Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Luísa Seuanes Serafim, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ana Maria Rebelo Barreto Xavier, Professora Auxiliar do Departamento de Química da Universidade de Aveiro

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palavras-chave

Polihidroxialcanoatos, Vinho, *Cupriavidus necator*, Indústria Vinícola, Resíduos Vinícolas.

resumo

Em resposta aos problemas e efeitos nocivos dos resíduos de plástico no ambiente, o desenvolvimento de materiais plásticos biodegradáveis, como os polihidroxialcanoatos (PHA), ganhou um interesse considerável. Os PHA são poliésteres de origem bacteriana e têm sido descritos como uma das alternativas sustentáveis mais promissoras aos plásticos à base de petróleo. De acordo com vários estudos, cerca de 20 a 50% dos custos totais de produção são atribuídos às matérias-primas, pelo que foram avaliados os resíduos da indústria vinícola, mais concretamente, o bagaço de uva (GB) como substratos para a produção de PHA por *Cupriavidus necator*. Os resíduos da indústria vitivinícola são uma potencial fonte de carbono natural, rica em açúcares que permitem o desenvolvimento de microrganismos. Todos os anos, são produzidos globalmente 279 milhões de hectolitros de vinho, sendo cerca de 6 milhões de hectolitros produzidos em Portugal, gerando assim grandes quantidades de resíduos.

Neste trabalho, o GB foi recolhido após a transformação de uvas brancas para a produção de espumantes comerciais da Bairrada, Portugal. Foram testados dois métodos de pré-tratamento do GB: hidrólise hidrotérmica e hidrólise ácida, obtendo-se dois hidrolisados ricos em glucose e frutose. Nos ensaios preliminares verificou-se que o hidrolisado proveniente da hidrólise hidrotérmica (com cerca de 13.12 g L^{-1} de açúcares) foi o que permitiu o melhor crescimento de *C. necator*. Para melhorar o crescimento microbiano e a produção de PHA, foram testadas diferentes fontes de azoto para avaliar a sua influência específica. A fonte de azoto para a qual se verificou um maior crescimento bacteriano foi a ureia que resultou numa concentração celular de 6.369 g L^{-1} com 86% de poli-3-hidroxibutirato, P(3HB). O polímero extraído destes ensaios foi caracterizado. No entanto, a fonte de azoto que promoveu um maior conteúdo em PHA foi o cloreto de amónia com 89,17%.

Este trabalho demonstrou pela primeira vez a utilização de hidrolisado bagaço de uva para a produção de PHA por *C. necator*.

keywords

Polyhydroxyalkanoates, Wine, *Cupriavidus necator*, Wine Industry, Wine Waste.

abstract

The development of biodegradable plastic materials such as Polyhydroxyalkanoates (PHA) gained considerable interest due to the problems and harmful effects of plastic waste on the environment. PHA are bacterial polyesters and were described as one of the most promising sustainable alternatives to petroleum-based plastics. According to several studies, about 20–50% of the total production costs are attributed to the raw materials, and therefore, different types of organic residues have been evaluated as substrates for PHA production. In this work, waste from the wine industry, grape bagasse (GB) was tested for the production of PHA by *Cupriavidus necator.* Every year, 279 million hectolitres of wine are globally produced being about 6 million hectolitres provided in Portugal, therefore generating large amounts of waste.

GB was collected after the transformation of white grapes for the production of commercial sparkling wines from Bairrada, Portugal. Two pretreatment methods were tested with GB: hydrothermal hydrolysis and acidic hydrolysis. Preliminary tests showed that the hydrolysate from hydrothermal hydrolysis (with about 13.12 g L^{-1} of sugars) allowed for the highest biomass concentration and the fastest growth of *C. necator*. To improve microbial growth and PHA production, different nitrogen sources were tested to assess their specific influence. The best nitrogen source for bacterial growth was urea, which resulted in a cell concentration of 6,369 g L^{-1} with 86% of poly-3-hydroxybutyrate, P(3HB). However, the nitrogen source that promoted the highest content in PHA was ammonia chloride with 89.17%. The polymer extracted from these assays was characterized.

This work demonstrated for the first time the use of hydrolyzed GB for PHA production *by C. necator*.

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Abbreviations

(%w/w)	% Weight per Weight
3HAs	3 Hydroxy Acids
AEH	Hydrolysate from Acidic extraction
CDW	Cell Dry Weight
CoA	Coenzyme A
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DO	Dissolved oxygen
DSC	Differential Scanning Calorimetry
DSMZ	German Collection of Microorganisms and Cell Cultures
Fru	Fructose
FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid equivalent
GC-FID	Gas chromatography with flame ionization detector
Glc	Glucose
HEH	Hydrolysate from hydrothermal extraction
HPLC	High performance liquid chromatography
Lcl	Long Chain Length
Mcl	Medium Chain Length
MS	Mineral Solution
P(3HB)	Poly (3-hydroxybutyrate)
P(3HB-co-3HV)	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

P(3HHx)	Poly (3-hydroxyhexanoate)
P(3HHx-co-3HO)	Poly (3-hydroxyhexanoate-co-3-hydroxyoctanoate)
P(3HO)	Poly (3-hydroxyoctanoate)
P(3HO)	Poly (3-hydroxyoctanoate)
P(3HV)	Poly (3-hydroxyvalerate)
P(4HB)	Poly (4-hydroxybutyrate)
PBS	Polybutylene Succinate
PHA	Polyhydroxyalkanoate
PHAs	Polyhydroxyalkanoates
PLA	Polylactic Acid
rCOD	Real chemical oxygen demand
rFru	Fructose consumption rate
rGlc	Glucose consumption rate
tCOD	Theoretical chemical oxygen demand
Tg	Glass transition temperature
TGA	Thermogravimetryc analysis
Tm	Melting temperature
$Y_{PHA/S}$	PHA product yield on substrate
$Y_{X/N}$	Growth Yield
$Y_{X/S}$	Biomass production yield on substrate
μ	Specific growth rate

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CHAPTER I – INTRUDUCTION

1. Circular Economy and Sustainability

Climate change is now affecting every country on every continent. It is disrupting national economies and affecting lives. Besides, urbanization, people's lifestyles, such as travel, intensive exploitation of natural resources, and land-use modification, may increase the likelihood of pandemics, such as that currently experienced. Since the industrial revolution, economic growth has accelerating negative environmental impact. The planet's resources are already getting scarce, and their use is increasing. Responsible consumption and production is an emerging concept for achieving sustainable development, and arouses more attention for the purpose of an efficient utilisation of resources, energy, and infrastructures (OECD 2011) (Karandinos et al. 2019).

Over the last century, human development has been based on fossil resources to produce fuels, energy, materials, chemicals, and precursors for several industries. More recently, the sustainability concerns associated with the massive petroleum exploration has led both governments and industry to pursue a biobased economy (Dabbert et al. 2009). Furthermore, recalcitrance of plastics also became a concern in waste management since the current solutions (e.g. incineration, landfilling) do not ensure a safe and sustainable return of carbon from plastics waste to the environment (Jambeck et al. 2015). Moreover, the accumulation of plastic debris in the environment and their adverse effects have been reported over the last decades (Gall and Thompson 2015).

Industrial biotechnology challenges this pattern and has the potential to break the cycle of resource consumption by allowing for a rethinking of traditional industrial processes. (OECD 2011).The transition from a dependence on fossil fuels to a situation where waste biomass is the main renewable raw material for industry will be the basis of the integrated bioeconomy (Lokko et al. 2018). The top challenges in this area are actions that aimed at minimizing the environmental impact of created products by choosing ingredients that will enable reuse. In this context, attention should be paid to 'The Concept of Circular Economy', schematized in Figure 1, which aims to 'closing the circle' of a product's life by increasing

recycling and reuse and will benefit both the environment and economy (Aguilar, Wohlgemuth, and Twardowski 2018).

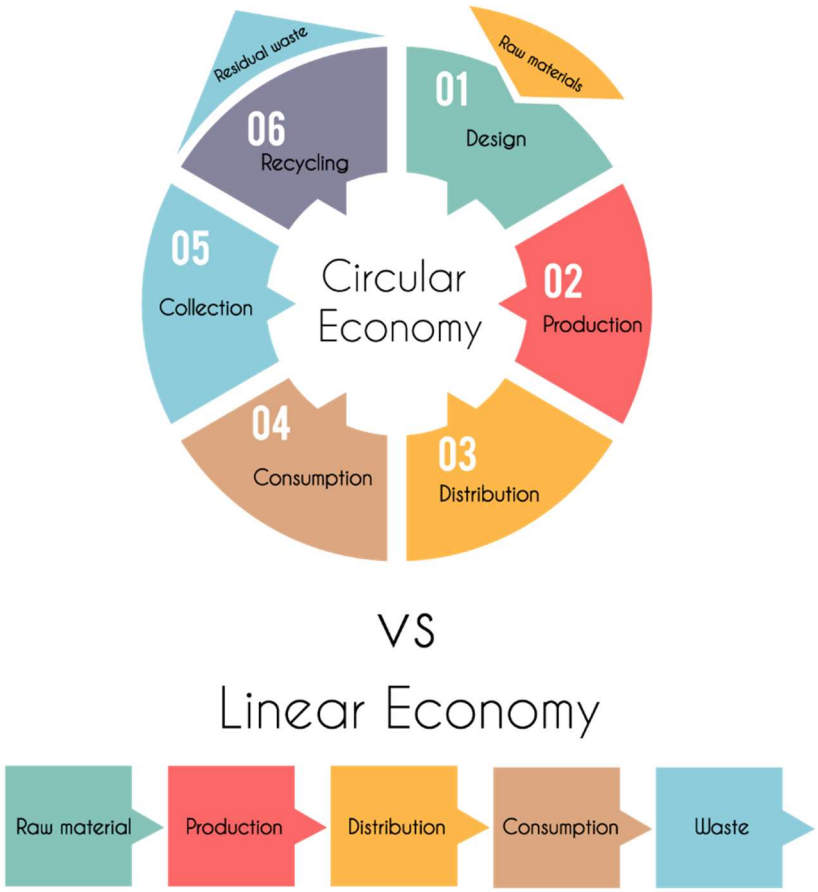


Figure 1 - Circular Economy vs Linear Economy.

Bioeconomy has been adopted by many industries, including agriculture, forestry, fisheries, food, feed, pulp, and paper production. Bioeconomy is a new paradigm whose aim is to create, build up and modernize economic system based on a sustainable use of renewable biological resources (Aguilar, Twardowski, and Wohlgemuth 2019). The bioeconomy can be the key to sustainable growth and, by nature, ensures a circular economy. It aims at the production and utilization of a wide variety of renewable biological resources, including industrial by-products and residues, and the efficient conversion of these resources in value-added products (De Besi et al. 2015). According to the European Commission, the main goals of the bioeconomy are to ensure food security for the increasing world population and stimulate economic growth in industrial, coastal, and rural areas while reducing the

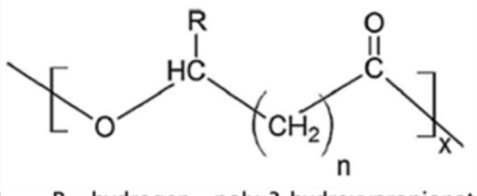
dependence on non-renewable and fossil resources, mitigating climate change, and preserving the environment.

2. Polyhydroxyalkanoates

Plastic materials are an integral part of contemporary life and are being used increasingly because of their durability, ease of moulding and resistance to biodegradation. This latter property, together with the fact that most common plastics are produced from non-renewable crude oil, causes a great concern. The annual synthesis of petroleum-derived plastics was reported as more than 300 million tons, and around 150 million tons of synthetic plastics and plastic-derived materials were consumed worldwide every year (Marichelvam, Jawaid, and Asim 2019). Petroleum-derived plastics take several decades to degrade and accumulate in the environment at a rate of 25 million tons per year (Marichelvam et al. 2019). A currently hot topic is the disastrous pollution of the oceans, as one of the consequences, "microplastics" endanger the complete food chain (Lebreton et al. 2017). Furthermore, plastic recycling offers limited possibilities and incineration can yield toxic compounds. In response to the problems and harmful effects of plastic waste on the environment, there has been considerable interest in the development of biodegradable plastic materials such as Polyhydroxyalkanoates (PHAs).(Cavalheiro et al. 2009).

PHAs are a class of renewable, biodegradable, and bio-based polymers, in the form of polyesters whose overall molecular structure is represented in Figure 2. Together with polylactic acid (PLA) and polybutylene succinate (PBS), they are expected to gradually substitute conventional plastics (C. Kourmentza and Kornaros 2016).

PHAs are attractive substitutes for conventional petrochemical plastics since they can naturally show, or they can be tailor-made to present, similar physical properties to various thermoplastics and elastomers. Plus, PHAs can be completely biodegraded under various conditions by a multiplicity of microorganisms within a period of 1 year. PHAs are naturally synthesized by a variety of different organisms using renewable resources (Solaiman et al. 2006).



n = 1	R = hydrogen	poly-3-hydroxypropionate
	methyl	poly-3-hydroxybutyrate
	ethyl	poly-3-hydroxyvalerate
	propyl	poly-3-hydroxyhexanoate
	pentyl	poly-3-hydroxyoctanoate
	nonyl	poly-3-hydroxydodecanoate
n = 2	hydrogen	poly-4-hydroxybutyrate
n = 3	hydrogen	poly-5-hydroxyvalerate

Figure 2 - General molecular structure of PHAs. Adapted from Mozejko-Ciesielska et al. (2016).

Given the potential of PHAs as new materials, research is being carried out globally in several scientific areas to reach technical and economic feasibility in large scale production (Devesa-Rey et al. 2011). The global bioplastics production volume is projected to be approximately 2.44 million tons in 2022 (Marichelvam et al. 2019). PHA are already produced at commercial scale. However, considering the entire production chain, it still lacks both efficiency and cost competitiveness with petrochemical-based plastics (Dietrich et al. 2017) (Wang et al. 2014). Currently, the production costs of PHA are estimated to be 3 to 4 times higher than petrochemical-based plastics (Koller et al. 2017). Low productivity and high costs hamper the wide production of PHA for low-value/high-volume applications (Keshavarz et al. 2010). As microbial polymers, there are several factors that need to be considered, developed and optimized for the sustainable production of PHA, such as the producing strains, feedstock and media ingredients, and production strategies (Raza et al. 2019).

