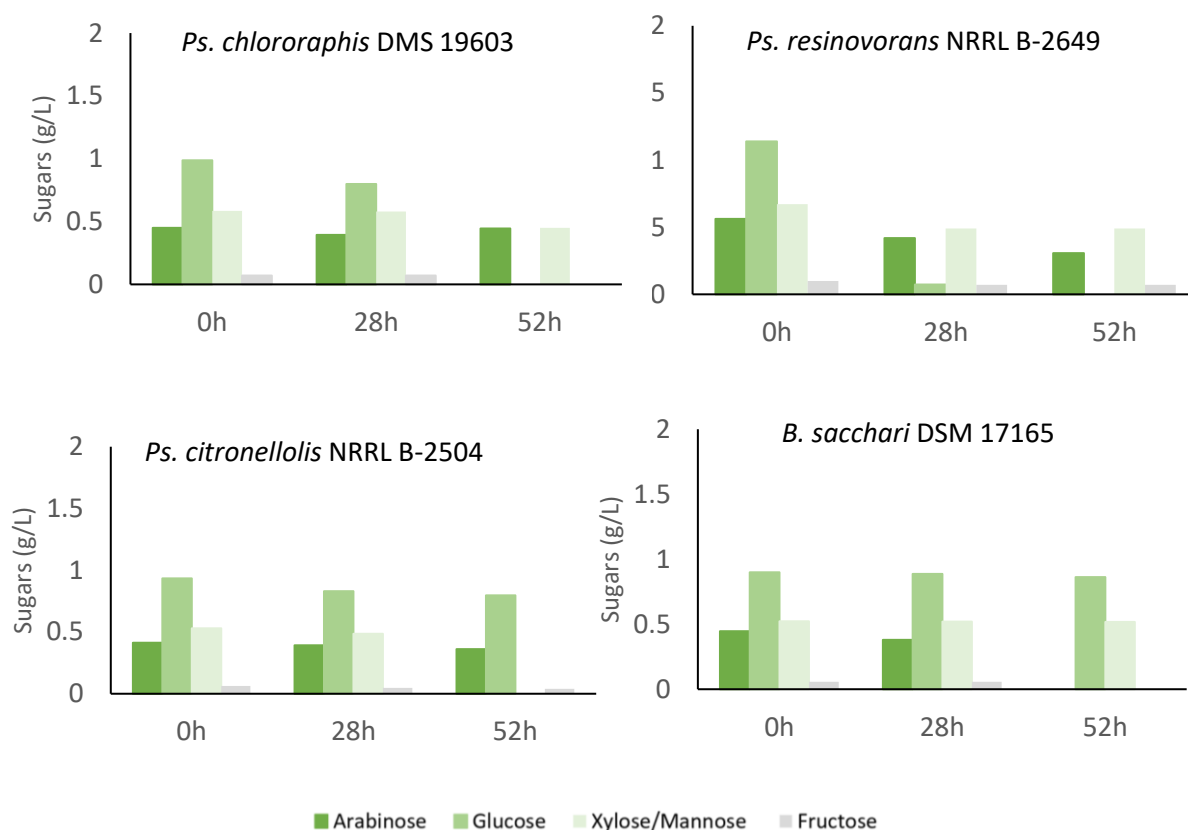
Figure 19 Sugar profiles, namely arabinose, glucose, xylose/mannose and fructose in the supernatant of each shake flask assay with different bacteria strains, with grape pomace HCW hydrolysate as the sole carbon source.

3.3 Bioreactor production of mcl-PHA

The screening assays allowed to notice that the *Ps. chlororaphis* DMS 19603*, Ps. citronellolis* NRRL B-2504 and *Ps. resinovorans* NRRL B-2649 were able to use the grape pomace aquous extract to growth and produce mcl-PHA, unlike with the hydrolysates, under the same tested conditions, that resulted in reduced cell growth and / or absence of polymer accumulation. So in this way, experiments in bioreactors were performed to optimize and characterize the mcl-PHA produced by *Ps. chlororaphis* DMS 19603*, Ps. citronellolis* NRRL B-2504 and *Ps. resinovorans* NRRL B-2649. The growth and polymer accumulation of these bacteria strains were evaluated in a bioreator with controlled conditions and using grape pomace aqueous extract as the sole source of carbon. First, the batch cultivation was tested and, to increase the amount of sugars, a second cultivation mode, using a fed solution, was performed.

3.3.1. Batch fermentation

3.3.1.1 Production of mcl-PHA by *Ps. chlororaphis* DMS 19603

The batch assay was perfomed with *Ps. chlororaphis* DMS 19603 in a 2 L Bioreactor using grape pomace aqueous extract as carbon source, during 24 h. Figure 20 ilustrates the batch cultivation profile.
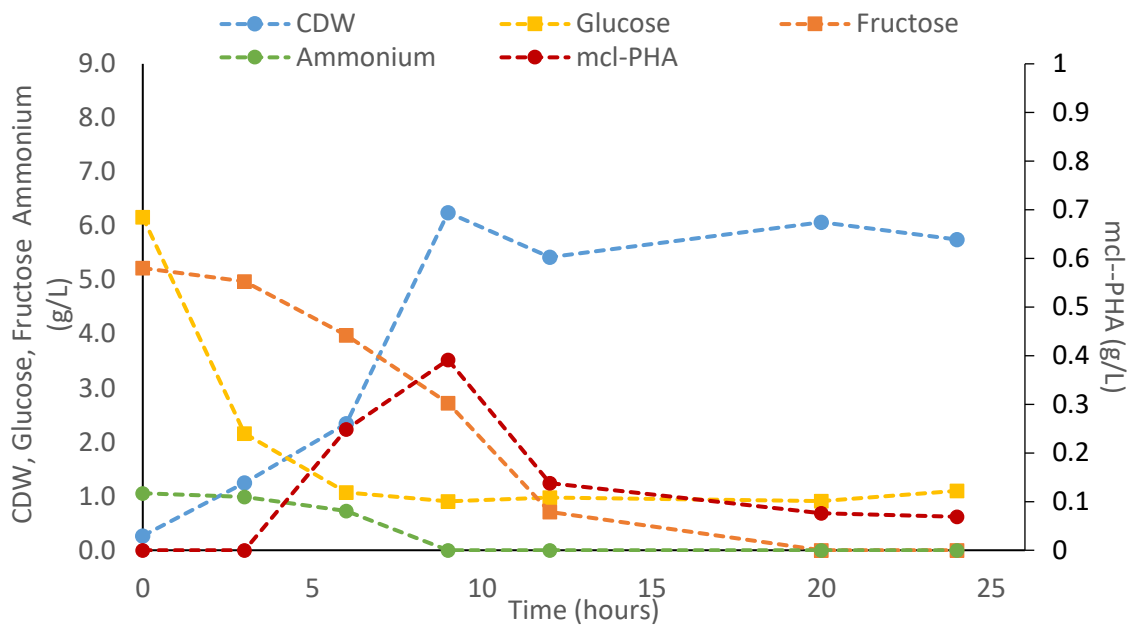
Figure 20 Cultivation profile of the batch bioreactor fermentation of *Ps. chlororaphis* DSM 19603 using aqueous extract of grape pomace as sole carbon source.

Figure 20 shows that the bacteria had a growth phase that lasted 9 h, reaching a CDW maximum of 6.25 g/L; at the same time, ammonium was exhausted, limiting cell growth. The specific cell growth rate was 0.33 h^{-1}. Thereafter, the CDW decreased to 5.75 g/L in the rest of the experiment. After 3 hours of batch fermentation, the bacteria started to accumulate mcl-PHA, at 9 h the maximum of polymer accumulation was achieved, with a concentration of 0.39 g/L, corresponding to a polymer content in the biomass of 7.7 wt.% and an active biomass of 10.09 g/L. Thereafter, the active biomass remained constant but there was a decrease of the mcl-PHA concentration to 0.08 g/L and only 1.4 wt.% polymer content in the biomass. This corresponds to a maximum volumetric productivity of 0.02 g/(L.h).

The grape pomace extract had a total sugars concentration of 11.3 g/L, wherein 6.2 g/L was glucose and 5.1 g/L was fructose. Both sugars decreased during the experiment, showing the bacteria consumed almost all the sugar content in the grape pomace extract. However, the consumption of glucose was faster than that of fructose that only started after glucose reached a concentration of 1.07 g/L (at 6h). The bacteria consumed a total of 10.3 g/L of sugar which

corresponds to a growth and polymer yield on substrate of 0.53 g_X/g_s and 0.01 g_p/g_s, respectively. The concentration of sugars was very low at 9 h, with a total sugar concentration of 3.63 g/L, at the same time, the mcl-PHA concentration started to decrease. This suggests that, at this point in time, the concentration of sugars in the grape pomace was not enough for *Ps. chlororaphis* DMS 19603 cellular maintenance, thus driving the cell to consume the accumulated polymer for survival.

3.3.2 Fed-Batch assay

3.3.2.1 Production of mcl-PHA by *Ps. chlororaphis* DMS 19603

Fed-batch assays were perfomed in order to increase the amount of sugar and consequently attempt to increase mcl-PHA production. These assays were tested, during 30 h, in a 2 L bioreactor, under the same conditions of the batch cultivations. However, after 9 hours of cultivation, a grape aqueous extract feeding concentrated solution, with a sugar concnetration of 215.80 g/L, was added. The cultivation profile of the fed-batch bioreactor cultivation of *Ps. chlororaphis* DSM 19603 is shown in Figure 21.

Figure 21 Cultivation profile of the fed-batch bioreactor fermentation of *Ps. chlororaphis* DSM 19603 using aqueous extract of grape pomace as sole carbon source.