2.1. Characteristics, Microorganisms, and Applications

PHAs are naturally produced by bacteria in general cultivated on agricultural raw materials. They can be processed to make a variety of useful products, where their biodegradability and naturalness are quite beneficial for application in single use packaging and agriculture.

2.1.1. Characteristics

Due to their fermentative synthesis, natural PHAs are strictly isotactic, featuring exclusively (R)-configuration However, PHAs vary in their mechanical properties and can be grouped into three subcategories: short chain length (scl), medium chain length (mcl), and long chain length (lcl) PHAs. Depending upon the monomeric configuration of polymer, PHAs can also be classified as homopolymer or heteropolymer. Scl-PHAs comprise of 3-5 carbon atoms, and include poly(3-hydroxybutyrate), P(3HB), poly(4-hydroxybutyrate) ,P(4HB), poly(3-hydroxyvalerate), P(3HV), and the heteropolymer including poly (3-hydroxybutyrate-copolymer-3-hydroxyvalerate), P(3HB-co- 3HV). Mcl-PHA polymers consist of 6-14 carbon atoms. Mcl polymers comprise of both homopolymers such as poly (3- hydroxyhexanoate), P(3HHx), and poly(3-hydroxyoctanoate), P(3HO), and heteropolymers such poly (3-hydroxyhexanoate- copolymer-3-hydroxyoctanoate), P(3HHx-co-3HO). More than 14 carbon atoms in the polymeric chain of PHAs come under the category of lcl (Winnacker et al. 2020). Approximately 150 constituents of PHAs have been identified. This large diversity of monomers provides a wide spectrum of polymers with varying physical and mechanical properties, from hard crystalline polymer to elastic rubber, depending on the incorporated monomeric units (Cavalheiro et al. 2009) (Mengxing Li et al. 2020).

Generally, PHA are insoluble in water, optically active and piezoelectric, and have a high degree of polymerization (Reddy et al. 2003). .

PHA types and corresponding characteristics are highly affected by the types of substrates and strains selected. For example, PHA synthase of *A. eutrophus* can polymerize scl-PHAs that has 3–5carbons whereas PHA synthase of *P. oleovorans* can synthesize mcl-PHAs that has 6–14 carbon atoms (Khanna and Srivastava 2005). Most scl-PHAs are very rigid and brittle thermoplastics, due to their high crystallinity (50–70%), while mcl-PHAs

are more elastic and viscous materials, characterized by low crystallinity degrees, glass transition temperatures and melting temperatures (Laycock et al. 2013)

As the monomeric composition of PHA varies, so may vary their physicochemical properties. For example, P(3HB) has a high melting temperature and is highly crystalline which makes it stiff and brittle, while P(3HB)-3HV has lower stiffness and brittleness and higher elongation to break. Conversely, mcl-PHA like polyhydroxyoctanoate (P3HO) have low melting temperature and crystallinity, resulting in higher elasticity (Mozejko-Ciesielska et al. 2019).

P(3HB) is a homo polymer and is the most prevalent and best characterized member of the PHA family. P(3HB), first discovered in bacteria by Lemoigne in 1925, is a linear polyester. It is an intracellular product, accumulated in granules by a wide variety of bacteria when exposed a nutrient deficit and an excess of carbon. (Varsha and Savitha 2011). P(3HB) is apparently produced by microorganisms (such as *Ralstonia eutrophus* or *Bacillus megaterium*) in response to conditions of physiological stress and can be produced either by pure culture than mixed culture of bacteria. The polymer is used as an energy reserve and is metabolized by microorganisms when there are no other energy sources. P(3HB) is a product derived from carbon assimilation (glucose or starch). (Laycock et al. 2013). The behaviour of P(3HB) is similar to polypropylene, which shows excellent gas entrapment characteristics and good moisture resistance (Luzi et al. 2019)

2.1.2. Microorganisms

Many groups of living organisms can synthesize PHAs and accumulate them intracellularly as granules when an excess of carbon source is available in the media, and it is deprived of nitrogen, phosphorous, or oxygen. PHAs act as a stored carbon source and provide energy during starvation conditions. Polymerization of soluble PHA intermediates to non-soluble PHA polymers occurs inside the bacterial system and does not affect the osmotic state of the bacterial cells. This phenomenon is advantageous to bacteria system, as it can prevent the leakage of PHAs from inside to outside the cells while the carbon and energy source remains available at a low maintenance cost (Kumar et al. 2020).

Nowadays, more than 90 genera of bacteria are known to produce PHA (Koller et al. 2017). PHA producers are naturally present in several ecological niches, from which they

can be collected and isolated. Natural environments with fluctuating nutrient availability are particularly minded to refuge PHA producing microorganisms, which is the case for marine and freshwater habitats and soil. PHA producers are also present in systems influenced by anthropogenic activities, such as industrial waste drainage sites and activated sludges from wastewater treatment plants (Koller et al. 2011). Several of the most studied Gram-negative bacteria, which comprise the majority of PHA producers, include *Alcaligenes latus*, *Azotobacter vinelandii*, *Burkholderia sp*., *Cupriavidus necator*, *Hydrogenophaga pseudoflava,* and *Pseudomonas spp*. (Kosseva et al. 2018). Regarding Gram-positive bacteria, the genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Rhodococcus*, *Staphylococcus* and *Streptomyces* have been reported as PHA producers. Commonly, Gram-positive bacteria accumulate PHA in lower contents (2 to 50% of CDW) than Gram-negative species which can reach up to 90% of CDW (Tan et al. 2014). PHA producing archaea are also reported in literature, namely *Haloferax*, *Haloarcula*, *Halobacterium*, *Halococcus*, *Haloquadratum*, *Halorubrum*, *Halostagnicola*, *Haloterrigena*, *Natronobacterium*, *Natronococcus* (Poli et al. 2011).

One of the most important factors for efficient production of PHA is the choice of the microorganisms, which will serve as the biocatalyst to convert the carbon source to PHA (Albuquerque et al. 2018). The choice of an efficient PHA producer must consider its nature and origin (genomic background, pathogenicity, and toxin production), the capacity to reach high cell density in a short time, the range of temperature and optimal pH for growth, even as high PHA accumulation rate and content. Moreover, the capacity to use different inexpensive substrates showing an high yield on carbon source and tolerance to inhibitors is desirable (Wang et al. 2014) (Dias et al. 2006). Preferably, microorganisms with large cell size and a fragile cell wall, with inducible flocculation would increase the efficiency of PHA recovery and purification (Wang et al. 2014).

2.1.3. Applications

Based on their physicochemical properties, PHAs were tested for many applications, summarized in Figure 3, such as packaging containers, bottles, wrapping films, bags, fibres, biofuels or fuel additives and medical devices such as surgical pins, bone screws, and controlled drug delivery carriers (Wang et al. 2010) (Min Li and Lai 2015) (Iwata et al.

2004). As piezoelectric polymers, PHA are eligible to produce pressure sensors for keyboards, microphones, stretch, and acceleration measuring instruments, as well as ultrasonic detectors (Ahammad et al. 2008). Moreover, therapeutic applications such as nerve repair were also reported (Ahammad et al. 2008). As non-toxic and biocompatible materials, PHA can be used for medical and therapeutic applications as drug carriers, tissue engineering scaffolds and medical devices (Ray et al. 2017) (Rai et al. 2011). Catabolic activity of microorganisms results in intermediate like 3-Hydroxy acids (3HAs). It primarily involves depolymerase enzyme resulting in monomers. These intermediates can be modified to synthesize antimicrobials (Kalia et al. 2019).

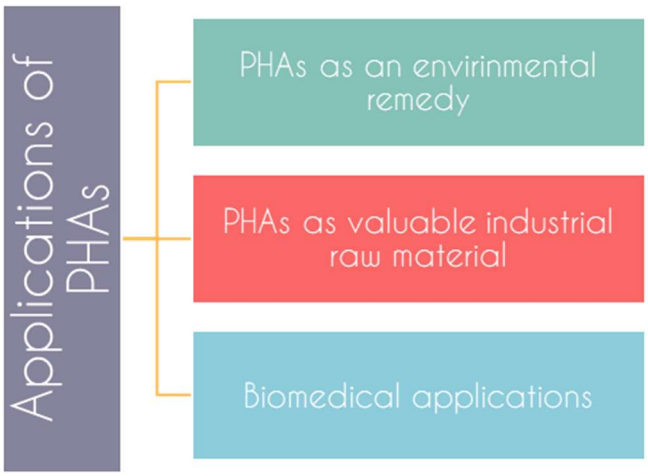


Figure 3 - Schematic representation of Applications of PHAs.

Over the last few decades, commercial scale production of PHA grew quickly, nevertheless the market share is still not very significant. In 2018, PHA products accounted for about 1.2% of the global production of bioplastics (total 2.11 million tons), which reflects the current state of low competitiveness of PHA among the bioplastics sector (Koller et al. 2018). Indeed, there are several factors still hampering the sustainable production of PHA, such as regarding techno-economic and environmental performance, as well as political constraints (Koller et al. 2017) (Álvarez-Chávez et al. 2012) (Y. Wang, Yin, and Chen 2014). Usually, PHA are claimed to potentially contribute to reduce dependence on fossil resources, greenhouse gas emissions, plastic accumulation in landfills, and resource waste, as well as

to create jobs in the biotechnology sector (Álvarez-Chávez et al. 2012). Moreover, given the need for new alternatives to conventional plastics, it is of great interest to attain a sustainable largescale production of PHA, along with other bioplastics, minding the acknowledged obstacles. As such, much research is being devoted to tackle techno-economic and environmental issues, which are yielding several tools and strategies (Koller et al. 2017).

2.2. Production at Industrial Scale

At industrial scale, the production of PHA is usually performed in large bioreactors where microorganisms convert the feedstock into biopolymer. Usually, the process is carried out in stirred tank reactors, under controlled conditions of pH, temperature, and dissolved oxygen, which are adjusted according to the requirements of microorganisms (Keshavarz and Roy 2010) (Koller and Braunegg 2018).

Since PHA accumulation is mainly regulated by nutrient levels, feeding strategies play a central role in process control. Several strategies were attempted for the optimization of accumulation levels of PHA in various microorganisms. The feeding regime may be carried out in batch, fed-batch, continuously or in derivation thereof (Koller et al. 2018).

Batch processes are usually employed for preliminary studies of the PHA producer, to study different operating conditions or bioconversion of novel feedstocks (Kaur et al. 2015). Batch processes naturally deliver low productivity, due to limitations of initial carbon and nitrogen concentrations. These are restricted by inhibitory levels for the production strain, with concentrations typically ranging from 10 to 30 g L^{-1} for carbon source and 2 to 3 g L^{-1} for nitrogen source (Koller et al. 2018). Furthermore, PHA accumulation is usuallynon-growth associated. Usually, the most adopted strategy for large scale production of PHA includes two stages of operation. The first aims at obtaining high cell density in the bioreactor by establishing optimal conditions for biomass growth. Then, growth-limiting conditions deriving from nutrient limitation are imposed in the second stage to induce PHA accumulation (Kaur et al. 2015) . Compared to low cell density processes, generally high cell cultivations result in higher productivity and lower production costs, resulting from the reduction of culture volume (Ienczak et al. 2013). High cell density cultures may be accomplished by repeated batch, fed-batch or continuous configuration. Repeated batch consists in operating an initial batch cultivation, with subsequent partial removal of the

fermentation broth and addition of fresh medium, when almost all the carbon source has been consumed (Koller and Braunegg 2018).

Fed-batch fermentations are initiated as batch and when the substrate concentration declines below a limit value, addition of substrate is initiated. There is no removal of medium and the product is recovered at the end of the process (Kaur et al. 2015) (Koller et al. 2018). This strategy is usually employed to prevent substrate inhibition, as may occur in batch fermentations. Moreover, the periodic substrate feeding avoids nutrient scarcity, which in turn allows a continued microbial growth and thus high cell density (Kaur and Roy 2015). The substrate addition may be administered in several forms: pulse feeding, constant rate feed and progressive rate feed, either increasing or decreasing. The monitoring of the substrate concentration as well as dissolved oxygen and pH is key to regulate feeding (Kaur et al. 2015). Along with the carbon source, pH regulation can be attached to nitrogen source feeding, since biomass growth is often associated with a decrease in pH that may further inhibit PHA production (Koller et al. 2018). (Koller et al. 2018). Both in batch and fed-batch processes, the process must be stopped before intracellular degradation of PHA occurs, while substrate loss should be prevented by avoiding premature termination of the process. Therefore, tight control of substrate concentration must be maintained, which can be performed either by periodic sampling and analysis or in situ control (Koller et al. 2018).

Continuous processes are usually performed in continuous stirred tank reactors (CSTR) and are characterized by a continuous substrate feeding and fermentation broth removal, keeping constant the working volume, as well as, the concentration of the components of the reactional mixture – steady-state process. The ratio between medium flow rate and the reactor working volume, the dilution rate, is a key parameter in these processes since it directly influences biomass growth rate and, consequently, PHA productivity. Insufficient substrate supply to cells will result from too low dilution rates, leading to low growth rates and productivity. On the other hand, too high dilution rates result in biomass wash out (Koller et al. 2018). With optimization of this parameter, continuous production processes typically yield high productivity for microorganisms with high maximum specific growth rates (Kaur et al. 2015). For PHA production under secondary metabolism, single-stage continuous processes are not as efficient as fed-batch fermentations since they fail to provide the required nutrient limitation to induce PHA accumulation (Koller et al. 2018). Multistage continuous processes were already studied to overcome this issue (Atlić et al.

2011). Nevertheless, the risk of contamination is one of the major aspects hampering industrial implementation of continuous processes (Koller et al. 2015).

Even though several works were done to reduce the major obstacles that hinder the production by using renewable low-cost substrates and constantly developing and assessing the sustainable production of the biopolymer, only few companies have come forward in the long run to produce PHAs in a commercial scale. The key market players profiled include Kaneka Corporation (Japan), Danimer Scientific. (U.S.), Shenzhen Ecomann Biotechnology Co., Ltd (China), Bio-On Srl (Italy), Newlight Technologies, LLC (U.S), and TianAn Biological Ma- terials Co. Ltd. (China); where mostly pure cultures are used (Sabapathy et al. 2020).

2.3. Main Difficulties in the production and marketing of PHAs

Considering the recalcitrance of conventional plastics in the environment, replacement of synthetic plastics with PHAs would have huge benefits for the society and the environment. Wide commercialization and industrialization of PHAs is still struggling due to their high production costs, resulting in higher prices compared to conventional polymers (Kourmentza et al. 2017). Figure 4 schematizes the main costs associated with the production of PHAs.

The main reasons for their high costs are the high prices of high purity substrates, such as glucose, production in discontinuous batch and fed-batch cultivation modes, and large amounts of solvents and/or labour regarding their downstream processing. For example, commercial PHA produced by microbes is still around three times more expensive than petroleum-based plastics. The PHA price, depending on polymer composition, ranges from 2.2 to 5.0 €/kg that is at least three times higher than the major petrochemical based polymers which cost less than 1.0 €/kg (Aramvash et al. 2018)(Sabapathy et al. 2020).

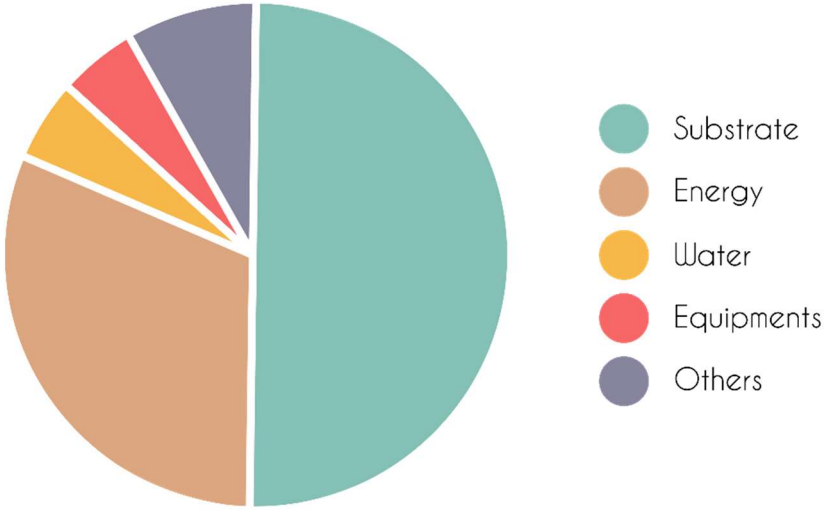


Figure 4 - Important factors contributing in PHA production cost. Adapted from Geeta Gahlawat (2019).

Raw materials correspond to about 50% of the production cost in which carbon sources account for 70–80% of the total cost, which include saccharides (e.g., glucose, lactose), short-chain organic acids (e.g. acetic acid, butyric acid,), alcohols (e.g. ethanol, glycerol), alkanes (e.g., hexane, octane) and gases (e.g., methane and carbon dioxide) (Tan et al. 2014) (Khosravi-Darani et al. 2013). Cheap and readily available feedstocks such as industrial, agricultural, municipal, and food-based wastes that are rich in carbon are desirable feedstocks for PHA production (Khanna and Srivastava 2005) (Mengxing Li et al. 2020). Particularly, wastewater treatment incorporated into PHA production has been studied intensively. For example, an analysis based on experimental results at laboratory scale and detailed data from German waste water treatment plants showed that 20% and 115% of current biopolymer production could be produced at German and EU treatment plants, respectively (Pittmann and Steinmetz 2016). The conversion of these wastes into PHA would create profit and solve waste disposal problems.

Generally, in recent years, PHAs have been produced from cheese whey (Colombo et al. 2016), municipal wastewater and solid waste (Bengtsson et al. 2017) (Sun et al. 2020), spend coffee grounds (Stanislav Obruca et al. 2014), waste cooking oil (Tufail, Munir, and Jamil 2017), agro-industrial waste (Elain et al. 2016), phenol (Y. Zhang et al. 2018), food waste (Ravindran and Jaiswal 2016), non-food crops such as ryegrass (Kataria et al. 2018) and Sweetwater, a by-product from sugar cane refining (Mohd Yatim et al. 2017).

So far, there are still some limitations in using low-cost feedstocks for PHA production since it results frequently in low PHA productivity (Koller et al. 2017). Extensive upstream processing and prior optimization are often required to deal with variation in compositions, low carbon concentration, carbon sources in inaccessible forms and presence of inhibitory compounds. These additional pre-treatment processes may in turn increase production costs, which counteracts with the main purpose of cost reduction (Koller et al. 2017) (Raza, Abid, and Banat 2018). Another constraint with using this kind of feedstocks is the likely contamination with DNA, endotoxins, proteins, lipids of viral or bacterial origin, which hinders the use of the resulting PHA in medical applications. Further extensive purification processes would increase the overall production cost (Raza, Abid, and Banat 2018).

The extraction of intracellular PHAs from cellular biomass creates a major drawback in the development of a commercially viable fermentation process. This could mainly be due to several reasons such as intracellular nature of product and less solubility in various classical non-toxic solvents. PHAs biopolymers are generally soluble in toxic halogenated solvents such as chloroform which are either expensive or not so easy to handle due to their toxicity. Therefore, simple, economical, and effective methods of isolating pure biopolymer from the cells are desperately needed. An ideal extraction protocol must result in high recovery and purity levels at reasonably low cost. Several recovery protocols have been used by different investigators for the extraction of PHAs granules from the cells. In recent years, researchers are now exploring various non-halogenated solvent such as propylene carbonate, ethanol, butyl acetate and ethyl acetate etc. as an environment friendly alternative to toxic halogenated solvents for the extraction of PHAs (Gahlawat 2019). Gahlawat and Soni reported that 1,2-propylene carbonate could be used as a solvent for the recovery of PHAs from cells of *C. necator* (Geeta Gahlawat and Soni 2017) (G. Gahlawat and Kumar Soni 2019). Another study aimed at developing an environmental-friendly and halogen-free approach for the extraction of PHA from genetically engineered *C. necator* using water and ethanol (Fiorese et al. 2009).

Another major limitation for the industrial production of PHAs is to maintain optimal bacterial growth conditions and to maximize PHA production, accumulation in cells and productivity. Optimization of culture conditions is important for empirical study, especially for large scale process in industry (Chen et al. 2015) (Alsafadi and Al-Mashaqbeh 2017).

For example, coupling PHA production to wine industry (Martinez et al. 2016), as well as lignocellulosic biorefineries (Khosravi-Darani et al. 2013) (Xu et al. 2010) was already reported. A close cooperation with industry will contribute to the development of an environmentally, economically feasible and scalable production of PHA (Koller et al. 2017). After all, the influence of decision-makers in politics and industry is essential for the shift from conventional plastics to sustainable bioplastics (Philp et al. 2013). Policy structure regarding PHA is vital for a successful implementation of large-scale processes, such as regarding incentives for production and acquisition of PHA derived products and guiding lines for research and innovation within academia and industry. For such, biopolymers like PHA have to be included in bioeconomy implementation strategies (Philp et al. 2013) (Dietrich et al. 2017).

3. PHAs production by *Cupriavidus necator* from wastes

Pure cultures are cultivated under strict sterile conditions to keep a single PHA-producing strain. This procedure is believed to result PHA with consistent characteristics, which is desirable for further manufacture of bioplastics that employ conventional thermal processing techniques (Koller et al. 2017). Additionally, these cultures usually provide relatively high productivities with simple substrates, which facilitates its optimization (Tan et al. 2014). In fact, the use pure cultures to produce PHA is the most common strategy, also because the results are more reproducible. On the other hand, the need for tight process control and sterility contributes to the increasing of operating costs (Dias et al. 2006).

C. necator is one of the most studied PHA-producing microorganisms for because of its easy cultivability, well understood biochemistry and physiology and capacity to accumulate higher amounts of PHAs from a wide range of substrates, including both purified substrates and cheap feedstocks carbon sources (such as glucose, fructose, and glycerol). (López et al. 2015) (Anjum et al. 2016) (Geeta Gahlawat 2019). *C. necator* is a non-pathogenic, Gram-negative bacterium, found in soil, as well as in freshwater habitats. *C. necator* can be both autotrophic, with CO_2 as carbon source and H_2 or formate as energy source, and heterotrophic using a vast range of substrates, such as monosaccharides, sugar acids, organic acids, amino acids and some alcohols and polyols (Berezina et al. 2015). This bacterium grows in the range of temperatures of 15 to 55ºC, with optimum temperature of

about 27ºC, while pH may range from 5.5 to 9.2, with optimum growth in 7.0 to 8.0 (Butlin 2002).

As PHA producer, *C. necator* is very robust with a broad metabolic activity. The main PHA produced is the P(3HB), but it also produces P(3HB)-3HV if co-substrates (e.g. propanol, valeric acid) are available (Gahlawat et al. 2017). Also, other polymers (e.g. P(3HB)-3HHx) can be obtained recurring to genetic engineering (Sato et al. 2013). *C. necator* can accumulate PHA in high contents (up to ca. 80% CDW) with simple substrates (G. Gahlawat et al. 2019).

To reduce production costs, much research is being dedicated to study the conversion of inexpensive feedstocks into PHA by *C. necator*. Some inexpensive feedstocks were used to produce PHA by *C. necator*, such as agricultural and food waste and lignocellulosic by-products derived from forestry. For example, cooking oil (Cruz et al. 2015), spent coffee grounds oil (Cruz et al. 2014), waste rapeseed oil (Stanislav Obruca et al. 2010), rapeseed oil (Verlinden et al. 2011), date seed oil (Yousuf 2017), kitchen waste (Farah et al. 2011), beer brewery wastewater and maltose (Amini et al. 2020), margarine waste (Morais et al. 2014), pineapple wastes (Sukruansuwan et al. 2018), orange juicing waste (Guzman et al. 2016), waste potato starch (Rusendi et al. 1995), waste animal fats (Riedel et al. 2015), pinewood hydrolysates (Kim et al. 2020), alkaline pre-treated lignin liquor from corn stover (Salvachúa et al. 2015), and green grass juice (Koller et al. 2005). On the other hand, crude glycerol resulting from biodiesel production has been extensively studied for PHA production by C. necator (Gahlawat et al. 2017) (Mothes et al. 2007) (Cavalheiro et al. 2009) (Cavalheiro et al. 2012) (García et al. 2013).

In summary, several studies present promising results concerning the use of inexpensive and renewable bio-based resources. Furthermore, *C. necator* is already used for commercial production of PHA in several companies, such as Bio-on (Italy), P(3HB) Industrial (Brazil) , SIRIM Bioplastics (Malaysia) and TianAn Biologic Materials (China) (Kourmentza et al. 2017).

In order to minimize production costs, many raw materials from waste industries have been used as a carbon source for PHA production. For example, coupling PHA production to wine industry (Martinez et al. 2016), as well as lignocellulosic biorefineries (Khosravi-Darani et al. 2013) (Xu et al. 2010) was already reported. In this work, waste from the wine industry will be used.

4. Wine Industry

All around the world, nearly 62 countries have wine industry (Bharathiraja et al. 2020). Every year, 279 million hectolitres of wine were globally produced of which 65% are produced in Europe (Da Ros et al. 2016) (Mendes et al. 2013).

The traditional wine production uses grape as a raw material. Production of wine is a multistage process, which starts from the cultivation and harvesting of raw materials, transportation of goods, followed by processing of wine production. Winemaking steps included washing of the grapes, stalks separation, crushing of grapes, addition of grape marc, followed by fermentation. In all these steps, a huge volume of water is used, which results on the generation of substantial quantities of wastewaters. At the end of fermentation process, separation of wine is performed, and further clarification and stabilization processes are carried out. At this stage, solid particles are generated as waste. Further, to improve the quality of wine, aging processes are performed at different time intervals. Finally, the produced wine is filtered and packed (Sirohi et al. 2020). Figure 5 summarizes the production of wine as well as the residues that form during the process.

In Europe, wineries are steeped in tradition and have a high economic value to the agricultural sector. At the same time, through the processes of wine production, they also have a considerable environmental footprint, including intensive use of soil, introduction of pesticides, significant water consumption, and production of high quantities of by-products and waste (Christ et al. 2013).

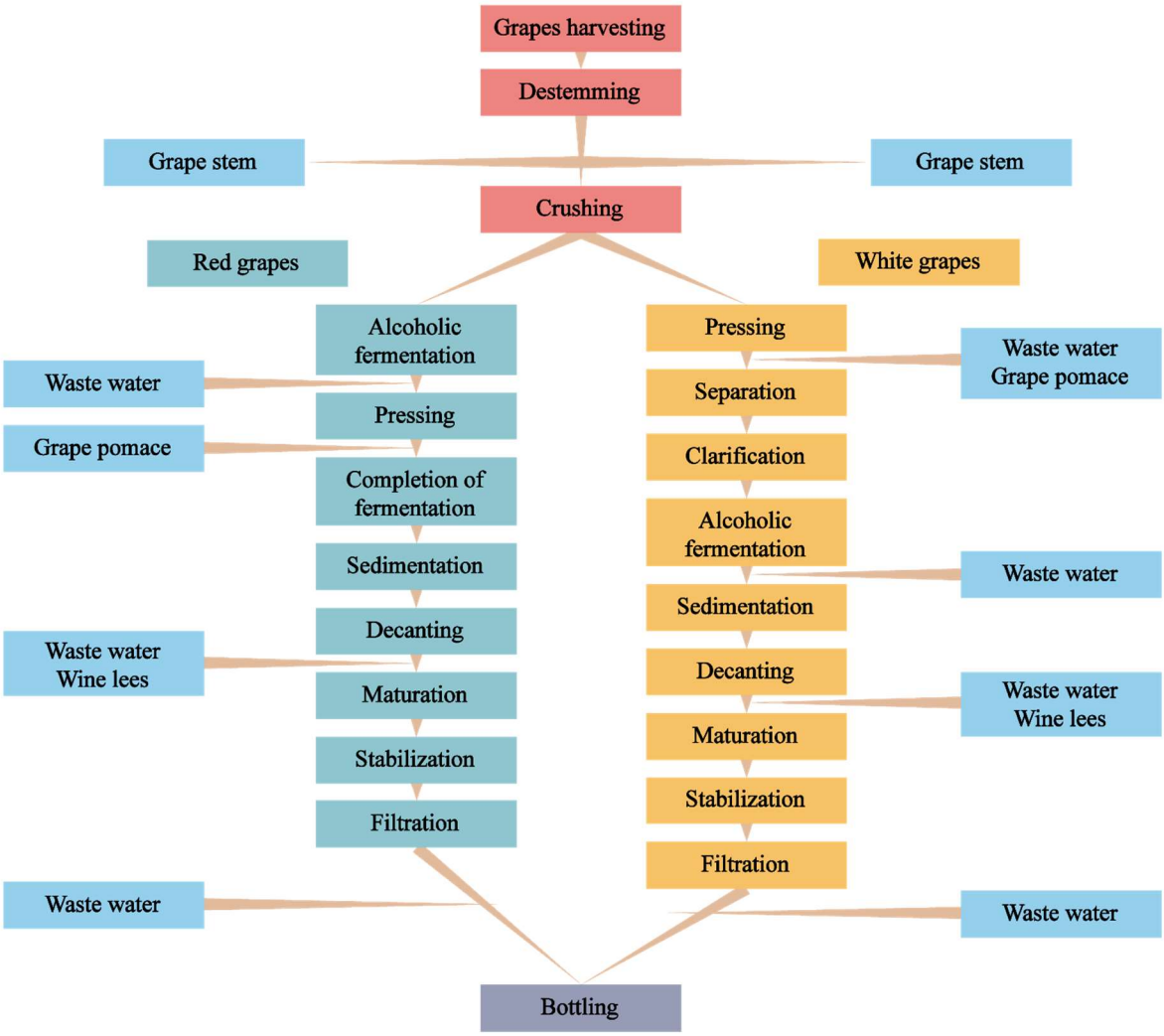


Figure 5 - Summary of wine making process. Adapted from Bharathiraja et al. (2020).