Figure 21 shows that *Ps. chlororaphis* DMS 19603 had a growth phase that lasted for 21 h, achieving a CDW concentration of 12.43 g/L. The specific cell growth rate was 0.31 h^{-1} (Table 3), similar to that obtained with the batch fermentation (0.33 h^{-1}). Both are considerably higher than the values reported for this strain when using substrates of animal waste (0.10 h^{-1}) [19], showing that the extract of grape pomace is a suitable carbon source for cell growth. The ammonium was exhausted at around 12 h, the bacteria growth stopped, keeping the same value of active biomass (10.0 g/L). At the time the ammonium was exhausted and cell growth stopped, polymer production got more evident (Figure 21), the increasing of CDW is caused by the mcl-PHA accumulation inside the bacterial cells. At the end of the experiment, the mcl-PHA concentration was 12.0 g/L, representing 16.7% of the total CDW produced. This value is significantly higher than the polymer accumulated in batch fermentation, where only 0.39 g/L

of mcl-PHA was accumulated, showing that the sugars added with the feeding increased polymer production. The percentage of mcl-PHA accumulated in fed-batch fermentation by *Ps. chlororaphis* was similar to the values reported in the literature (between 10% to 17%), except when using Palm Kernel Oil as substrate, which produced 45 % of mcl-PHA, although the final product concentration was lower (1.49 g/L) [65]. The maximum volumetric productivity obtained was 0.07 g/(L.h), at 21h, being in the range of the reported values: 0.052-0.1 g/(L.h) (Table 6). The higher value of volumetric productivity was obtained using substrate from animal waste in a pulse feeding fermentation where a higher CDW was reached [65].

The initial amount of total sugar was 25.15 g/L, 11.80 g/L of glucose and 13.35 g/L of fructose (Figure 21). After 6 h the amount of fructose had been reduced to 10.15 g/L and there is no glucose in the broth, which provided a notable preference of bacteria to consume glucose instead of fructose. At that exact moment, a feeding solution was given, so the bacteria consumed more 27.50 g/L of sugar till the end of the assay, consuming during all the experiment, a total amount of sugars of 42.50 g/L. Considering the monosaccharide sugars, glucose and fructose, presents in the substrate, there was a consumption of 33.09 g and 18.41 g, respectively. However, at the end of the experiment, still remained 13.16 g/L of fructose and some vestigial glucose. The bacteria were consuming the glucose that was being fed, because it preferred this sugar more than fructose. The growth and polymer yield of, 0.29 g_X/g_s and 0.03 g_P/g_s, respectively (Table 6).

Table 6 Kinetic and stoichiometric parameters for mcl-PHA production by *Ps. chlororaphis* using several wastes and by-products as feedstocks.

Strains	Carbon source	μ_{max} (h^{-1})	CDW (g/L)	mcl-PHA (%)	mcl-PHA (g/L)	rp (g/L.h)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	References
Ps. chlororaphis DSM 19603	**Grape pomace (batch fermentation)**	0.33	5.75	1.39	0.08	0.02	0.53	0.01	This study
	Grape Pomace (fed-batch fermentation)	0.31	12.0	16.7	1.8	0.07	0.29	0.03	This study
	Crude glycerol* (batch fermentation)	n.a.	6.71	17.1	n.a.	0.052	0.27	0.06	[63]
Ps. chlororaphis DSM 50083	SFAE (pulse feeding fermentation)	0.08	30	15.2	n.a.	0.071	0.62	0.75	[19]
		0.10	41.3	10.0	n.a.	0.094	0.73	0.07	
		0.13	41.2	15.2	n.a.	0.138	0.66	0.101	
Ps. chlororaphis HS21	PKO (batch fermentation)	n.a.	3.3	45	1.49	n.a	0.67	n.a.	[65]
Ps. chlororaphis IMD555	EGPJ (Fed-Batch)	n.a.	37.5	10	3.75	0.1	n.a.	n.a.	[68]

μmax, maximum specific cell growth rate; CDW, cell dry weight; rp, volumetric productivity; Yx/s, active biomass yield on grape pomace extract; Yp/s, polymer yield on grape pomace extract; n.a. data not available; SFAE - substrates derived from animal waste; PKO - Palm Kernel Oil; EGPJ - Ensiled Grass Press Juice) *from biodiesel production

3.3.2.2 Production of mcl-PHA by *Ps. citronellolis* NRR B-2504

A fed-batch strategy was perfomed in a 2L bioreactor during 30 h. After 9 hours of cultivation, a concentrated feeding solution, composed of grape aqueous extract with a sugars concentration of 182.3 g/L, was added. The cultivation profile of the fed-batch bioreactor cultivation of *Ps. citronellolis* NRR B-2504 is shown in Figure 22.
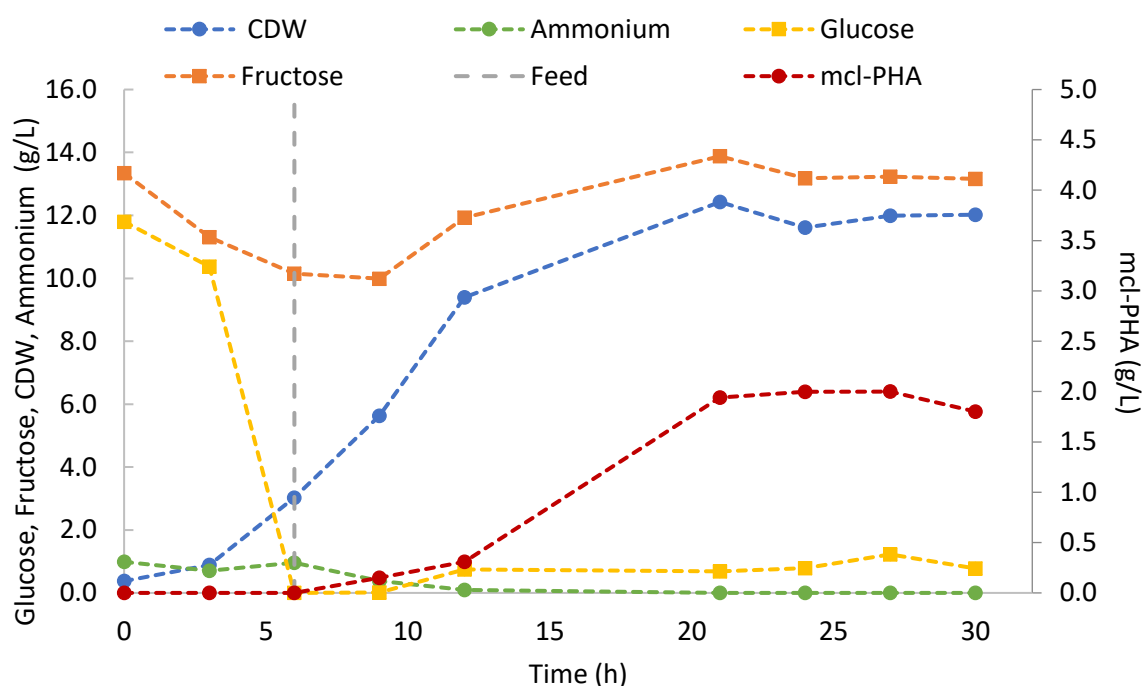

Figure 22 Cultivation profile of the fed-batch bioreactor fermentation *of Ps. citronellolis NRR B-2504* using aqueous extract of grape pomace as sole carbon source.

The available ammonia was exhausted within 12 h of cultivation and at this moment the cell growth stopped. After an initial lag phase of 3 h, the culture grew at a specific cell growth rate of 0.69 h^{-1}. This value is higher than the values reported using tallow based biodiesel [16] and waste apple pulp [64], with specific cell growth rate of 0.08 h^{-1} and 0.24 h^{-1}, respectively (Table 7). This result demonstrates that grape pomace extract was a more sustainable carbon source for microbial growth of *Ps. citronellolis* NRR B-2504, as previous also observed for *Ps. chlororaphis* DMS 19603. After 30 h of cultivation, a CDW of 9.30 g/L was attained (Table 7). This parameter is higher than most of the values reported in the literature, except using tallow based biodiesel as feedstock in a fed-batch fermentation (11.2-14.1 g/L) [69]. The mcl-PHA accumulation started around 6 h, increasing to a concentration of 1.3 g/L, corresponding to a polymer content in the biomass of 14.3 wt.%, by the end of the assay. The polymer content in the biomass is within to the values obtained using fatty acids by-product [64] and tallow based biodiesel [16] as carbon source for *Ps. citronellolis* (0.1-2.9 g/L) (Table 7). The corresponding

maximum volumetric productivity was 0.04 g/(L.h). The values reported are lower than those obtained in this study (Table 7), except using Tallow based biodiesel (0.06-0.1 g/(L.h)).

The grape pomace had a total concentration sugars of 21.0 g/L, with similar amounts of glucose (10.8 g/L) and fructose (10.2 g/L). At the end of 9 h of the cultivation, 26.4 g of sugars were added to the bioreactor. After 6 h, when the accumulation of mcl-PHA started, the consumption of sugars was more intense (Figure 21). At the end of the experiment, a total concentration of sugars (60 g/L) was consumed. However, there was still 9.0 g/L of sugars left, 3 g/L were glucose and 6 g/L were fructose (Figure 21). As observed before in *Ps. chlororaphis* DMS 19603 cultivation (Figure 20), *Ps. citronellolis* NRR B-2504 consumed more glucose than fructose (Figure 20). The growth and polymer yield with grape pomace extract were 0.16 gX/gs and 0.02 gp/gs, respectively (Table 7).

A previous study reported the use of glucose and fructose from apple pulp waste in batch cultivations by *Ps. citronellolis* NRR B-2504 used in this study with grape pomace extract [64]. In that study a *rp* of 0.0025 g/(L.h) and growth rate was lower of 0.24 h^{-1}, lowers values when compared with using grape pomace aqueous extract [64].

Table 7 Kinetic and stoichiometric parameters for mcl-PHA production by *Ps. citronellolis* NRR B-2504 using several wastes and by-products as feedstocks.

Carbon source	μ_{max} (h^{-1})	CDW (g/L)	mcl-PHA (%)	mcl-PHA (g/L)	rp (g/L.h)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	References
Grape Pomace (fed-batch fermentation)	**0.69**	**9.3**	**14.3**	**1.3**	**0.04**	**0.16**	**0.02**	This study
Olive oil distillate (shake flask)	n.a	4.8	10	0.5	0.008	n.a.	0.08	[36]
Fatty acids by-product (shake flask)	n.a	3.5	3	0.1	0.004	n.a.	0.02	[36]
Margarine waste (shake flask)	n.a	6.3	8	0.5	0.007	n.a.	n.a.	[70]
Tallow free fatty acids (shake flask)	n.a	1.7	3	0.05	0.0008-0.0012	n.a.	n.a.	[71]
apple pulp waste (batch fermentation)	0.24	4.0	30.0	1.2	0.025	0.27	0.12	[64]
Tallow based biodiesel (fed-batch fermentation)	0.08-0.10	11.2-14.1	20-27	2.8-2.9	0.067-0.1	n.a.	n.a.	[16]

μ_{max}, maximum specific cell growth rate; CDW, cell dry weight; rp, volumetric productivity; Yx/s, active biomass yield on grape pomace extract; Yp/s, polymer yield on grape pomace extract; n.a. data not available.