Despite being associated with a large number of environmental problems, the wine industry has traditionally been subjected to a low amount of regulations probably due to the fact wine has generally been considered an environmentally 'safe' product (Ene et al. 2013) (Ruggieri et al. 2009). Regulators from around the world are becoming increasingly aware of the environmental implications of wine production and industry associations are responding through the development of proactive environmental initiatives that appear to be

designed to support environmental self-regulation (Cordano et al. 2010) (Pullman, Maloni, and Dillard 2010).

4.1. Wine Waste

During the winemaking process residues (Figure 6) such as winery wastewater, grape stalks, grape bagasse, wine lees and vine shoot are generated, which eventually do not have any major application or economic value and their disposal in the environment is of serious concern. Despite tremendous improvement and growth in wine industry over the past several decades, the waste generated has not been still properly utilized for environmental sustainability because conventional methods available for the treatment of winery industry waste are expensive and energy intensive (Østergaard 2012).

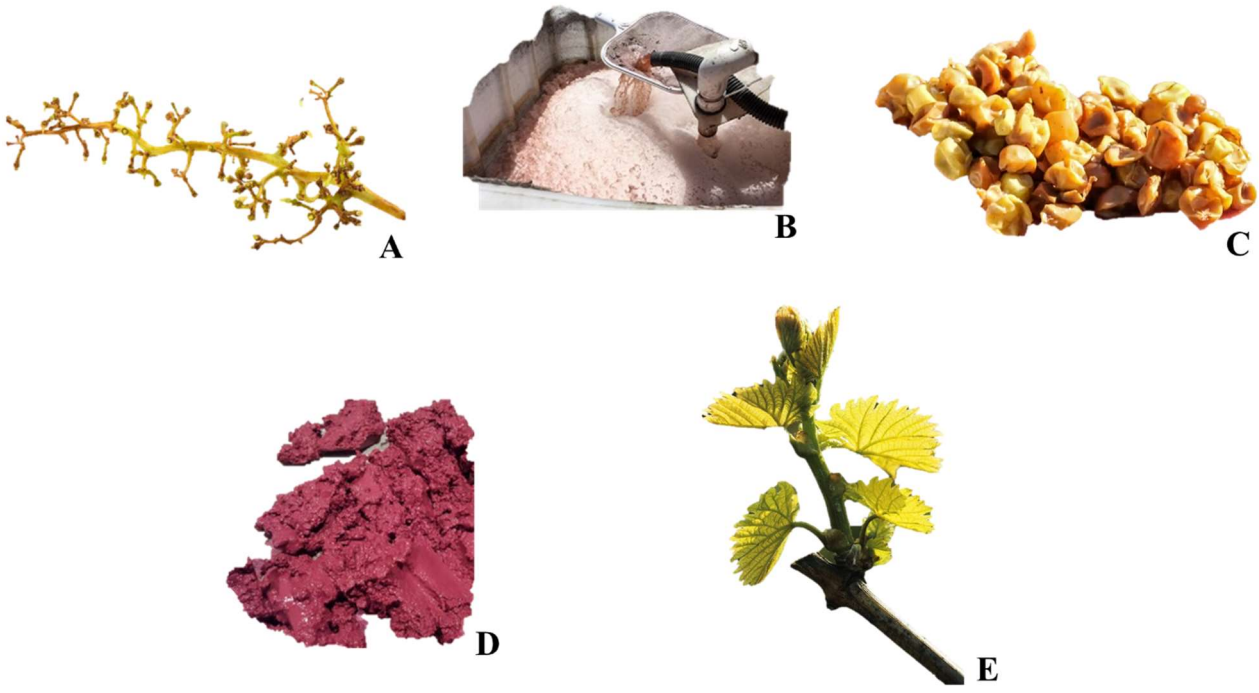


Figure 6 - Winemaking process residues. A) Grape Stalks; B) Wastewater; C) Grape Bagasse; D) Wine Lees; E) Vine Shoot.

The composition of winery waste (Table 1) depends on the harvest conditions and period (Ene et al. 2013). About 0.5–14 L of winery water is generated per litre of wine produced. Winemaking process residues are acidic, phytotoxic and contains high level of biological oxygen demand and contains salts, organic matters, trace elements such as magnesium, calcium and sodium, herbicide and pesticides and has low pH (less than 5.5). They also contains a notable amount of sugars, organic acids, glycerol, and alcohols along with the microbial population of yeasts and bacteria (Lucas et al. 2009). The presence of sulphur, moderate salinity and inorganic particles has also been reported in winery effluents (Kyzas, Symeonidou, and Matis 2016).

Table 1 - Overview of winery waste compositions. Adapted from Bharathiraja et al. (2020).

Winery Waste	Compositions
Winery wastewater	Inorganic salts, carbohydrates, organic acids, glycerol, alcohols, microorganism, magnesium, calcium, sodium, sulfur, and inorganic particles.
Grape stalks	Proteins, hemicellulose, cellulose and lignin, lignocellulose, and lipid.
Grape bagasse	Protein, cellulose, pectin, monosaccharides, polysaccharides, and oligosaccharide.
Wine lees	Inorganic salts. Organic acids, phenolic compounds, cellulose, hemicellulose, lignin, and microorganism.
Vine shoot	Lignin, hemicelluloses, and celluloses.

The solid residues generated from winemaking industry include grape stalks (leaves and shoots), grape seed, wine lees and grape bagasse whose chemical composition differed based on the source (Table 2). Grape stalks contain a notable number of proteins,

hemicellulose, cellulose, and lignin. Grape bagasse is generated as solid waste during the initial stages of grape juice production and contains water-soluble and water-insoluble contents. Its moisture content varies from 40 to 81% and contains notable number of insoluble residues and protein along with cellulose and pectin compounds. Water-soluble contents are due to the presence of monosaccharides (glucose and fructose), polysaccharides, and oligosaccharides whereas cell wall participating polysaccharides are the water-insoluble in nature.

Table 2 - Characterization of different solid winery waste. Adapted from Bharathiraja et al.

Parameter	Grape pomace	Grape stalks
Moisture (%w/w)	73.6	7.09
Reducing sugars (%w/w)	1.5	-
Ash (%w/w)	4.6	6.11
Cellulose (%w/w)	22.7	12.19
Proteins (%w/w)	18.8	6.1
Tannins (%w/w)	13.8	15.9
Hemicellulose (%w/w)	22.2	25.7
Glucose (%w/w)	-	13.35
Fructose (%w/w)	-	13.08
Lignin (%w/w)	5.12	32.35

Wine lees are formed at the end of the fermentation process. Wine lees are composed of inorganic components, organic acids, and phenolic compounds. Both liquid and solid fractions are present in wine lees. Solid fractions of wine lees contain cellulose, hemicellulose, lignin, seeds, grains, organic and inorganic salts. The liquid fraction of wine lees is called as vinasse, which consists of the spent fermentation broth. It contains about 58% of water content on a weight basis with low pH 3.5 and is the major sources of polyphenol compounds.

Circular bioeconomy deals with the processes in which residues generated in different stages of bioprocesses are simultaneously utilized in the same cycle with the formation of

some other products. In bioeconomy, biomass-based processes have attained greater relevance in which renewable biomass are used as feedstock to produce fuels and chemicals with a view to replace fossil-based fuels and chemicals (Mak et al. 2020). Therefore, aadoption the concept of biorefinery for the treatment and management of winery waste could offer potential benefits for the environmental sustainability and generate additional economic benefits.

4.2. Potential of Wine Waste

The winemaking industry has been majorly positively portrayed, due to its socioeconomic and cultural benefits (Rončević et al. 2019). Regardless of the vast amounts of waste generated, the great use of water resources and the exhaustive land usage, the industry has not been viewed negatively by the public. This, in turn, has encouraged its development and consequent generation of higher amounts of waste.

Conventional treatment of waste is becoming increasingly expensive, demanding significant amounts of effort, resources, and energy for safe waste discharge into the environment (Østergaard 2012). Winery industry waste can be effectively used to produce commercially important products by applying biorefinery concept. The wastes generated in winemaking process can be effectively used in bioprocesses to produce commercially important enzymes, biobased organic acids, biofuels, and other products.

The phenolic compounds of wine, and particularly the flavanols, have been the focus of recent studies as their relation with the beneficial effects attributed to a moderate consumption of wine was found. These compounds have their origin in grape, and only a part of them is transferred to the must. Their extractability mainly depends on the employed technological conditions during vinification. For this reason, important quantities of phenolic compounds still remain in the wine by-products and there is great interest in the exploitation of this type of grape by-products to obtain potentially bio-active phenolic compounds (Kammerer et al. 2004). Grape bagasse can also be used to produce bioenergy and xylanase (Díaz et al. 2012). Hot water extracts of grape skin pulp can serve as a good substrate to produce pullulan, an industrially important polysaccharide. Wastewater can be used to produce lipase, tannase and protease (Salgado et al. 2016). Recently, wine waste was tested as substrate for the production of citric acid (Papadaki and Mantzouridou 2019).

Summing up, winery waste could be used to produce many value-added products and several of them are described in table 3.

Table 3 – Value-added products from different winery wastes.

Product	Source	Microorganisms	Reference
Lipase, Protease, Tannase	Winery wastewater	*Aspergillus species*	(Salgado et al. 2016)
Citric acid	Grape bagasse	*Aspergillus niger*	(Papadaki et al. 2019)
Tartaric acid	Wine less	-	(Kontogiannopoulos et al. 2016)
Lipid	Winery wastewater	*Chlorella species*	(Ganeshkumar et al. 2018)
Biooil	Grape seed	-	(Fernández et al. 2010)
Bioethanol	Grape bagasse	-	(Corbin et al. 2015)
Methane	Grape bagasse	-	(El Achkar et al. 2018)
Methane	Grape bagasse and wine lees	-	(Da Ros et al. 2016)
Xanthan	Winery wastewater	*Xanthomonas campestris*	(Rončević et al. 2019)
Biocompost	Winery waste sludge and grape stalks	-	(L. Zhang and Sun 2016)
Xylanase	Grape bagasse	*Aspergillus awamori*	(Díaz et al. 2012)
Ethanol	Grape bagasse	*Escherichia coli*	(Zheng et al. 2012)

Table 3 (continuation) - Value-added products from different

Product	Source	Microorganisms	Reference
Biosurfactant	Vine shoot	*Bacillus tequilensis*	(Cortés-Camargo et al. 2016)
Phenyllactic acid, Biosurfactants	Vine shoot	*Lactobacillus species*	(Rodríguez-Pazo et al. 2013)
Lactic acid, Biosurfactants	Vine shoot	*Lactobacillus pentosus*	(Bustos et al. 2007)
Lactic acid, Biosurfactants	Grape bagasse	*Lactobacillus pentosus*	(Rivera et al. 2007)
Xylanase, Pectinase	Grape bagasse	*Aspergillus awamori*	(Díaz et al. 2012)
Acetone – butanol – ethanol	Grape bagasse	*Clostridium beijerinckii*	(Jin et al. 2018)
Biogas	Wine lees	-	(Da Ros et al. 2014)

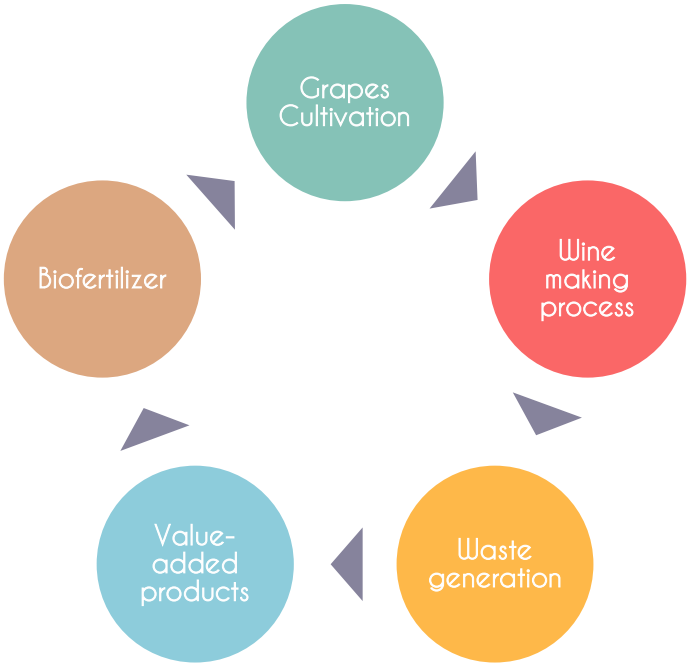


Figure 7 - Projected circular bioeconomy model for Wine Waste.

Technologies have been developed to produce value-added products from winery industry waste (Figure 7), which can successfully handle the waste generated during winemaking process. These technologies can be integrated with the wine making process. However, there are still challenges related to process and economic aspects, including the commercialization of these technologies. Currently, various pre-treatment processes are available for winery waste treatment, but the technologies combined with the treatment of winery wastewater and production of commercially important products can offer dual benefits on eco-friendly treatment and add market value. The seasonal availability of the waste demands judicious handling and treatment to achieve economic viability and efficiency. Further research and practical experimentation are necessary since, in the case of winery waste, limited studies have been conducted and life cycle analysis regarding full economic costing of the use wine waste as a resource is needed (Zacharof et al.2017). The currently available results on the biotechnological use of winery waste are a promising alternative to the current treatment techniques that are focusing on the waste remediation and treatment, rather than resource recovery.

4.2. Potential of Grape Bagasse

The production of 1 hL of wine requires, on average, 1.325 kg of fresh grape (Kovalcik et al. 2020). It is estimated that about 25% of the weight of the grapes used in the production of wine result in grape bagasse (skins, stems and seeds) (Figure 8) (Dwyer et al. 2014). About 11.1 million tons of grape bagasse are generated annually (Kovalcik et al. 2020).

Figure 8 - White grape bagasse.

This grape bagasse is a raw material with potential to be used in a biorefineries. The grape bagasse generated in wine production contains high contents of fermentable sugars (mainly glucose and fructose as can be seen in table 2). Several studies report that grape bagasse is a valuable residue for the production of value-added products with broad applications, for example, in biopolymer production, food industry, cosmetics (Wittenauer et al. 2015), pharmacy, agriculture and chemical industry , biomethane (Allison and Simmons 2018) or biodiesel (Bolonio et al. 2019).

Only a few scientific papers report on the use of grape bagasse as a carbon source for the biosynthesis of PHAs. Follonier et al., in their study, which evaluated the potential of grape bagasse as a carbon source to produce medium-chain-length PHA, reported about 106 g fermentable sugars (per kg grape bagasse). Follonier using *Pseudomonas resinovorans* in the batch process on 0.1 L of hydrolysed bagasse and reported a PHA content in biomass and the volumetric productivity being, 23.3 % and 0.05 g L^{-1} h^{-1}, respectively (Follonier et al. 2014). The same research group increased the mcl-PHA volumetric productivity into 0.10 g_{PHA} L^{-1} h^{-1} by employing *Pseudomonas putida* KT2440 in 100 L bioreactor growing on Gewürztraminer bagasse (Follonier et al.2015).

5. Objectives of this Work

From a perspective of economic and environmental sustainability and based on the revised literature this work had the following objectives:

- To define a pre-treatment that allow obtaining aqueous extracts with high concentration of sugars from grape bagasse;
- Test the consumption of the obtained sugars present in the substrate by *C. necator*;
- Study the effect of using different nitrogen sources on PHAs production by *C. necator*;
- Characterize the obtained PHAs.

CHAPTER II – MATERIALS AND METHODS

1. Grape bagasse

Grape bagasse (GB) was supplied by Manuel Alves Ribeiro de Almeida & Filhos, Bairrada, Portugal. GB was collected after the processing of white grapes used to produce sparkling wine. GB were stored at a temperature of -18 ºC until pre-treatment.

1.1. Pre-treatment of Grape Bagasse

To obtain solutions of monosaccharides, two types of pretreatments were applied to GB: mild acid extraction and hydrothermal extraction.

The hydrothermal extraction was performed following the procedure described by Mendes et al. (2013). GB was mixed with distilled water to a 1:10 solid/liquid ratio (Mendes et al. 2013) without pH control. The mixture was autoclaved at 100 ºC and 2 bar for 1 h, 2 h, 3 h, and 4 h. After cooling, the hydrolysate was filtered to remove the suspended solid and was stored at -18 ºC for future use.

The acidic extraction was performed using the same solid/liquid ratio, temperatures, and pressure, but pH was adjusted at 1.0 with the manual addition of 2.0 M H_2SO_4 solution. The proceeding was also tested for 1 h, 2 h, 3 h, and 4 h.

2. Microorganisms

Cupriavidus necator DMS 545 was acquired from the German Collection of Microorganisms and Cell Cultures GmbH (DMSZ). The lyophilized culture was revived according to the recommended procedure by the DMSZ using the DSMZ medium 1.

The microorganisms were maintained in agar plates with Luria-Bertani (LB) medium, through monthly streaking of 1-3 colonies to new plates, following incubation at 28 ºC, for 48 to 72 hours and subsequent storage at 4 ºC.

3. Media

3.1. DMSZ medium 1 and LB medium

The DMSZ medium 1 was used to revive *C. necator* DMS 545. Per litre of distilled water, it included 5.0 g of peptone and 3.0 g of meat extract.

LB medium was used for agar plates and pre-inoculum. Its composition was as follows: 10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of sodium chloride and 15.0 g agar per litre of distilled water.

Prior to sterilization in autoclave for 20 minutes at 121ºC, pH was adjusted to 7.0 for both media.

3.2. Supplementary medium

The mineral solution (MS), described in Table 4, was used as supplementation of the GB hydrolysate. All media were autoclaved for 20 min at 121 ºC, and the pH was adjusted to 7.0 before inoculation.

Carbon sources (hydrolysate) and components indicated in Tables 4 and 5 were autoclaved separately and mixed before inoculation. The concentration of each component in Tables 4 and 5 corresponds to the final concentration in fermentation medium.

Table 4 - Composition of the MS.

MS	
Component	**Per L of fermentation medium**
KH_2PO_4 [a]	2.30 g
Na_2HPO_4 [a]	2.30 g
$MgSO_4.7H_2O$	0.50 g
NH_4Cl	1.00 g
$NaHCO_3$	0.50 g
$CaCl_2.2H_2O$	10.0 mg
$C_6H_6O_7(NH_4)_2$	22.0 mg
$FeCl_3$	30.0 mg
Micronutrient Solution [a]	5.00 mL

[a]Autoclaved separately

Table 5 - Composition of the micronutrient's solution.

Micronutrient Solution	
Component	**Per L of fermentation medium**
$ZnSO_4.7H_2O$	0.10 g
$MnCl_2.4H_2O$	0.030 g
H_3BO_3	0.30 g
$CoCl_2.6H_2O$	0.20 g
$CuCl_2.2H_2O$	0.010 g
$NiCl.6H_2O$	0.020 g
Na_2MoO_4	0.030 g

4. Assays with *C. necator*

The pre-inocula and inocula preparation, as well as the flask assays were carried out at 30 ºC (Arumugam et al. 2018) and 160 rpm in an orbital shaker (Carromata).

4.1. Pre-inocula and inocula preparation

For both flask and reactor assays, the pre-inocula were prepared by transferring 2-3 colonies from LB plates to 50 mL of LB medium in 100 mL Erlenmeyers and incubated for 24 h.

In a first phase, to prepare the inocula for flask assays, 2 mL from the pre-inoculum was transferred to 100 mL of LB medium in 250 mL Erlenmeyers and incubated for 12-14 hours, to ensure that the culture would be in early exponential phase upon the beginning of the assays.

In a second phase of this study, to decrease the lag phase of microbial growth, the inoculum was prepared in Erlenmeyers of 250 mL (100 mL working volume) containing 80% (v/v) of the carbon sources solution (hydrolysate), 10% (v/v) of LB or supplementary medium and 10% (v/v) of pre-inoculum.

For reactor assays, 20 mL of pre-inoculum were transferred to 500 mL Erlenmeyers with 200 mL of culture medium ((80% (v/v) of the carbon sources solution, 10% (v/v) of supplementary medium (MS) and 10% (v/v) of pre-inoculum), which were incubated for 12-14 hours before inoculation of the reactor. In both pre-inocula and inocula, the optical density was monitored (650 nm) over time to ensure a similar cell concentration at the beginning of each assay.

4.2. Preliminary flask assays

Before studying the capacity of *C. necator* 545 to use the obtained hydrolysates, AEH and HEH, for PHA production, the supplementation media that contained the nutrients required for the bacterial growth were tested. Two tests with glucose and fructose, the monosaccharides dominating both hydrolysates, where performed with two different sources of nutrients (LB medium and MS). These assays were carried out in 500 mL Erlenmeyers (250 mL working volume) with 80% (v/v) of the carbon sources solution, 10% (v/v) of LB

or supplementary medium and 10% (v/v) of inoculum. The flasks were incubated for 3-4 days.

4.3. Flask assays for hydrolysate selection

Flask assays were performed with both hydrolysates (from hydrothermal and acidic extraction) to select the best and were carried out in 500 mL Erlenmeyers (250 mL working volume) with 80% (v/v) of the carbon sources solution, 10% (v/v) of LB medium or supplementary medium and 10% (v/v) of inoculum. The flasks were incubated for 3-4 days.

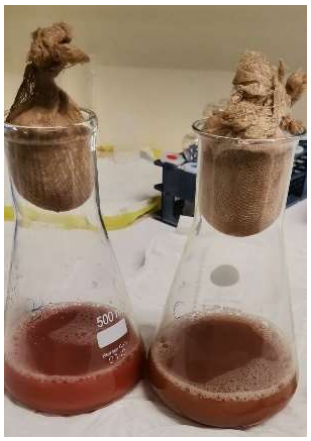


Figure 9 - Flasks from the assays for hydrolysate selection.

4.4. Flask assays with hydrolysate for nitrogen source selection

Tests were performed to select the nitrogen source that promoted the best growth of *C. necator*. In these trials, the medium was supplemented with MS varying the nitrogen source, keeping it at a final concentration of 1g L^{-1}. The nitrogen sources tested were urea, ammonia sulfate, ammonia chloride and yeast extract. These assays were carried out in 500 mL Erlenmeyers (250 mL working volume) with 80% (v/v) of the carbon sources solution, 10% (v/v) of supplementary medium and 10% (v/v) of inoculum. The flasks were incubated for 3-4 days.

4.5. Reactor assays

Two batch assays were performed with *C. necator* DSM 545 in a 5 L Sartorius Biostat Aplus reactor (Figure 10), with a working volume of 2 L and equipped with two six-bladed disk-turbine impellers. Control of temperature, pH and dissolved oxygen (DO) concentration was performed using a micro DCU system, and data acquisition by a MFCS/DA 3.0 system (Sartorius).

The reactor started with an initial volume of 2 L with 80% (v/v) of the carbon sources solution, 10% (v/v) of supplementary medium and 10% (v/v) of inoculum. The pH was monitored with a pH sensor (Mettler Toledo) and controlled at 7.00 ± 0.05 with H_2SO_4 (1 M) and KOH (5 M) solutions. Temperature was maintained at 30 ºC with a thermostatic jacket. Air supplying was performed resorting to a ring sparger.

In the first test, the control of the DO (about 30%) was done by automatically adjusting the agitation. This control led to the test taking place with very low agitation (about 120 rpm). In the second test, to monitor the oxygen uptake rate, a respirometer consisting of a 45 mL vessel was coupled to the bioreactor, to which the medium was periodically circulated by a Watson-Marlow SCI 400 peristaltic pump, under magnetic stirring (IKA Topolino). An oxygen electrode (Mettler Toledo InPro 6800) was inserted into the respirometer and connected to an Oxygen meter Transmitter M300 (Mettler Toledo). In this assay, DO was also monitored using an O_2 sensor (Mettler Toledo InPro 6800) but it was not controlled and an agitation of 250 rpm was maintained throughout the trial.

Figure 10 - Sartorius Biostat A plus reactor used for the assays with C. necator DSM 545.

5. Sampling

To monitor the assays, samples of 3-5 mL were collected every 1.5 h for pH measurement, quantification of biomass, sugars, PHA, and COD. After collection, the sample was used to measure pH and optical density at 650 nm (OD650), with the adequate dilution to monitor cell growth. Then, the sample was centrifuged at 13000 rpm for 10 min (Eppendorf MiniSpin). The supernatant was stored at -18 °C for further analyses. The biomass pellet was stored at -18 °C for polymer analysis.

6. PHA extraction

At the end of the assays, the biomass was separated by centrifugation (ThermoFisher Scientific Heraeus Megafuge 16R) at 5000 rpm and 4 ºC, for 20 minutes. The supernatant was discarded, and the biomass pellet was lyophilized (VirTis benchtop K) for 48 hours. The lyophilized biomass was resuspended in chloroform (30 mL per gram of biomass) in a covered 100 mL Erlenmeyer and incubated in an orbital shaker (Certomat) for 24h at 180 rpm and 28 ºC. After the incubation period, the solution was filtered under vacuum, with Glass microfiber filter 629 (1 µm pore diameter; VWR) to remove cell residues. In a glass,

the filtrate was left to evaporate the chloroform and the PHA was then collected for characterization by FTIR.

Figure 11 - Extracted PHA.

7. Analytical methods

7.1. Quantification of biomass and determination of cell dry weight

In the preliminary flask assays with *C. necator*, OD650 was related with biomass concentration in a calibration curve. For that, samples of 5 mL were retrieved in triplicate from the culture in stationary phase. These were then filtered by vacuum filtration in cellulose nitrate membrane filters (0.2 μm pore; 47 mm diameter Whatman). The filters with biomass were left to dry in an oven (Memmert) at 100 ºC during 72 h, after which they were weighed. Additionally, the optical density of several dilutions of the same culture was measured and related with biomass concentration to prepare the calibration curve.

7.2. pH

The pH was measured for the flasks and bioreactor samples using a benchtop meter Hach sensION+ MM340.

7.3. Chemical oxygen demand

COD measurements were used to characterize the hydrolyzed GB as well as to monitor the COD variation during the assays. The solutions for COD determination were prepared

according to the APHA Standard Methods for the Examination of Water and Wastewater, namely a digestive aqueous solution with $K_2Cr_2O_7$ and H_2SO_4, and an acid solution with H_2SO_4 and AgSO4 (Johnson and Donald, 1997).

COD was measured with Spectroquant TR 620 Kit (Merck). 1.2 mL of digestive solution and 2.8 mL of acid solution were added to 2 mL of sample properly diluted and the mixture was incubated at 150 ºC for 2 h. After cooling, the absorbance was measured and the COD was calculated according to the calibration curve, which was done with glucose in COD concentrations between 0-1 g L^{-1}.