3.3.2.3 Production of mcl-PHA by *Ps. rsesinovorans* NRRL B-2649

A fed-batch strategy was perfomed in a 2 L bioreactor during 54 h. After 20 hours of cultivation, a feeding concentrated solution, with a sugars' concentration of 180 g/L, was added. The cultivation profile of the fed-batch bioreactor cultivation of *Ps. rsesinovorans* NRRL B-2649 is shown in Figure 23.
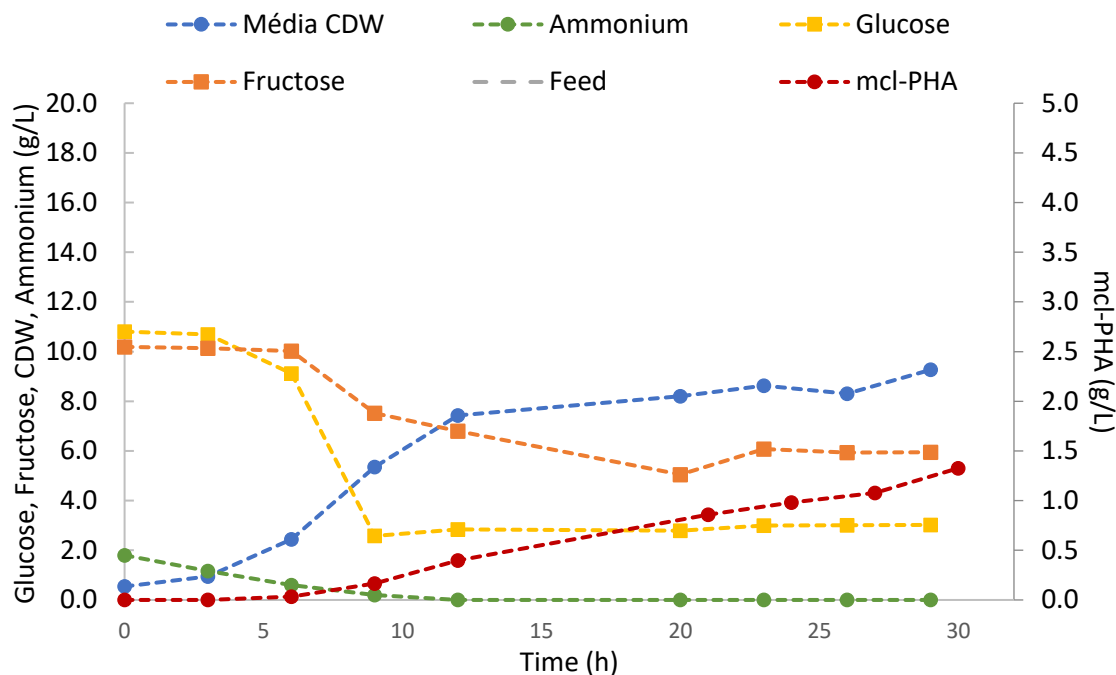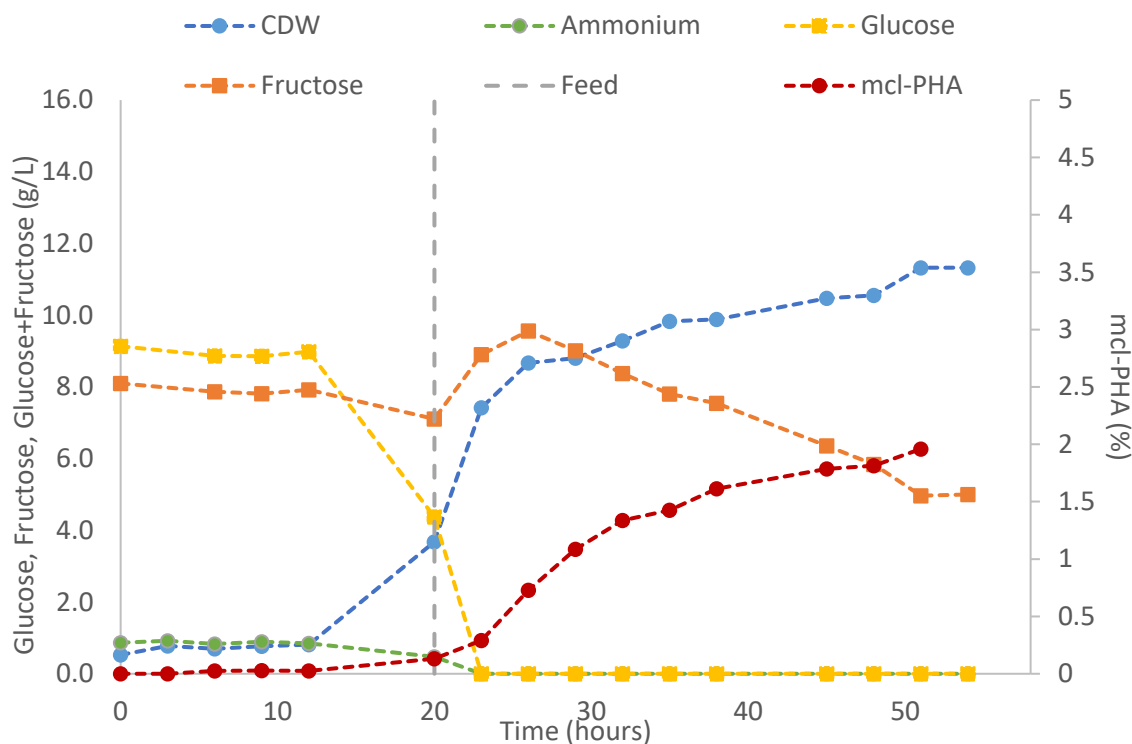

Figure 23 Cultivation profile of the fed-batch bioreactor fermentation of *Ps. resinovorans* NRRL B-2649 using aqueous extract of grape pomace as sole carbon source.

Figure 23 shows that *Ps. resinovorans* NRRL B-2649 had a lag phase that lasted for 12 h, unlike *Ps. chlororaphis* DMS 19603 (Figure 21) that started immediately the growth. Afterwards, the culture entered a growth phase with a cell growth rate of 0.19 h^{-1} that lasted till 23 h of cultivation. Among all the strains tested using grape pomace extract, Ps. *resinovorans* had the lowest specific cell growth rate. The available ammonia was exhausted at this point, limiting the growth of *Ps. rsesinovorans* NRRL B-2649, concomitantly, the mcl-PHA accumulation increased significatly and the concentration of sugars present in the grape pomace extract decreased (Figure 23). During the exponential phase, a CDW of 7.4 g/L was obtained within 23 h of cultivation, however until the end of the experiment the CDW increased to 11.3 g/L, as a result of the polymer accumulation inside the bacterial cells. The polymer accumulation increased significantly by the time the ammonia was exhausted and cell growth stopped (Figure 21). The total concentration of mcl-PHA accumulated was 2.0 g/L, corresponding to a maximum volumetric productivity of 0.08 g/(L.h) (Table 8). This value is within the ones reported in the

literature (0.02-0.24 g./(L.h)). The mcl-PHA content in the biomass was 17.4 wt.%, which is within the reported values for *Ps. resinovorans* using different substrates, (2%-53.2%). This result was close to the ones obtained with *Ps. chlororaphis* (Table 6) and *Ps. citronellolis* (Table 7) using grape pomace extract and the same cultivation mode.

The experiment started with a total sugar concentration of 17.2 g/L, wherein 9.1 g/L where glucose and 8.1 g/L fructose. The culture only started to consume the sugars present in the grape pomace extract in the exponential phase, at 12 h. At approximately 23 h of cultivation, glucose was all consumed, including the glucose added in feeding (Figure 23). On the other hand, fructose was consumed significantly only when glucose was exhausted. Similar to the other bacterial strains used in this study, *Ps. resinovorans* showed preference for glucose instead of fructose. A total of 28.5 g/L were consumed during the entire experiment, corresponding to an active biomass and polymer yield of 0.37 g_X/g_s and 0.08 g_p/g_s, respectively. A different strain, *Ps. resinovorans* (DSMZ 21078), was previously reported to use glucose and fructose from apricots and solaris grapes [49]. In this study, the CDW values reported were 10.2 g/L and 6.1 g/L, and the *r*p were 0.03 g/(L.h) and 0.05 g/(L.h), for apricots and solaris grapes, respectively. These results are more modest than the ones obtained in the present study, showing that the NRRL B-2649 strain of *Ps. resinovorans* is able to grow and produce PHA using as carbon source the glucose and fructose present in grape pomace waste.

Table 8 Kinetic and stoichiometric parameters for mcl-PHA production by *Ps. resinovorans* using several wastes and by-products as feedstocks.

Strains	Carbon source	μ_{max} (h^{-1})	CDW (g/L)	mcl-PHA (%)	mcl-PHA (g/L)	*r*p (g/L.h)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	References
Ps. resinovorans NRRL B-2649	**Grape Pomace (fed-batch fermentation)**	**0.19**	**11.3**	**17.4**	**1.8**	**0.08**	**0.37**	**0.08**	This study
	Olive oil distillate (shake flask)	n.a.	7.1	31	2.2	**0.04**	n.a.	0.29	[36]
	Used cooking oil (shake flask)	n.a.	3.2	28	0.9	0.02	n.a.	0.29	[36]
	Biodiesel fatty acids by-product (shake flask)	n.a.	2.6	>2	0	0	n.a.	0	[36]
	Olive oil deodorizer distillate (fed-batch fermentation)	n.a.	12.7	36	4.7	**0.24**	0.28	0.21	[66]
	Crude Pollock oil (batch fermentation)	n.a.	4.7	53.2	2.5	0.03	n.a.	0.18	[14]
Ps. resinovorans DSMZ 21078	Apricots Solaris grapes (batch fermentation)	n.a. n.a.	10.2 6.1	12.4 23.3	n.a n.a	0.03 0.05	n.a. n.a.	n.a. n.a.	[49]

μmax, maximum specific cell growth rate; CDW, cell dry weight; rp, volumetric productivity; Yx/s, active biomass yield on grape pomace extract; Yp/s, polymer yield on grape pomace extract; n.a. data not available.

3.3.3 mcl-PHA Characterization

3.3.3.1 Composition

The polymers produced by *Ps. chlororaphis*, *Ps citronellolis* and *Ps. resinovorans* in the fed-batch assay using grape pomace extract as feedstock were characterized to identify their monomeric composition. The mcl-PHA produced by *Ps. chlororaphis* was mainly composed of 3-hydroxydecanoate (HD), 61.9 wt% and 3-hydroxydodecanoate (HDd), 18.1 wt% followed by 3-hydroxyoctanoate (HO), 10.2 and 3-hydroxytetradecanoate (HTd), 9.8 wt% and the monomer 3-hydroxyhexanoate (HHx) was not detected (Table 9). *Ps. chlororaphis* produced a different mcl-PHA, the monomers were the same, but the relative content was different using different substrate as carbon source. The mcl-PHA produced using EGPJ is similar to that produced from grape pomace extract, but contained HHX [68]. The differences observed in the relative content of the monomers may result from the use of different substrates. *Ps. citronellolis* and *Ps. resinovorans* also produced mcl-PHA with different monomer composition, using different substrates. The mcl-PHA produced by *Ps. citronellolis* using grape pomace extract was manly composed by HD (64 wt%) and HO (19 wt%). It had a minor content of HDd (12 wt%) and HTd (5 wt%) and did not had HHx. The content of this polymer was similar to the one produced using the sugars of the apple pulp waste, composed by 68 wt% of HD, 22 wt% of HO, 5 wt% of HDd, 4 wt% of HTd and 1% of HHx. [49]. The mcl-PHA produced in a similar manner but with olive oil distillate [49], fatty acids by-product [49], tallow fatty acids [71] or tallow based biodiesel [16] was manly composed by HO (between 36 wt% to 48 wt%) and HD (12 wt% to 40 wt%) and had a higher content of HHx (5 wt% to 14 wt%). The monomeric content of the mcl-PHA produced by *Ps resinovorans* was similar to the mcl-PHA produced by *Ps. citronellolis*, both using grape pomace extract (Table 9). The same could be observed using olive oil distillate [36]. These results demonstrated that the composition of the polymer produced is highly dependent on the feedstock used.

Overall, all the mcl-PHAs produced with grape pomace extract were mainly composed of HD (61.9 wt% to 64 wt%) and had none or a vestigial content of HHx. (Table 9). However, there was a considerable difference between the mcl-PHA produced by *Ps. chlororaphis* and the mcl-PHA produced by *Ps citronellolis* and *Ps. Resinovorans. The* mcl-PHA produced by *Ps. chlororaphis* had more HDd than HO, while the opposite was true for the two other strains. All the three selected bacteria had a low content of HTd (between 5 wt% and 9.8 wt%). The bacterial strain also had an impact in the magnitude of the differences observed in the mcl-PHA produced.

Table 9 Monomer composition of the mcl-PHA produced by *Ps. chlororaphis*, *Ps. citronellolis* and *Ps. resinovorans* using different carbon sources.

Bacterial strains	Carbon source	mcl-PHA composition					References
		HHx	HO	HD	HDd	HTd	
Ps. chlororaphis DMS 19603	**Grape Pomace**	**n.d.**	**10.2**	**61.9**	**18.1**	**9.8**	**This study**
	Crude glycerol	6	10	29	43	12	[63]
	SFAE	15.5	50.6	26.1	5.0	n.d.	[19]
		14.6	45.7	27.3	6.7	n.d.	
		10.2	47.9	31.4	6.2	n.d.	
	PKO	4.7	34.7	32.5	1.4	n.d.	[65]
	EGPJ	n.d.	10	49	39	2	[68]
Ps. citronellolis NRRL B-2504	**Grape Pomace**	**n.d.**	**19**	**64**	**12**	**5**	**This study**
	Olive oil distillate	14	43	32	12	<1	[36]
	Fatty acids by-product	10	36	40	14	<1	[36]
	Tallow free fatty acids	10	48	28	10	4	[71]
	Waste apple pulp	1	22	68	5	4	[64]
	Tallow based biodiesel	5-6	40-46	36-40	7-9	n.d.	[16]
Ps. resinovorans NRRL B-2649	**Grape Pomace**	**2**	**20**	**62**	**11**	**5**	**This study**
	Olive oil distillate	19	44	33	12	<1	[36]
	Used cooking oil	11	43	33	12	<1	[36]
	Olive oil deodorizer distillate	12	48	31	8	<1	[66]
	Apricots	12.5	32.3	37.4	9.6	10.0	[49]
	Solaris grapes	16.3	33.8	30.4	9.5	8.2	
	Crude Pollock oil	3	27	48	15	7	[14]

SFAE - substrates derived from animal waste; PKO - Palm Kernel Oil; EGPJ - Ensiled Grass Press Juice) HHx, 3-hydroxyhexanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate; HDd, 3-hydroxydodecanoate; HTd, 3-hydroxytetradecanoate; n.a., data available

3.3.3.2 Molecular Mass Distribution

The SEC chromatogram (Figure A in Appendices) highlights that the mcl-PHA synthesized *by Ps. chloraphis* using grape pomace extract as carbon source exhibited a main single peak. The polymer had an average molecular weight (Mw) of 1.0×10^5 Da, with a polydispersity index (PDI) of 1.75 (Table 10). The low PDI value shows it was a highly homogeneous material. The polymer's Mw was within the range reported for the mcl-PHA produced by *Ps. chloraphis* strains (0.8×10^5 – 1.2×10^5 Da) using other feedstocks, as well as for polymers synthesized by others *Pseudomonas* sp. such as *Ps. citronellolis* and *Ps. resinovorans*.

The Mw of the mcl-PHA produced by *Ps. citronellolis* cultivated with grape pomace extract was 1.4×10^5 Da and had a PDI of 1.89 (Table 10) (SEC chromatogram in Appendices, Figure B). These values are in accordance with those obtained for mcl-PHA produced by the same strain but using different carbon sources, such as: tallow based biodiesel (0.7×10^5-2.0×10^5 Da) [16] and tallow fatty acids (0.9×10^5-1.6×10^5 Da) [71]. This can reflect similar polymers composition and properties [71].

The average molecular weight of the mcl-PHA produced by *Ps. resinovorans* with grape pomace extract (SEC chromatogram in Appendices, Figure C) is 3.1×10^5 Da (Table 10), with a PDI of 2.17. The Mw obtained was similar to the one obtained using Crude Pollock oil (3.1×10^5 Da) and higher than the values obtained with the mcl-PHA produce by the same strain but growing in feedstocks such as olive oil distillate (0.2×10^5 Da) [36] or used cooking oil (0.3×10^5 Da) [36] or olive oil deodorizer distillate (0.3×10^5 Da) [66]. The observed differences in the composition of the mcl-PHA may be due to different: production conditions, specifically the composition of the substrate; cultivation mode; or stage of growth when the cells were harvested [16]. Overall the molecular weight and PDI values obtained with *Ps. resinovorans* strain were higher than with *Ps. 3chloraphis* or *Ps. citronellolis*, when all were cultivated with grape pomace extract.

Table 10 Physical-chemical properties of mcl-PHA produced by different Pseudomonas, namely *Ps. chlororaphis*, *Ps. citronellolis* and *Ps. resinovorans*.

Bacterial Strains	Carbon source	Mw (10⁵Da)	Mn (10⁵Da)	PDI	References
Ps. chlororaphis DMS 19603	**Grape Pomace**	**1.0**	**0.6**	**1.75**	**This study**
	Crude glycerol*	1.1	0.4	1.5	[63]
	PKO	0.8	n.a.	1.5	[65]
	EGPJ	1.2	0.5	2.3	[68]
Ps. citronellolis NRRL B-2504	**Grape Pomace**	**1.4**	**0.8**	**1.89**	**This study**
	Olive oil distillate	0.3	0.2	1.5	[36]
	Tallow free fatty acids	0.9-1.6	0.4-0.7	2.2-2.6	[71]
	Waste apple pulp	3.7	1.7	2.1	[64]
	Tallow based biodiesel	0.7-2.0	0.4-0.8	1.9-2.5	[16]
Ps. resinovorans NRRL B-2649	**Grape Pomace**	**3.1**	**1.4**	**2.17**	**This study**
	Olive oil distillate	0.2	0.3	1.5	[36]
	Used cooking oil	0.3	0.4	1.3	[36]
	Olive oil deodorizer distillate	0.3	n.a.	1.5	[66]
	Crude Pollock oil	3.4	1.5	2.2	[14]

Mn, molecular number; Mw, molecular weight; PDI, polydispersity index; n.a., data not available

3.3.3.3 Thermal Properties

The thermal properties of the mcl-PHA produced in this study were determined by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Regarding the polymers' thermal stability, the decomposition of the polymers involved a fast one-step process. Their decomposition showed a single weight loss of approximately 91%, 98% and 99% (Figure D, F and G). The maximum degradation temperature (T_{deg}) for the polymers produced by *Ps. chloraphis, Ps. citronellolis* and *Ps. resinovorans* from grape pomace extract were 291.1, 292.5 and 292.6 °C, respectively (Table 10). Although the mcl-PHA was produced by different strains, the three values obtained were similar when using the same residue as carbon source. The values of T_{deg} exhibited by the *mcl*-PHA produced from grape pomace extract with *Ps. chloraphis* was higher than the one produced by the same strain growing in crude biodiesel (285 °C) [63]. Comparing the same strains of *Ps. citronellolis* NRRL B-2504, the culture that growth with Waste apple pulp had a higher T_{deg} (296 °C). The observed difference may be associated with the fact that this mcl-PHA had a significantly higher Mw (3.7×10^5 Da) than that produced by *Ps. citronellolis* from grape pomace extract (1.4×10^5 Da). *Ps. mendocina* NK-01 produced a mcl-PHA with a lower T_{deg} (283.94 °C).