7.4. Quantification of sugars by high performance liquid chromatography

High performance liquid chromatography (HPLC) was used to determine the concentration of sugars, in hydrolysate and in samples from flask and bioreactor assays. The hydrolysate and samples supernatant were diluted with distilled water. Every sample with the appropriate dilution were filtered in Spin-X polypropylene centrifuge tube filter (0.22 µm pore; VWR) in a Eppendorf MiniSpin centrifuge for 15 minutes at 8000 rpm. Then the filtrate was subsequently analysed in a Hitachi HPLC apparatus with a Biorad Aminex HPX-87H column, oven Gecko 2000 and detector RI Hitachi U-2490. Furfural was detected by a diode array detector (DAD) Hitachi L-2455. The runs were performed for 30 min, at 60 ºC, with an eluent of 0.01 N H2SO4 in Milli-Q water as mobile phase at a flow rate of 0.5 mL min-1.

Standards with known concentrations of glucose, and fructose were used as standards to prepare a calibration curve in the range of 0 to 5 g L^{-1}.

7.5. Folin-Ciocalteu method

The Folin-Ciocalteu reagent (Panreac) was used to determine total phenols in hydrolysates, according to the following method: 0.02 mL of sample were mixed with 1.5 mL of distilled water, 0.3 mL of a sodium carbonate solution (7.5% w/v) and 0.1 mL of Folin-Ciocalteu reagent. The mixture was incubated at 28 oC for 1 h in the dark, after which the absorbance at 765 nm was measured in the UV-Vis spectrophotometer (Shimadzu UVmini - 1240). A calibration curve was prepared with several dilutions of gallic acid,

ranging from 0 to 1 g L^{-1}. The total phenols content was expressed in grams of gallic acid equivalent per litter (g_{GAE} L^{-1}).

7.6. Quantification of PHA by gas chromatography

Gas chromatography with flame ionization detector (GC-FID) was performed to quantify the amount and type of PHA according to an adapted procedure from Moita and Lemos (Moita and Lemos 2012). The biomass was lyophilised (VirTis benchtop K) for 48 hours and was incubated for 3.5 hours, at 100 ºC, with 1 mL of acidic methanol (20%) and 1 mL of a solution of heptadecane (internal standard) dissolved in chloroform (1:1). After cooling, the chloroform phase was extracted with 0.5 mL of water and injected into GC-FID (Konik Instruments HRGC-3000C) equipped with a Restek Stabilwax MS (30x0.25x0.25 cm) column and with hydrogen as carrier gas (50 kPa). An injection volume of 0.5 µL, at 280 °C was used. The initial temperature of the oven was 60 °C and the temperature program was the following: 20 °C min^{-1} until 100 °C; 3 °C min^{-1} until 175 °C; 20 °C min^{-1} until 220 °C. The detector temperature was set at 250 °C. The calibration of 3HB and 3HV concentrations was done using standards of commercial P(3HB)-3HV (88%/12%, Aldrich).

7.7. PHA characterization

7.7.1. Fourier-transform infrared spectroscopy

FTIR was used to characterize the extracted PHA, and the analyses were performed in a Perkin Elmer Spectrum BX FTIR spectrometer. The scanning was performed with a spectral range of 4000-500 cm^{-1}, 64 scans and a resolution of 4 cm-1. Baseline correction and vector normalization were applied to the raw signal, using Spectra software. Commercial P(3HB) (Sigma-Aldrich) was used as reference to identify and compare functional groups of the extracted polymer.

7.7.2. Thermogravimetric analysis (TGA)

TGA experiments were performed using a TGA instrument (Seteram, model Settsys Evolution 1750, TGA mode, S sensor) to determine the thermal stability of the extracted

PHA. Nitrogen was supplied at 50 mL min^{-1}. The furnace temperature was set from 0 °C to 800 °C with a heating rate of 10 °C min^{-1}.

7.7.3. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (PerkinElmer, model Diamond DSC) was used to determine the thermal characteristics of the PHA extracted. Around 3 mg of each PHA sample was placed in the DSC cell aluminium pan, the chamber was heated from - 50 °C to 180 °C at a rate of 5 °C.min^{-1}, the temperature was then held at 180 °C for 1 min and cooled to - 50 °C at a rate of 10 °C.min^{-1}, where it was held for another minute. Finally, the samples were reheated up to 180 °C at a 5 °C.min^{-1} rate. An inert nitrogen atmosphere was used during the purge. The melting temperature (Tm) and the melting enthalpy (ΔH) were obtained considering the values from the second heating in the DSC curve and the glass transition temperature (Tg) considering the values from the cooling ramp. The crystallinity (Eq. 1) was calculated according to:

$$Crystalinity = \frac{\Delta H\ (J\ g^{-1})}{\Delta H_0 . w} \cdot 100 \qquad (1)$$

where ΔH_0 is melting enthalpy of the 100% crystalline P(3HB), which is assumed to be 146 $J.g^{-1}$ and w is the weight fraction of P(3HB) in the sample.

8. Calculations

8.1. Specific growth rate (μ)

The specific growth rate of biomass (μ) was calculated by integration and linearization of the Equation 2, resulting in Equation 3, where t corresponds to time (h) and *X* to biomass concentration (g L^{-1}) at a given time. For specific growth rate (μ), only the exponential phase of growth was considered, so *0* and *i* corresponded to the beginning and end of this phase. The slope in the linearization (Equation 3) corresponds to μ.

$$\mu = \frac{1}{X} \cdot \frac{dx}{dt} (h^{-1}) \quad (2)$$

$$lnX_i = \mu \cdot t + lnX_0 \quad (3)$$

8.2. Volumetric substrate uptake rate (r_s)

To determine the volumetric substrate uptake rate, rs (g L^{-1} h^{-1}), the variation of substrate concentration (ΔS; g L^{-1}), over time (Δt; h) was considered (Equation 4). More specifically, r_s was calculated for glucose (rGlc), and fructose (rFru).

$$\boldsymbol{r_s = \frac{\Delta S}{\Delta t} (gL^{-1}h^{-1})} \quad (4)$$

8.3. Growth yield, $Y_{X/S}$ and $Y_{X/N}$

The growth yield, $Y_{X/S}$, is given by Equation 5, in which the amount of biomass (mol L^{-1}) in the end of the process is divided by the substrate consumption (mol L^{-1}), which can refer to glucose and fructose. An estimate biomass chemical formula of $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$ was used for the calculations with *C. necator*.

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \ (mol\ mol^{-1}) \quad (5)$$

The growth yield, $Y_{X/N}$, is given by Equation 6, in which the amount of biomass (mol L^{-1}) in the end of the process is divided by the substrate consumption (mol L^{-1}), which can refer to nitrogen. An estimate biomass chemical formula of $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$ was used for the calculations with *C. necator.*

$$Y_{X/N} = \frac{\Delta X}{\Delta N} \ (mol\ mol^{-1}) \quad (6)$$

8.4. PHA content per cell dry weight (%PHA)

The PHA content per cell dry weight (CDW) was calculated according to Equation 7, from the concentrations of total PHA (g L^{-1}) and total biomass, X (g L^{-1}).

$$\%PHA = \frac{PHA}{X} \cdot 100 \quad (7)$$

8.5. Oxygen uptake rate (OUR)

To determine the oxygen uptake rate (OUR), the DO (%) data from the respirometer was converted to mg L^{-1}, considering an oxygen saturation of 7.8 mg L^{-1}, at 28 oC (*Standard Methods For the Examination of Water and Wastewater,* 2018). At each sampling time, the OUR corresponded to the slope of the linear regression of DO (mg L^{-1}) versus time (s).

8.6. COD

The COD (g_{O2} g_S^{-1}) of each compound was calculated by Equation 8, in which n corresponds to the number of oxygen moles required for oxidation of the substrate, S, and Mw corresponds to the molecular weight of oxygen (Mw(O_2)) or substrate (Mw(S)).

$$COD_s = \frac{n \times Mw(O_2)}{Mw(s)} \tag{8}$$

To determine the oxygen moles required for oxidation of glucose and fructose, the oxidation reaction was considered (Equation 9).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \tag{9}$$

As such, the COD of each substrate is:

$$COD_{Glc,Fru} = \frac{6 \times 32}{180} = 1.07\ g_{O2}\ g_{Glc,Fru}^{-1} \tag{10}$$

The theoretical COD, tCOD (g_{COD} L^{-1}) was calculated by multiplying the concentration of each substrate (g L^{-1}) by the respective COD (g_{O2} g_S^{-1}). The real COD, rCOD (g_{COD} L^{-1}) corresponded to the value obtained by the protocol of Section 7.3.

8.7. PHA product yield on substrate ($Y_{PHA/S}$)

The PHA product yield on substrate ($Y_{PHA/S}$) was calculated by diving the amount of PHA (mol L^{-1}) by the consumption of substrate, i.e. glucose and fructose (mol L^{-1}) (Equation 11).

$$Y_{PHA/S} = \frac{\Delta PHA}{\Delta S} \ (mol\ mol^{-1}) \qquad (11)$$

CHAPTER III – RESULTS AND DISCUSSION

1. Grape bagasse pre-treatment

GB represents a potential low-cost and renewable carbon source (Brodin et al. 2017). To generally obtain enough fermentable sugars for the bioprocess, GB needs to be subjected to pre-treatments (TAEHrzadeh and Karimi 2008) (Obruca et al. 2015). An advantageous pre-treatment allows a high recovery of sugar, low presence of inhibitory compounds, as well as prevent the formation of undesirable compounds to avoid a step of purifying the substrate (Nguyen et al. 2019). In addition, it is desirable that the pretreatment is low cost and has a low energy consumption, so that it does not represent a high additional cost in the process.

In this study, hydrothermal extraction and acidic extraction were chosen because they are considered low-cost pretreatments.

1.1. Hydrothermal and acidic extraction

Hydrothermal extraction is a very appealing method due to its simplicity, since no acidic or other substance are added (Conde and Mussatto 2016). On the other hand, acidic extraction is considered one of the most successful methods for obtaining free sugars (Andler et al. 2021). In addition, an extraction with acid at pH 1.0 is beneficial for the storage of large amounts of substrate, since it prevents the growth of microorganisms. However, this requires a pH neutralization step before using the substrate (Juarez et al. 2018).

In a first phase, hydrothermal and acidic extractions were carried out at 100 ºC with solid/liquid ratios of 1:10 for 1h, 2h, 3h, and 4h. The results obtained are summarized in Table 6.

Table 6 - Hydrolysate composition determined by COD method.

Time (h)	Acidic extraction		Hydrothermal extraction	
	[Monosaccharides] (g_{COD} L^{-1})	COD (g_{COD} L^{-1})	[Monosaccharides] (g_{COD} L^{-1})	COD (g_{COD} L^{-1})
1	12.56	14.02	12.40	13.70
2	16.16	25.43	13.99	19.19
3	16.26	26.13	14.15	21.19
4	16.33	27.20	14.30	21.35

Concerning acidic extraction, the amount of organic matter extracted varied from 14.02 g_{COD} L^{-1} to 27.20 g_{COD} L^{-1}. On the other hand, in hydrothermal extraction, the amount of organic matter varied from 13.70 g_{COD} L^{-1} to 21. 35 g_{COD} L^{-1}. It is noteworthy the presence of a considerable amount of simple sugars with only 1 h of pre-treatment, 12.56 g L^{-1} and 12.40 g L^{-1}. This may be due to the fact that the grape skin already contains large amounts of simple sugars before the hydrolysis stages, according to Sousa et al (2014). After 2 h of pre-treatment the amount of monosaccharides and COD available increased over time in both methods, although not so significantly as the increase observed between 1 h and 2 h. COD increased more markedly with the time of hydrolysis than monosaccharides.

One hypothesis to increase the sugar content would be using dry GB before extractions, which was found to favor the breaking of the lignocellulosic structure (Mendes et al. 2013) (Andler et al. 2021). However, additional steps can represent additional costs for the process.

Another possibility to increase the sugar content in the substrates could also be the increase in temperature and/or the increase in the percentage of acid solution. However, several studies have found that increasing these factors from certain values may lead to the opposite effect, a decrease in monosaccharides, in particular glucose (Abubakar et al. 2016) (Xiang et al. 2004) (Lenihan et al. 2010) (Yoon et al. 2014).

Finally, it is important to note that the contribution of monosaccharides to COD in hydrothermal extraction (66-90%) is generally higher than in acid extraction (60-85%).

Thus, it can be assumed that in acid extraction other compounds are being extracted, such as inhibitors and phenolic compounds in a greater extent.

HPLC showed that the main sugars present on the hydrolysate were fructose and glucose. The results obtained are summarized in Table 7.

Table 7 - Hydrolysate composition determined by HPLC and COD methods.

	Acidic extraction		**Hydrothermal extraction**	
Time (h)	**[Fructose] (g L^{-1})**	**[Glucose] (g L^{-1})**	**[Fructose] (g_{COD} L^{-1})**	**[Glucose] (g_{COD} L^{-1})**
1	6.07	5.71	5.95	5.68
2	**7.56**	**7.59**	**6.64**	**6.48**
3	7.60	7.65	6.75	6.52
4	7.63	7.69	6.81	6.60

Considering acidic extraction, the concentration of monosaccharides ranged from 11.78 g L^{-1} to 15.32 g L^{-1}. In hydrothermal extraction, the variation was from 11.63 g L^{-1} to 13.41 g L^{-1}.

As observed before, in both extractions the greatest increase of monosaccharides in hydrolyzed occurred when the reaction time increased from 1h to 2h and the increase observed for 3h and 4h was not significant as well as no changes on monosaccharides composition were observed. For this reason, 2 h of extraction were chosen to hydrolyse GB for the assays of this work. The acidic extraction, carried out for 2h at 100 ºC, produced a hydrolysate (AEH) with 7.56 g L^{-1} fructose and 7.59 g L^{-1} glucose. Hydrothermal extraction under the same conditions produced a hydrolysate (HEH) with 6.64 g L^{-1} fructose and 6.48 g L^{-1} glucose. This was the time chosen for the pre-treatment since the prolongation of the reactions was not justified. This increase had already been reported in the literature by Andler et al. (Andler et al. 2021). The decline in the formation of monosaccharides in the 3h and 4h of reaction may be due to the existence of unwanted side reactions or even some degradation of monosaccharides (Kang et al. 2018).

Phenolics can be recovered from white GB through acidic extraction and they often occur combined with sugars (de la Rosa et al. 2018).