The melting enthalpy (ΔHm) of the polymer produced by *Ps. chloraphis*, *Ps. citronellolis* and *Ps. resinovorans* was 56.6, 26.3 and 14.3 J/g, respectively. Showing that the mcl-PHA produced in this study was not completely amorphous but was, to some extent, crystalline, since has characteristics similar to other rubber-latex materials. The mcl-PHA produced in this study by *Ps. chloraphis*, *Ps. citronellolis* and *Ps. resinovorans* had significantly higher ΔHm and, consequently, higher X_c (56.6%, 26.3% and 14.9%, respectively) when compared with the mcl-PHA produced by the same strains but using different substrates, implying that the mcl-PHAs produced by these strains growing in grape pomace extract were less amorphous than those produced using other carbon sources. Examples are the mcl-PHA produced by: *Ps. chloraphis* using crude biodiesel (37%); *Ps. citronellolis* using olive oil distillate or apple pulp waste (1% and 15%, respectively); or *Ps. resinovorans* using olive oil distillate (6%-7%). *Ps. mendocina* NK-01 mcl-PHA had the lowest ΔHm (0.366 Jg^{-1}).

Table 11 Thermal properties and degree of crystallinity of the mcl-PHA produced by different bacteria strains from the Genus *Pseudomonas*.

Bacterial Strains	Tm (°C)	Tdeg (°C)	Xc (%)	ΔHm (Jg^{-1})	References
Ps. chlororaphis DMS 19603	**50**	**291.1**	**38.8**	**56.6**	**This study**
	43	285	37	n.a.	[63]
Ps. chlororaphis 555	38	284	n.a.	n.a.	
Ps. citronellolis NRRL B-2504	**58**	**292.5**	**18.0**	**26.3**	**This study**
	25.2	n.a.	1	1.9	[36]
	53	296	15	21.3	[64]
Ps. resinovorans NRRL B-2649	**53**	**292.6**	**10.2**	**14.9**	**This study**
	35.6-43.3	n.a.	6-7	8.3-9.9	[36]
Ps. mendocina NK-01	54.9	283.94	n.a.	0.366	[72]

T_m, melting temperature; T_{deg}, degradation temperature; X_c, crystallinity fraction; ΔHm, melting enthalpy;

n. a. data not available

4. Conclusion and Future Work

Regarding the acid hydrolysate, *Ps. resinovorans* was the strain that demonstrated to use more efficiently this carbon source to grow and produce mcl-PHA. So, in order to optimize this production, tests in bioreactors, trying different feed strategies and different conditions should be done. To maximize lignocellulose hydrolysis and minimizing monosaccharides' degradation, different strategies of acid hydrolysis should be established, like different percentages of acid, different acids or the use of enzymes after the hydrolysis to understand which approach is more efficient.

The hydrolysate obtained by HCW demonstrated to be an ineffective carbon source for microbial biopolymer production, probably due the production of degradation products that may inhibit microbial growth and, consequently, polymer accumulation.

This work has demonstrated that grape pomace aqueous extract is a suitable and prospective feedstock for microbial growth and biopolymers production, allowing the valorisation of this waste. All the strains tested had the ability to use the grape pomace extract for polymer accumulation being an indicative of the potential of this waste. *Ps. resinovorans* showed to have the highest maximum volumetric productivity among all the strains tested *Ps. citronellolis* have the highest volumetric productivity. However, this process needs to be optimized regarding the maximum valorisation of the sugars into PHA accumulation. In this way, different strategies of cultivation should be applied and different set of condition and test their impact in microbial growth and polymer production.

In conclusion, the use of grape pomace as sole carbon source for different bacteria has a high potential regarding the biopolymer accumulation, highlighting the advantages of lowering the cost of producing biopolymers, since it was an inexpensive substrate and on the other the grape pomace became a value-added product.

5. References

[1] N. Raddadi and F. Fava, "Science of the Total Environment Biodegradation of oil-based plastics in the environment : Existing knowledge and needs of research and innovation," *Sci. Total Environ.*, vol. 679, pp. 148–158, 2019.

[2] Choi S, Rhie M, Kim H, Joo J, Cho I, Son J, Jo S, Sohn Y, Baritugo K, Pyo J, Lee Y, Lee S and Park S, "Metabolic engineering for the synthesis of polyesters : A 100-year journey from polyhydroxyalkanoates to non-natural microbial polyesters," *Metab. Eng. *, 2019.

[3] A. Anjum, M. Zuber, K. M. Zia, A. Noreen, M. N. Anjum, and S. Tabasum, "International Journal of Biological Macromolecules Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements," *Int. J. Biol. Macromol.*, vol. 89, pp. 161–174, 2016.

[4] I. Mutiara, N. Razaad, Y. Li, L. Zhao, Y. Mo, and J. Wang, "Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review," *Polymers*, vol. 6, pp. 706–754, 2014.

[5] Y. Zheng, J. Chen, Y. Ma, and G. Chen, "Engineering biosynthesis of polyhydroxyalkanoates (PHA) for diversity and cost reduction," *Metab. Eng.*, vol. 13, no. 6 pp. 612-632, 2019.

[6] C. S. K. Reddy, R. Ghai, and V. C. Kalia, "Polyhydroxyalkanoates: an overview," *Bioresource Technology*., vol. 87, pp. 137–146, 2003.

[7] K. Sudesh, H. Abe, and Y. Doi, "Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters," *Elsevier*, vol. 25, pp. 1503–1555, 2000.

[8] Z. Junyu, E. I. Shishatskaya, T. G. Volova, L. Ferreira, and G. Chen, "Polyhydroxyalkanoates (PHA) for therapeutic applications," *Mater. Sci. Eng. C*, vol. 86, pp. 144-150, 2017.

[9] T. Keshavarz and I. Roy, "Polyhydroxyalkanoates: bioplastics with a green agenda," *Curr. Opin. Microbiol.*, vol. 13, no. 3, pp. 321–326, 2010.

[10] A. K. Singh and N. Mallick, "Enhanced production of SCL-LCL-PHA co-polymer by sludge-isolated *Pseudomonas aeruginosa* MTCC 7925," *Letters in Applied Microbiology*, vol. 46, pp. 350–357, 2008.

[11] Wang S, Chen W, Xiang H, Yang J, Zhou Z, Zhu M, "Modification and Potential Application of Short-Chain-Length," *Polymers*, vol.8, no. 8, pp. 273, 2016.

[12] R. A. J. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams, and I. Radecka, "Bacterial synthesis of biodegradable polyhydroxyalkanoates," *Journal of Applied Microbiology*, vol. 102, pp. 1437–1449, 2007.

[13] Loo C and Sudesh K, "Polyhydroxyalkanoates: Bio-based microbial plastics and their properties," *Malaysian Polymer Journal*, vol. 2, pp. 31-57, 2007.

[14] R. D. Ashby and Æ. D. K. Y. Solaiman, "Poly(hydroxyalkanoate) Biosynthesis from Crude Alaskan Pollock (Theragra chalcogramma) Oil," *J. Polym. Environ.*, vol. 16, pp. 221–229, 2008.

[15] Parveen K Sharma, Nisha Mohanan, Ravinder Sidhu and David B. Levin, "Colonization and degradation of polyhydroxyalkanoates by lipase producing bacteria," *Canadian journal of microbiology* vol. 65 pp. 1–44, 2019.

[16] A. Muhr, E. Maria, A. Salerno, A. Reiterer, and M. Schiller, "Reactive & Functional Polymers Biodegradable latexes from animal-derived waste : Biosynthesis and characterization of mcl-PHA accumulated by *Ps . citronellolis*," *Elsevior*, vol. 73, pp. 1391–1398, 2013.

[17] M. Kumar, A. Singhal, P. K. Verma, and I. S. Thakur, "Production and Characterization of Polyhydroxyalkanoate from Lignin Derivatives by *Pandoraea sp*. ISTKB," *ACS Omega*, vol. 2, pp. 9156-9163, 2017.

[18] R. Rai, T. Keshavarz, J. A. Roether, A. R. Boccaccini, and I. Roy, "Medium chain length polyhydroxyalkanoates , promising new biomedical materials for the future," *Mater. Sci. Eng. R*, vol. 72, no. 3, pp. 29–47, 2011.

[19] Superb K. Misra, Sabeel P. Valappil, Ipsita Roy and Aldo R. Boccaccini "Polyhydroxyalkanoate (PHA)/Inorganic Phase Composites for Tissue Engineering Applications," *BioMacromolecules*, vol. 7, no. 8, pp. 2249-2258, 2006.

[20] Muhr A, Rechberger E, Salerno A, Reiterer A, Malli K, Strohmeier K, Schober S, Mittelbach M and Koller M, "Novel Description of mcl -PHA Biosynthesis by *Pseudomonas chlororaphis* from Animal-Derived Waste," *J. Biotechnol.*, vol. 165, no. 1, pp. 45–51, 2013.

[21] Parveen K Sharma, Riffat I. Munir, Teresa de Kievit and David B. Levin "Synthesis of Polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *Pseudomonas chlororaphis*," *Canadian Journal of Microbiology,* vol. 63, no.12, pp. 307-319, 2017.

[22] Parveen K. Sharma, Riffat I. Munir, Warren Blunt, Chris Dartiailh, Juijun Cheng, Trevor C. Charles and David B. Levin, "Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE (PhaC1 16) Enzyme," *Applied sciences,* vol. 7 no. 3, pp. 242, 2017.

[23] M. Tortajada, L. Ferreira, and M. A. Prieto, "Second-generation functionalized medium-chain-length polyhydroxyalkanoates : the gateway to high-value bioplastic applications," *Internaciol Microbiology,* vol. 16, pp. 1–15, 2013.

[24] G. Chen and X. Jiang, "Engineering bacteria for enhanced polyhydroxyalkanoates (PHA) biosynthesis," *Synth. Syst. Biotechnol.*, vol.2, pp. 1–6, 2017.

[25] H. Pakalapati, C. Chang, P. L. Show, S. K. Arumugasamy, and J. C. Lan, "Development of polyhydroxyalkanoates production from waste feedstocks and applications," *J. Biosci. Bioeng.*, vol. 126, no. 3, pp. 282-292, 2018.

[26] C. Du, J. Sabirova, W. Soetaert, and S. Ki Carol Lin, "Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials," *Curr. Chem. Biol.*, vol. 6, no. 1, pp. 14–25, 2012.

[27] Cruz M, Freitas F, Paiva A, Mano F, Dionísio M, Ramos A, Reis M, "Valorization of fatty acids-containing wastes and byproducts into short- and medium-chain length polyhydroxyalkanoates," *New Biotechnology*, vol. 33, no. 1, pp. 206-2015, 2015.

[28] S. Rodriguez-Perez, A. Serrano, A. A. Pantión, and B. Alonso-Fariñas, "Challenges of scaling-up PHA production from waste streams. A review," *J. Environ. Manage.*, vol. 205, pp. 215–230, 2018.

[29] P. Kumar and B. S. Kim, "Valorization of polyhydroxyalkanoates production process by co-synthesis of value-added products," *Bioresour. Technol.*, vol. 269, pp. 544-556, 2018.

[30] B. Kessler, R. Weusthuis, B. Witholt, and G. Eggink, "Production of Microbial Polyesters: Fermentation and Downstream Processes," *Managing*, vol. 71, pp. 159-182, 2001.

[31] B. Diwan, P. Parkhey, and P. Gupta, "From agro-industrial wastes to single cell oils: a step towards prospective biorefinery," *Folia Microbiol. (Praha).*, vol. 63, no. 5, pp. 547–568, 2018.

[32] Carreira P, Mendes J, Trovatti E, Serafim L, Freire C, Silvestre A and Neto C, "Utilization of residues from agro-forest industries in the production of high value bacterial cellulose," *Bioresour. Technol.*, vol. 102, no. 15, pp. 7354–7360, 2011.

[33] A. G. Fallis, "*Biotechnology for Agro-Industrial Residues Utilisation*," *Springer*, vol. 53, no. 9, pp. 1689-1699, 2013.

[34] Fava F, Totaro G, Diels L, Reis M, Duarte J, Carioca O, Poggi-Varaldo H and Ferreira B, "Biowaste biorefinery in Europe: Opportunities and research & development needs," *N. Biotechnol.*, vol. 32, no. 1, pp. 100–108, 2015.

[35] R. S. Singh, N. Kaur, and J. F. Kennedy, "Pullulan production from agro-industrial waste and its applications in food industry: A review," *Carbohydr. Polym.*, vol. 217, pp. 46–57, 2019.

[36] S. Bhushan, K. Kalia, M. Sharma, B. Singh, and P. S. Ahuja, "Processing of apple pomace for bioactive molecules," *Crit. Rev. Biotechnol.*, vol. 28, no. 4, pp. 285–296, 2008.

[37] R. Devesa-Rey, X. Vecino, J. L. Varela-Alende, M. T. Barral, J. M. Cruz, and A. B. Moldes, "Valorization of winery waste vs. the costs of not recycling," *Waste Manag.*, vol. 31, no. 11, pp. 2327–2335, 2011.

[38] S. Antunes, F. Freitas, C. Sevrin, C. Grandfils, and M. A. M. Reis, "Production of FucoPol by *Enterobacter* A47 using waste tomato paste by-product as sole carbon source," *Bioresour. Technol.*, vol. 227, pp. 66–73, 2017.

[39] M. A. Henrique, H. A. Silvério, W. P. Flauzino Neto, and D. Pasquini, "Valorization of an agro-industrial waste, mango seed, by the extraction and characterization of its cellulose nanocrystals," *J. Environ. Manage.*, vol. 121, pp. 202–209, 2013.

[40] OIV, "OIV Statistical Report on World Vitiviniculture," *Int. Organ. Vine Wine*, 2018.

[41] http://www.infovini.com/index.php#tab2 (August 2019).

[42] A. R. Fontana, A. Antoniolli, and R. Bottini, "Grape pomace as a sustainable source of bioactive compounds: Extraction, characterization, and biotechnological applications of phenolics," *J. Agric. Food Chem.*, vol. 61, no. 38, pp. 8987–9003, 2013.

[43] R. A. Muhlack, R. Potumarthi, and D. W. Jeffery, "Sustainable wineries through waste valorisation: A review of grape marc utilisation for value-added products," *Waste Manag.*, vol. 72, pp. 99–118, 2018.

[44] *El Achkar J, Lendormi T, Hobaika Z, Salameh D, Louka N, Maroun R* and *Lanoisellé J*, "Anaerobic digestion of grape pomace: Biochemical characterization of the fractions and methane production in batch and continuous digesters," *Waste Manag.*, vol. 50, pp. 275–282, 2016.

[45] Beres C, Costa G, Cabezudo I, da Silva-James N, Teles A, Cruz A, Mellinger-Silva C, Tonon R, Cabral L and Freitas S, "Towards integral utilization of grape pomace from winemaking process: A review," *Waste Manag.*, vol. 68, pp. 581–594, 2017.

[46] Sousa E, Uchôa-Thomaz A. Carioca J, de Morais S, de Lima A, Martins C, Alexandrino C, Ferreira P, Rodrigues A, Rodrigues S, Silva J and sRodrigues L, "Chemical composition and bioactive compounds of grape pomace (*Vitis vinifera L.)*, Benitaka variety, grown in the semiarid region of Northeast Brazil," *Food Sci. Technol.*, vol. 34, no. 1, pp. 135–142, 2014.

[47] S. Nair, P. Pullammanappallil, and S. Street, "Value added products from vineyards wastes - a review," *Proceedings of ORBIT*, pp. 108-118, 2003.

[48] Q. Deng and Y. Zhao, "Physicochemical, nutritional, and antimicrobial properties of wine grape (cv. Merlot) pomace extract-based films," *J. Food Sci.*, vol. 76, no. 3, pp. 309–317, 2011.

[49] Martinez G, Rebecchi S, Decorti D, Domingos J, Natolino A, Del Rio D, Bertin L, Da Porto C and Fava F, "Towards multi-purpose biorefinery platforms for the valorisation of red grape pomace: production of polyphenols, volatile fatty acids, polyhydroxyalkanoates and biogas," *Green Chem.*, vol. 18, no. 1, pp. 261–270, 2016.

[50] S. Follonier, R. Riesen, and M. Zinn, "Pilot-scale production of functionalized mcl-PHA from grape pomace supplemented with fatty acids," *Chem. Biochem. Eng. Q.*, vol. 29, no. 2, pp. 113–121, 2015.

[51] Follonier S, Goyder M, Silvestri A, Crelier S, Kalman F, Riesen R and Zinn M, "Fruit pomace and waste frying oil as sustainable resources for the bioproduction of medium-chain-length polyhydroxyalkanoates," *Int. J. Biol. Macromol.*, vol. 71, pp. 42-52, 2014.

[52] U. Bornscheuer, K. Buchholz, and J. Seibel, "Enzymatic degradation of (ligno)cellulose," *Angew. Chemie - Int. Ed.*, vol. 53, no. 41, pp. 10876–10893, 2014.

[53] Tomás-Pejó E, Oliva J and Ballesteros M, "Realistic approach for full-scale bioethanol production from lignocellulose: A review," *J. Sci. Ind. Res.*, vol. 67, no. 11, pp. 874–884, 2008.

[54] Haghighi Mood S, Hossein Golfeshan A, Tabatabaei M, Salehi Jouzani G, Najafi G, Gholami M and Ardjmand M, "Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment," *Renew. Sustain. Energy Rev.*, vol. 27, pp. 77–93, 2013.

[55] S. Obruca, "Use of Lignocellulosic Materials for PHA Production," *Chem. Biochem. Eng. Q.*, vol. 29, no. 2, pp. 135–144, 2015.

[56] Xiao-Jun Ji, He Huang, Zhi-Kui Nie, Liang Qu, Qing Xu and George T. Tsao, "Fuels and Chemicals from Hemicellulose Sugars," *Advances in Biochemical Engineering Biotechnology*, vol. 128, pp. 199-224, 2012.

[57] M. J. Taherzadeh and K. Karimi, "Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review," *International Journal of Molecular Sciences*, vol. 9, no. 9, pp. 1621-1651, 2008.

[58] F. M. Gírio, F. Carvalheiro, and L. C. Duarte, "Hemicelluloses biorefinery: a review on biomass pretreatments," *J. Sci. Ind. Res.*, vol. 67, pp. 849–864, 2008.

[59] B. Pedras, M. Salema-Oom, I. Sá-Nogueira, P. Simões, A. Paiva, and S. Barreiros, "Valorization of white wine grape pomace through application of subcritical water: Analysis of extraction, hydrolysis, and biological activity of the extracts obtained," *J. Supercrit. Fluids*, vol. 128, pp. 138–144, 2017.

[60] A. Meillisa, H. C. Woo, and B. S. Chun, "Production of monosaccharides and bio-active compounds derived from marine polysaccharides using subcritical water hydrolysis," *Food Chem.*, vol. 171, pp. 70–77, 2015.

[61] H. M. Liu, F. Y. Wang, and Y. L. Liu, "Hot-compressed water extraction of polysaccharides from soy hulls," *Food Chem.*, vol. 202, pp. 104–109, 2016.

[62] T. Powell, S. Bowra, and H. J. Cooper, "Subcritical Water Hydrolysis of Peptides: Amino Acid Side-Chain Modifications," *J. Am. Soc. Mass Spectrom.*, vol. 28, no. 9, pp. 1775–1786, 2017.

[63] J. R. Vergara-Salinas, M. Vergara, C. Altamirano, Á. Gonzalez, and J. R. Pérez-Correa, "Characterization of pressurized hot water extracts of grape pomace: Chemical and biological antioxidant activity," *Food Chem.*, vol. 171, pp. 62–69, 2015.

[64] M. Herrero, M. Castro-Puyana, J. A. Mendiola, and E. Ibañez, "Compressed fluids for the extraction of bioactive compounds," *TrAC - Trends Anal. Chem.*, vol. 43, no. 2, pp. 67–83, 2013.

[65] Pereira J, Araújo D, Marques A, Neves L, Grandfils C, Sevrin C, Alves V, Fortunato E, Reis M and Freitas F, "Demonstration of the adhesive properties of the medium-chain-length polyhydroxyalkanoate produced by *Pseudomonas chlororaphis* subsp. aurantiaca from glycerol" *Int. J. Biol. Macromol.*, vol.122 , pp. 1144-1151, 2018.