The phenolic compounds present in the hydrolysates from the extractions, carried out at 2h, were analyzed using the Folin-Ciocalteu method and quantified as galic acid (GAE). The AEH showed a phenolics concentration of 0.448 gGAE L^{-1}, while the HEH, 0.282 gGAE L^{-1}.

This is in accordance with the literature since acidic extraction usually results in a higher concentration of phenolic compounds(Juarez et al. 2018).

2.Preliminary flask assays for the selection of hydrolysate and supplementary medium

Before studying the capacity of *C. necator* 545 to use the obtained hydrolysates, AEH and HEH, for PHA production, the supplementation media that contained the nutrients required for the bacterial growth were tested. Two tests with glucose and fructose, the monosaccharides dominating both hydrolysates, where performed with two different sources of nutrients: assay A with LB medium and assay B with the mineral solution (MS). Figure 12 shows the evolution of pH and concentrations of carbon sources and biomass.

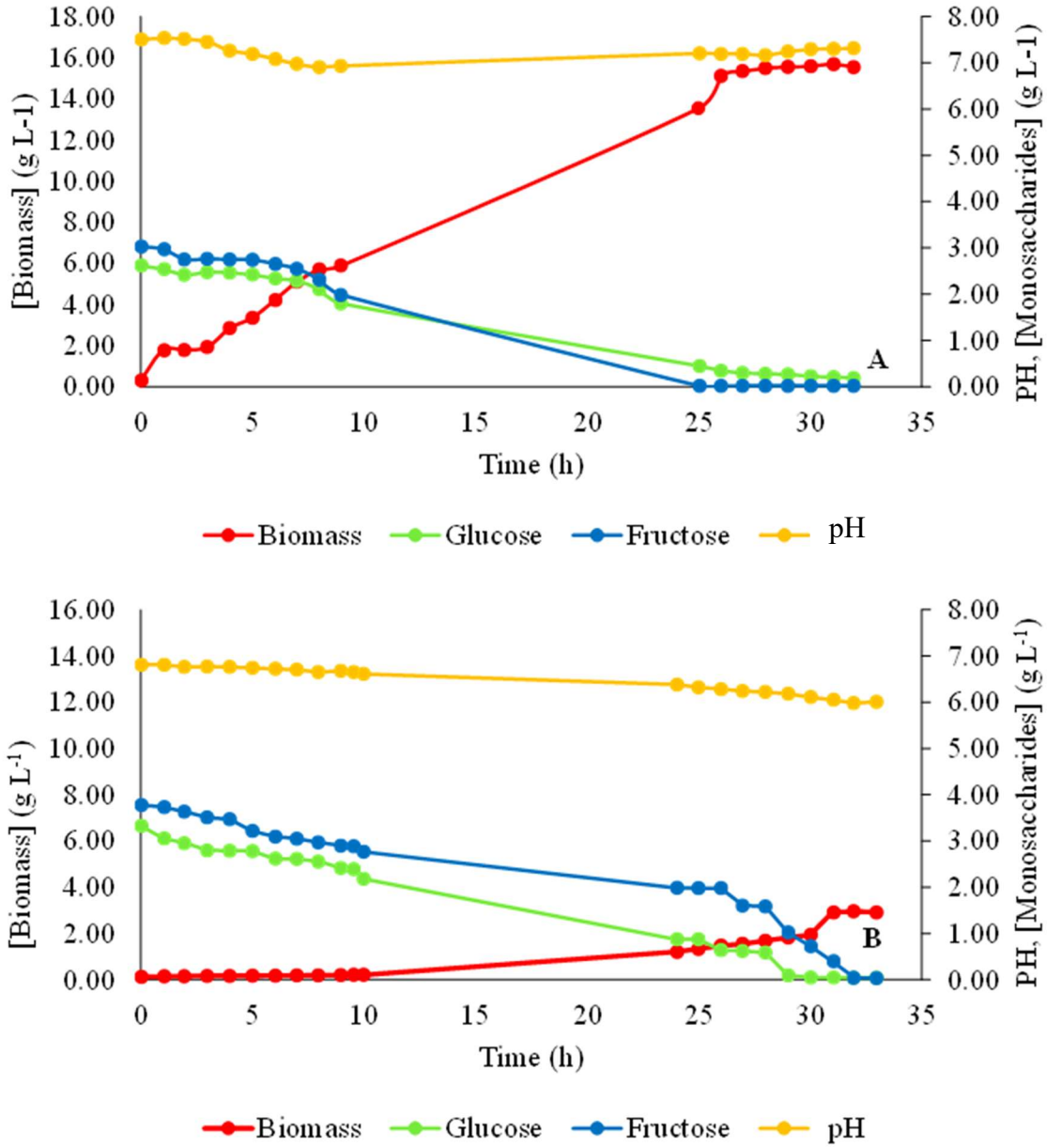


Figure 12 - Evolution of pH and concentration of biomass and carbon sources of the preliminary assays with *C. necator*. A) Pure glucose and fructose supplemented with LB medium. B) Pure glucose and fructose supplemented with MS.

In these assays it was possible to observe, as expected, that *C. necator* preferred the LB medium. In test A the biomass grew about 5 times more than in test B to 15.532 g L^{-1}. Also, the lag phase of assay A, 3 h; was lower than in assay B (10 h). In both assays glucose and fructose were totally consumed. This might result from the fact that LB is an extremely rich medium, providing optimal conditions for the growth *of C. necator*.

Then the same supplementary media were tested with AEH and HEH hydrolysates, instead of fructose and glucose. Figure 13 shows the evolution of pH and concentrations of carbon sources and biomass for assays C (LB with HEH), D (HEH with MS), E (AEH with LB), and F (AEH with MS). For these four experiments and for the two previous ones (A and B), some parameters were calculated and summarized in Table 8, namely the maximum cell concentration ($[X]_{max}$), specific growth rate (μ), volumetric consumption rate of glucose and fructose (r_{Glc} and r_{Fru}, respectively) and biomass yield ($Y_{X/S}$).

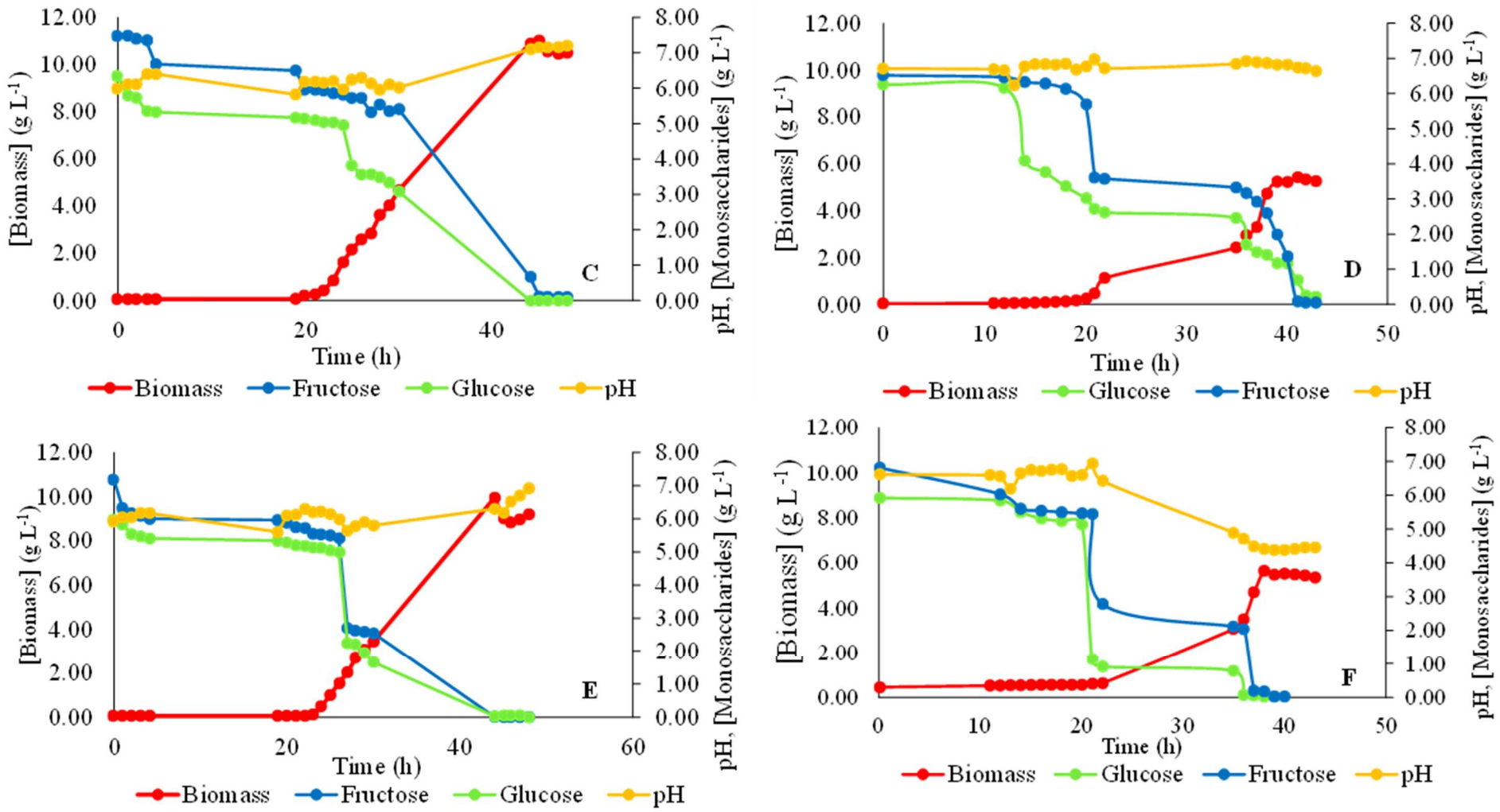


Figure 13 - Evolution of pH, concentration of biomass and carbon sources of the preliminary assays in synthetic medium with *C. necator*. C) HEH supplemented with LB, D) HEH supplemented with MS, E) AEH supplemented with LB, F) AEH supplemented with MS.

Table 8 - Summary of results from the preliminary assays with *C. necator*.

Assay	Medium	$[X]_{max}$ (g L^{-1})	μ (h^{-1})	r_{Glc} (g L^{-1} h^{-1})	r_{Fru} (g L^{-1} h^{-1})	$Y_{X/S}$ (mol mol^{-1})
A	Synthetic+ LB	15.532	0.072	0.076	0.094	5.190
B	Synthetic+MS	2.908	0.104	0.100	0.113	0.736
C	HEH + LB	10.500	0.102	0.132	0.154	1.411
D	HEH + MS	5.260	0.083	0.140	0.151	0.773
E	AEH + LB	9.200	0.124	0.124	0.149	1.288
F	AEH + MS	5.350	0.128	0.160	0.170	0.688

All the assays with hydrolysates presented very long lag phases (about 24h). In all assays the fructose and glucose were fully consumed, with exception of D, and the LB medium showed better results than MS medium. However, the biomass concentrations obtained were lower with hydrolysates (10.500 g L^{-1} and 9.200 g L^{-1} for C and E, respectively) than with pure fructose and glucose (15.532 g L^{-1} for A).

Despite its long lag phase, in assay C the exponential phase occurred with a specific growth rate of 0.102 h^{-1}. In assay E, the exponential phase occurred with a μ of 0.124 h^{-1} and there was also total consumption of glucose and fructose. Interestingly, the value of the biomass yield in these assays was higher than 1 (Table 8), meaning that besides fructose and glucose other carbon sources present in the LB medium have served as carbon sources for *C. necator.*

The assays supplemented with MS generally showed lower biomass concentrations. Moreover, in these assays the hydrolysates resulted in higher biomass concentrations with 5.260 g L^{-1} and 5.350 g L^{-1}, for D and F assays respectively, than assay B, 2.908 g L^{-1}. In assay D, the exponential phase occurred with a specific growth rate of 0.083 h^{-1}, while in assay F with 0.128 h^{-1}. These values were in the same range of assays C and E.

In all tests, glucose and fructose are consumed almost simultaneously. However, fructose consumption rates (0.094 to 0.170 g L^{-1} h^{-1}) are slightly higher than glucose consumption rates (0.076 to 0.160 g L^{-1} h^{-1}). This behavior was already reported in the literature. *C. necator* was described of being capable of metabolizing fructose as the dominant saccharide present in grape sugar extract (Volodina et al. 2016), its capacity to

utilize glucose is limited, less productive and requires an adaptation to this sugar (Franz et al. 2012).

The pH of tests A and B decreased over time, probably resulting from the release of CO_2 from the respiratory metabolism of *C. necator*. However, in the tests with the hydrolyzed, this was not the case, except for assay F. In assays, C, D and E, the pH tended to remain stable, around 7.0 or to slightly increase.

In general, the assays supplemented with LB medium showed a better microbial growth. Regarding the assays with hydrolysates, the growth of *C. necator* was quite similar in both as well as the consumption rates of carbon sources. Thus, these tests were not enough to select the best medium or the hydrolysate for the growth of *C. necator*.

3. Flask assays for hydrolysate selection

To decrease the duration of the lag phase observed in the previous assays, the incubation time of the pre-inocula was increased from 12h to 24h and the composition of the culture medium of the inoculum was changed to a composition similar to that of the assay to obtain a faster adaptation of microorganisms to hydrolysates. Also, the initial biomass concentration used in the assays increased and, consequently, the duration of the lag phase decreased. Table 9 summarizes the effects of these changes.

Table 9 - Effects observed on bacterial growth after changes made to pre-inoculum incubation time and inoculum culture medium composition.

Medium	Assay	[X]initial (g L^{-1})	lag phase duration (h)
HEH + LB	C	0.062	24
	G	0.572	6
HEH + MS	D	0.041	21
	H	0.399	6
AEH + LB	E	0.061	25
	I	0.769	7.5
AEH + MS	F	0.457	22
	J	0.424	8.5

In the tests performed with HEH, G and H the lag phase decreased about four times. On the other hand, in the tests performed with AEH, the decrease in the lag phase was about three times. Initial biomass concentration increased about 10 times in all assays except for test J. However, the fact that the inoculum was performed in a culture medium already containing hydrolysate contributed to the decrease on the lag phase. Thus, the changes made to the pre-inoculum and inoculum preparation were maintained in the following assays.