[66] Rebocho A, Pereira J, Freitas F, Neves L, Alves V, Sevrin C, Grandfils C and Reis M "Production of Medium-Chain Length Polyhydroxyalkanoates by *Pseudomonas citronellolis* Grown in Apple Pulp Waste," *Applied Food biotechnology,* vol. 6, no. 1, pp. 71–82, 2019.

[67] H.S. Yun, D.Y. Kim, Chung-Wook Chung and H.W. Kim "Characterization of a Tacky Poly(3-Hydroxyalkanoate) Produced by *Pseudomonas chlororaphis* HS21 from Palm Kernel Oil," *Journal of Microbiology and Biotechnology,* vol. 13, no.1 pp. 64-69, 2003.

[68] M. V Cruz, D. Araújo, V. D. Alves, F. Freitas, and M. A. M. Reis, "Characterization of medium chain length polyhydroxyalkanoate produced from olive oil deodorizer distillate," *Int. J. Biol. Macromol.*, vol. 82, pp. 243-248, 2015.

[69] Cerrone F, Davis R, Kenny S, Woods T, O'Donovan A, Gupta V, Tuohy M, Babu R, O'Kiely P and O'Connor K, "Use of a mannitol rich ensiled grass press juice (EGPJ) as a sole carbon source for polyhydroxyalkanoates (PHAs) production through high cell density cultivation," *Bioresour. Technol.*, vol. 191, pp. 45-52, 2015.

[70] Mun Hwan Choi and Sung Chul Yoon "Polyester Biosynthesis Characteristics of *Pseudomonas citronellolis* Grown on Various Carbon Sources , Including 3-Methyl-Branched Substrates," *Applied and Environmental Microbiology,* vol. 60, no. 9, pp. 3245–3254, 1994.

[71] C. Morais, F. Freitas, M. V. Cruz, A. Paiva, M. Dionísio, and M. A. M. Reis, "Conversion of fat-containing waste from the margarine manufacturing process into bacterial polyhydroxyalkanoates," *Int. J. Biol. Macromol.*, vol. 71, pp. 68–73, 2014.

[72] A. M. Cromwick, T. Foglia, and R. W. Lenz, "The microbial production of poly(hydroxyalkanoates) from tallow," *Appl. Microbiol. Biotechnol.*, vol. 46, no. 5–6, pp. 464–469, 1996.

[73] M. S. G. Lopes, R. C. S. Rocha, S. P. Zanotto, J. G. C. Gomez, and L. F. da Silva, "Screening of bacteria to produce polyhydroxyalkanoates from xylose," *World J. Microbiol. Biotechnol.*, vol. 25, no. 10, pp. 1751–1756, 2009.
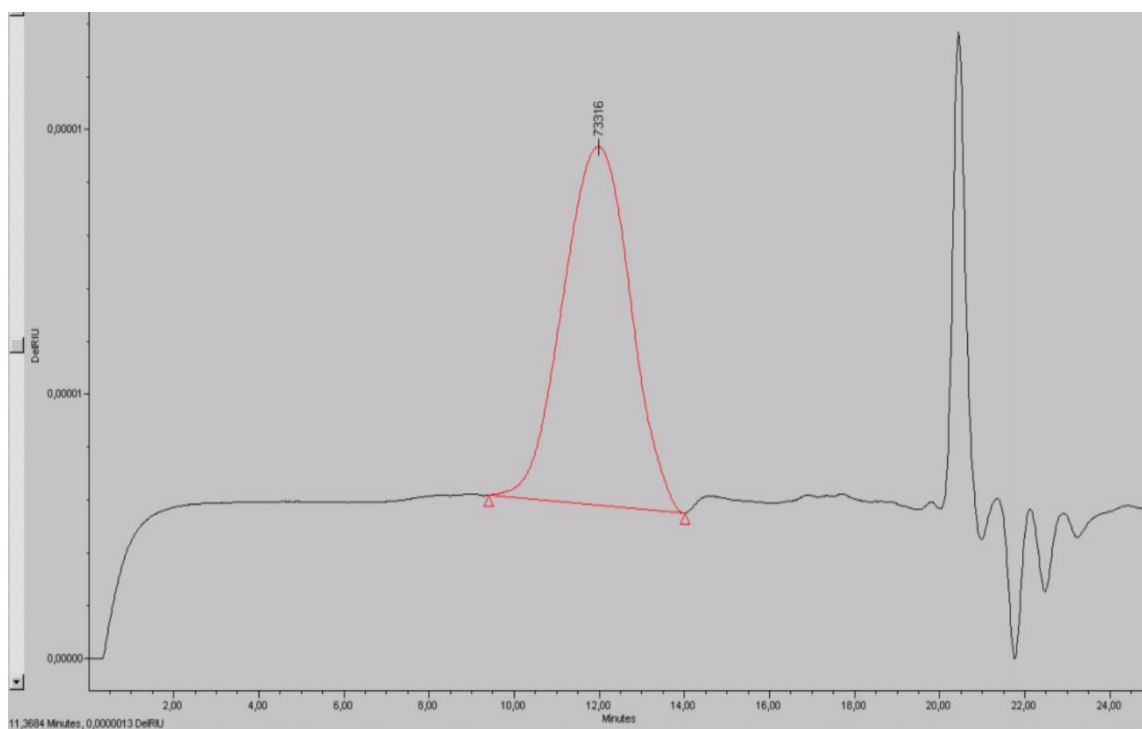
6. Appendices

Figure 24 Size exclusion chromatograms (SEC) of the mcl-PHA polymer produced by *Ps. chlororaphis* DMS 19603 from grape pomace aqueous extract.
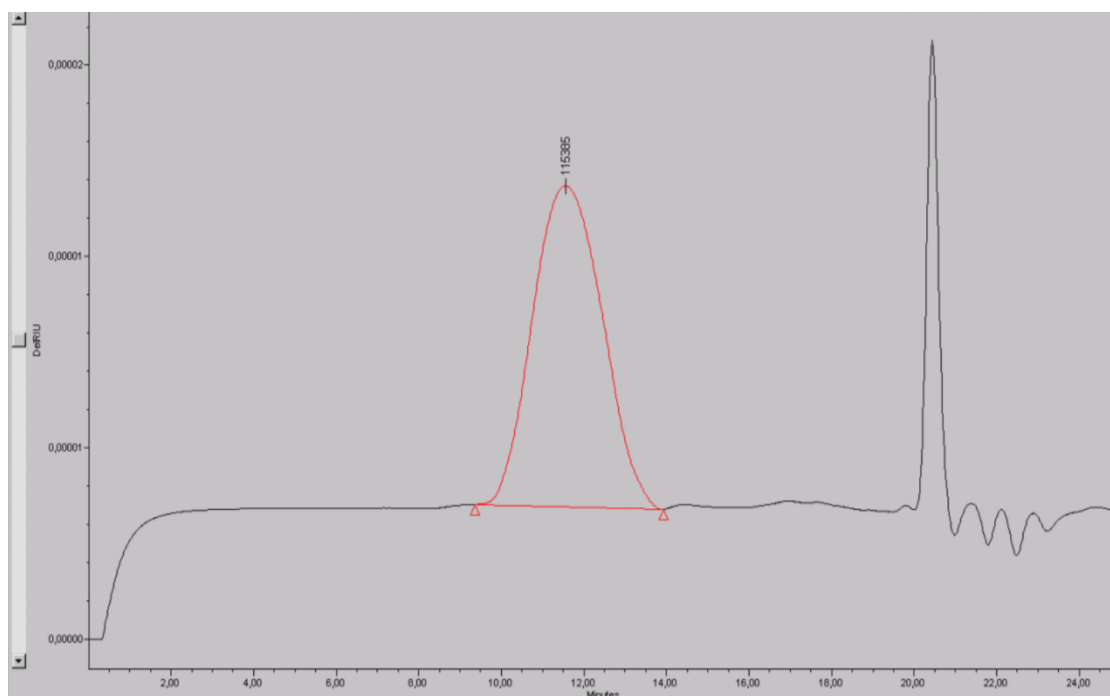

Figure 25 Size exclusion chromatograms (SEC) of the mcl-PHA polymer produced by *Ps. citronellolis* NRRL B-2504 from grape pomace aqueous extract.
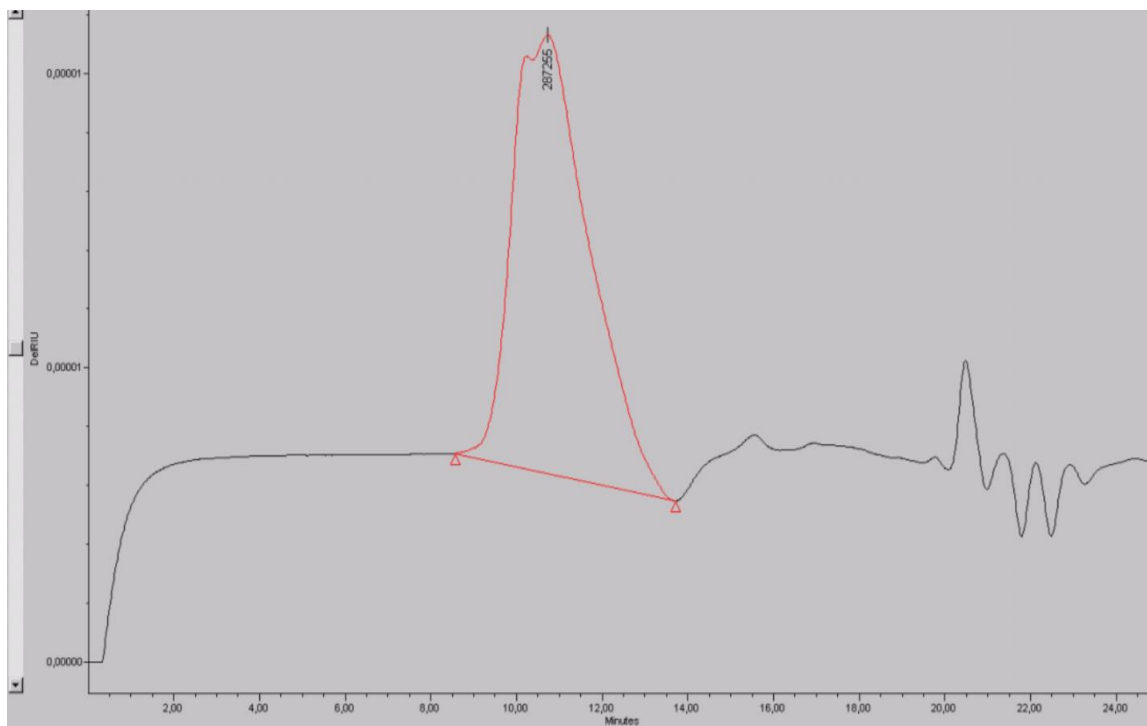
Figure 26 Size exclusion chromatograms (SEC) of the mcl-PHA polymer produced by *Ps. resinovorans* NRRL B-2649 from grape pomace aqueous extract.
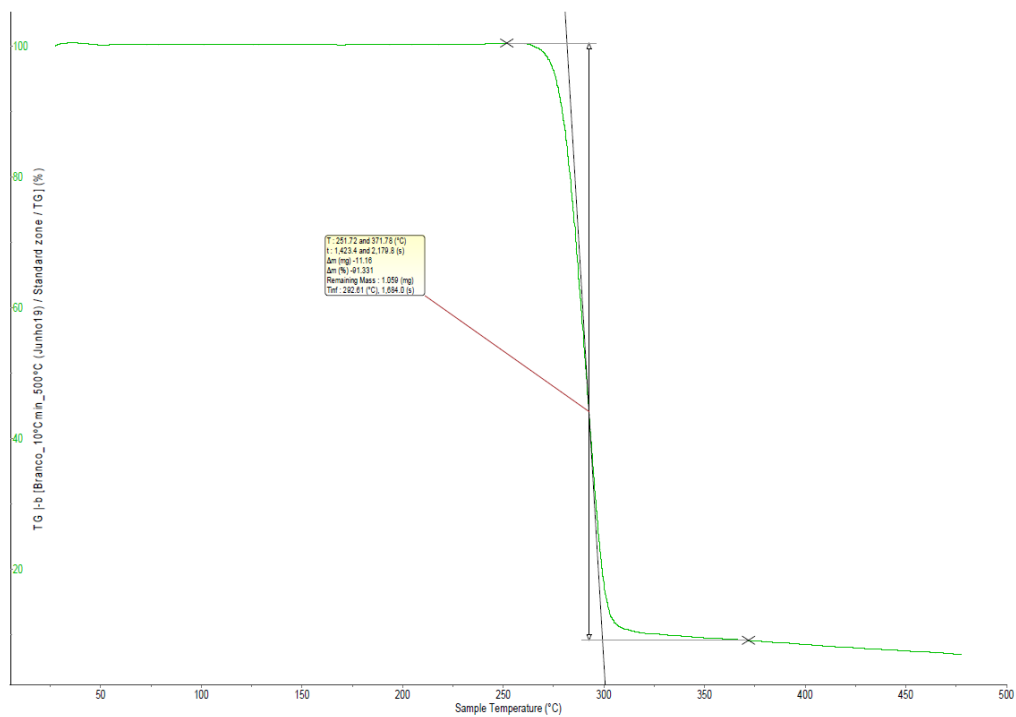

Figure 27 Thermogravimetric analysis (TGA) curve of the mcl-PHA polymer produced by *Ps. chlororaphis* DMS 19603 from grape pomace aqueous extract.
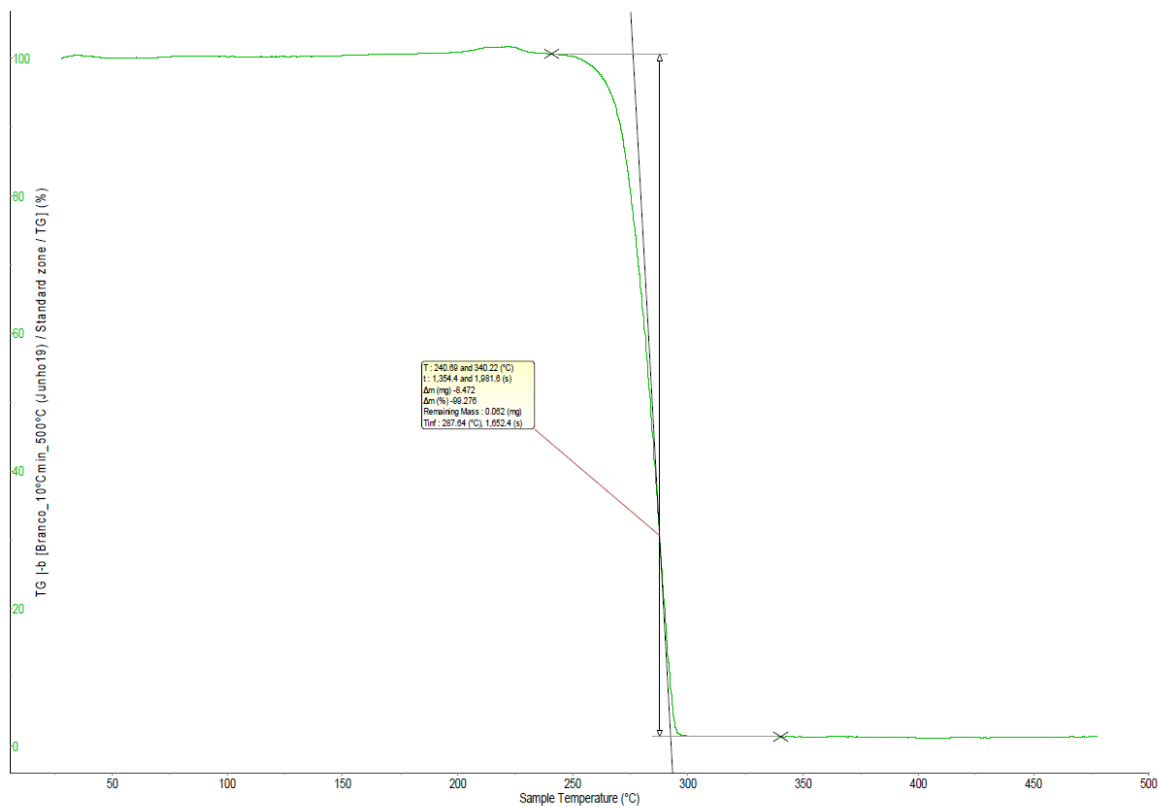
Figure 28 Thermogravimetric analysis (TGA) curve of the mcl-PHA polymer produced by *Ps. citronellolis* NRRL B-2504 from grape pomace aqueous extract.
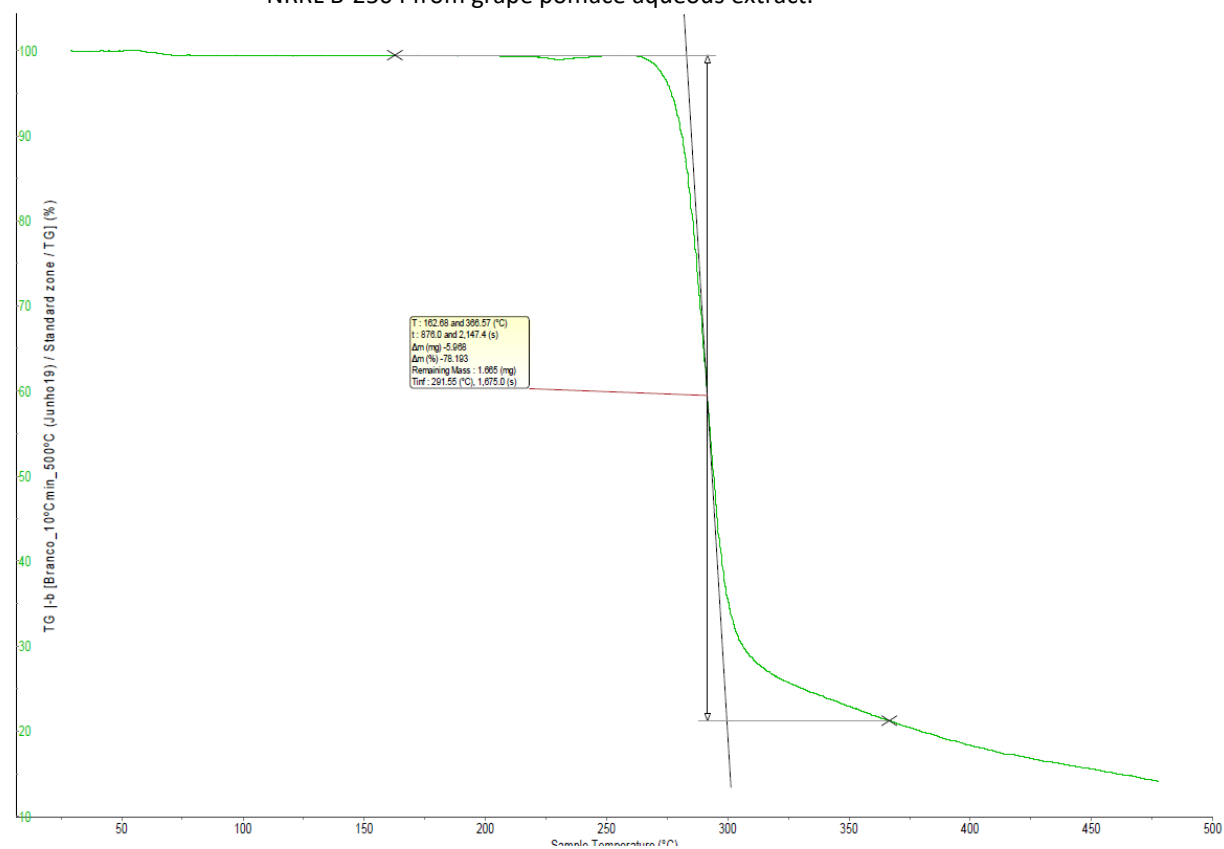

Figure 29 Thermogravimetric analysis (TGA) curve of the mcl-PHA polymer produced by *Ps. resinovorans* NRRL B-2649 from grape pomace aqueous extract.