Tests G, H, I and J were also carried out with the objective of selecting the hydrolysate that provides a better microbial growth and, consequently, a better production of PHA. These assays were performed with both hydrolysates and both supplementations (LB medium and MS). In Figure 14, it is possible to see the evolution of biomass and monosaccharides concentration and the evolution of pH throughout the assays

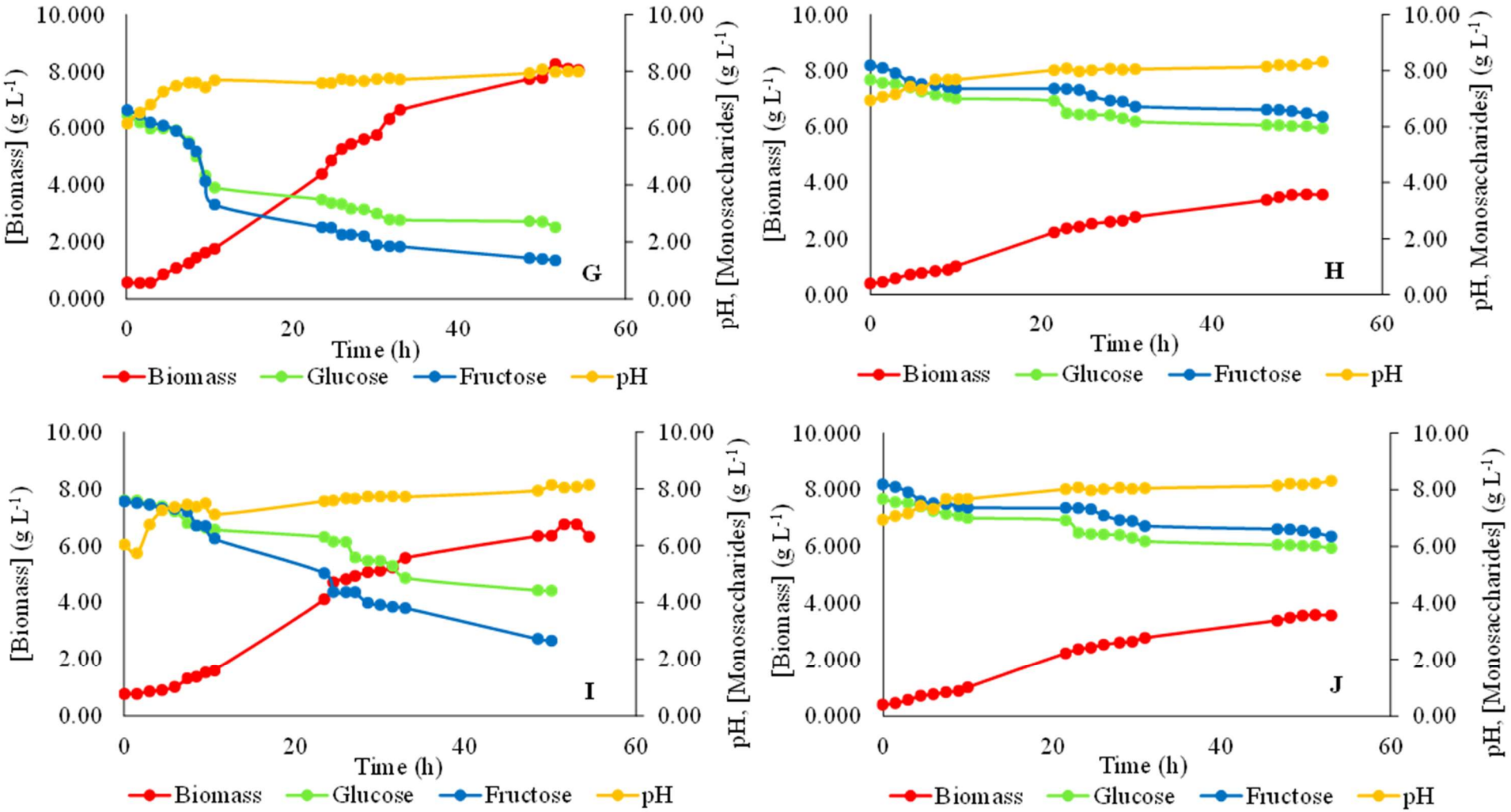


Figure 14 - Evolution of pH, concentration of biomass and carbon sources of the assays for hydrolysate selection. G) HEH supplemented with LB medium. H) HEH supplemented with MS. I) AEH supplemented with LB medium. J) AEH supplemented with MS.

Table 10 details the various parameters calculated for these assays, namely the maximum cell concentration ($[X]_{max)}$, specific growth rate (μ), volumetric consumption rate of glucose and fructose (r_{Glc} and r_{Fru}, respectively), biomass yield ($Y_{X/S}$) and the evolution of theorical (tCOD) and real COD (rCOD).

Table 10 - Summary of results from the assays for hydrolysate selection.

Assay	Medium	[X]max (g L^{-1})	μ (h^{-1})	rGlc (g L^{-1} h^{-1})	rFru (g L^{-1} h^{-1})	YX/S (mol mol^{-1})	tCOD consumed (g_{COD} L^{-1})	rCOD consumed (g_{COD} L^{-1})
G	HEH + LB	8.055	0.059	0.077	0.103	1.498	9.865	12.436
H	HEH + MS	3.566	0.030	0.033	0.035	1.646	3.802	8.946
I	AEH + LB	6.313	0.034	0.064	0.098	1.271	8.616	10.862
J	AEH + MS	4.289	0.032	0.055	0.059	1.185	6.443	8.864

As previously observed, the assays in LB medium showed the best bacterial growth, 8.055 g L^{-1} (G) and 6.313 g L^{-1} (I). In assay G the specific growth rate was 0.059 h^{-1} while in assay I, 0.034 h^{-1}.

In the assays with MS (H and J) bacterial growth was lower, as observed in previous assays and might indicate that this medium was poor in nutrients, which limited the growth of the microorganism. In the assay H the $[X]_{max}$ achieved was 3.566 g L^{-1} while in assay J it was 4.289 g L^{-1}. Despite having a lower $[X]_{max}$, the specific growth rate was 0.030 h^{-1}, while in assay J was 0.032 h^{-1}.

In these assays, the pH increased to approximately 8. This might result of some kind of buffering effect from the hydrolysates.

Glucose and fructose were not fully consumed in any of the assays G, H, I and J. This was not expected, since the only difference for the previous assays was the inocula preparation. Also, the microbial growth was quite similar to that of previous assays and the

biomass yield in these assays was again higher than 1.0, which could indicate that other compounds in the culture medium may have served as carbon sources for *C. necator*. Several studies indicate that *C.necator* only consumed the available sugars after consuming other carbon sources available in the medium (Yu et al. 2008) (Wang et al. 2014). The phenolic compounds were also analyzed throughout the tests and no consumption by *C.necator* was observed. The results for this analysis are shown in annex in section B, Figure 22. Moreover, the real COD was analyzed and compared to the theorical value corresponding to the monosaccharides consumed. This comparison allowed assessing whether the microorganism has effectively used other carbon sources present in the medium.

In all assays, the total rCOD consumed was higher than the total tCOD consumed. Indeed, this is coherent with the values of biomass yields higher than 1.0, which can confirm the consumption of other components of the hydrolysates by *C. necator.* These results also show that the hydrothermal extraction resulted in a higher amount of organic matter that could be used by *C, necator* since in both assays with HEH more rCOD was consumed than in assays with AEH.

4. Flask assays with hydrolysate for the selection of nitrogen source

In the previous assays, LB medium resulted in a better growth of *C. necator* with a higher biomass concentration and specific growth rate. However, despite the good results, LB medium is too costly to be used at industrial level. Also, the good results could also be a result of the other carbon sources present in this medium (tryptone and yeast extract). For this reason, in the following assays, MS medium was used. Regarding the hydrolysate, HEH was chosen because not only more COD was available for the microorganisms, but also because it led to the best results with LB medium, despite the choice of MS for the following assays.

Tests L, M, N and O were performed with the objective of optimizing MS. This optimization aims to improve microbial growth when MS is used with HEH. Based on the work of Azizi et al. (Azizi et al., 2017), four different nitrogen sources, yeast extract, urea, ammonium chloride, and ammonium sulfate were selected at a concentration of 1 g/L. The nitrogen sources were added to the medium to assess the influence of different nitrogen sources on bacterial growth. An assay supplemented with LB medium (K) was performed simultaneously as a control.

In these assays, the PHA production was measured at the end of each one.

Table 11 details the various parameters calculated for these assays, namely the maximum cell concentration ($[X]_{max)}$, specific growth rate (μ), volumetric consumption rate of glucose and fructose (r_{Glc} and r_{Fru}, respectively), PHA content and biomass and polymer yields on substrate ($Y_{X/S}$, $Y_{PHA/S}$), tCOD and rCOD consumption as well as the biomass yield on nitrogen source.

Figure 15 shows the influence of the chosen nitrogen sources on bacterial growth as well as the consumption of carbon sources and pH.

Table 11 - Summary of results from the assays with hydrolysate for nitrogen source selection.

Assay	Medium	[X]max (g L^{-1})	μ (h^{-1})	rGlc (g L^{-1} h^{-1})	rFru (g L^{-1} h^{-1})	$Y_{X/S}$ (mol mol^{-1})	$Y_{X/N}$ (mol mol^{-1})	$\%PHA_{final}$	$Y_{PHA/S}$ (mol mol^{-1})	tCOD consumed (g_{COD} L^{-1})	rCOD consumed (g_{COD} L^{-1})
K	HEH + LB	9.090	0.077	0.061	0.071	2.267	-	70.06	1.134	7.364	10.193
L	HEH + Urea in MS	6.369	0.045	0.055	0.058	1.826	9.052	86.97	1.223	6.334	9.742
M	HEH + Ammonium sulfate in MS	3.300	0.042	0.052	0.062	0.927	8.695	89.12	0.640	6.732	9.571
N	HEH + Ammonium chloride in MS	3.761	0.043	0.052	0.046	1.234	12.298	89.17	0.854	5.775	8.367
O	HEH + Yeast extract in MS	2.257	0.027	0.027	0.051	0.869	13.316	89.13	0.601	4.618	11.239

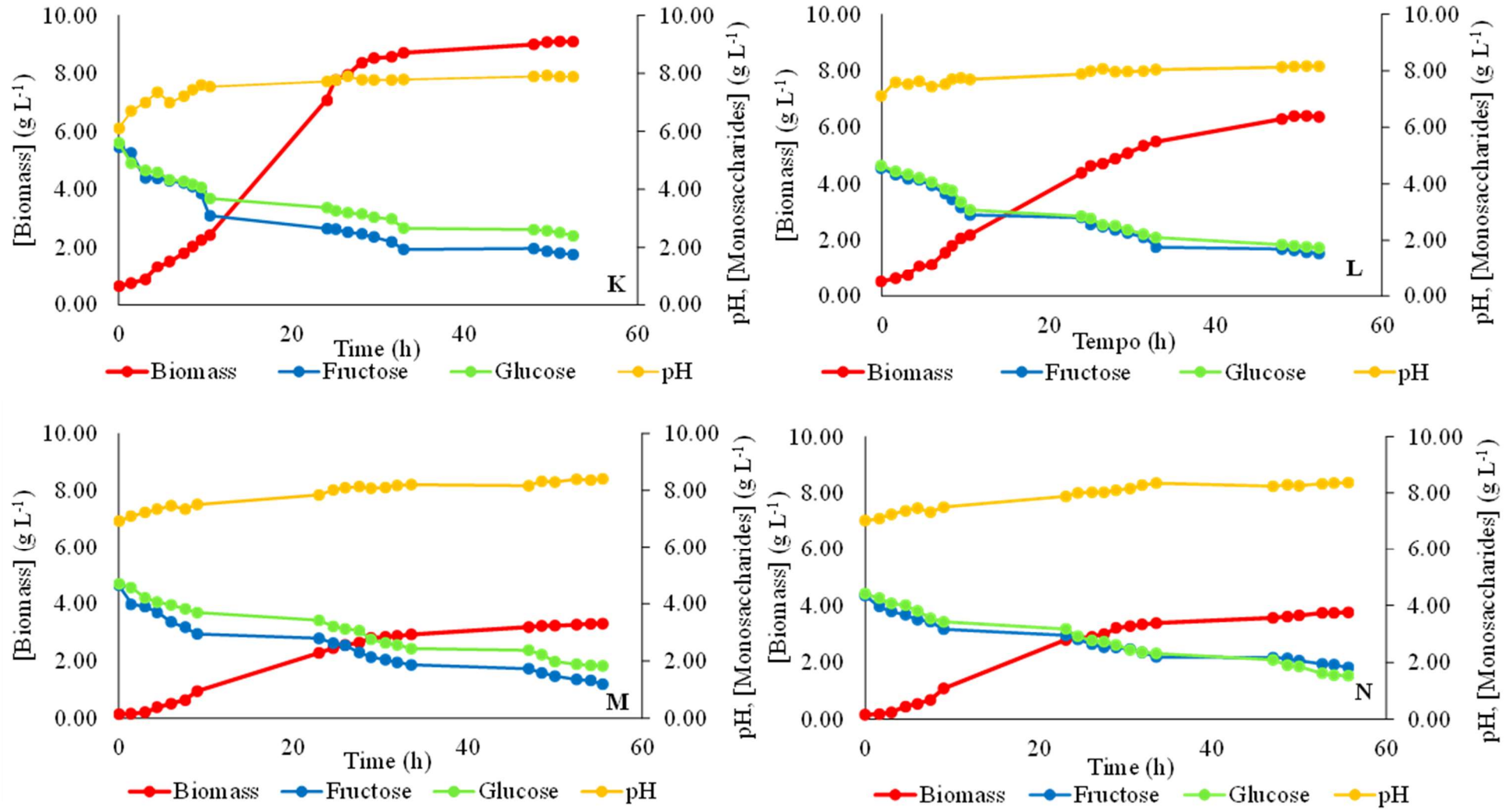


Figure 15 - Evolution of pH and concentration of biomass and carbon sources of the assays for nitrogen source selection. K) HEH supplemented with LB medium. L) Urea as nitrogen source. M) Ammonia sulfate as nitrogen source. N) Ammonia chloride as a source of nitrogen. O) Yeast extract as nitrogen source.

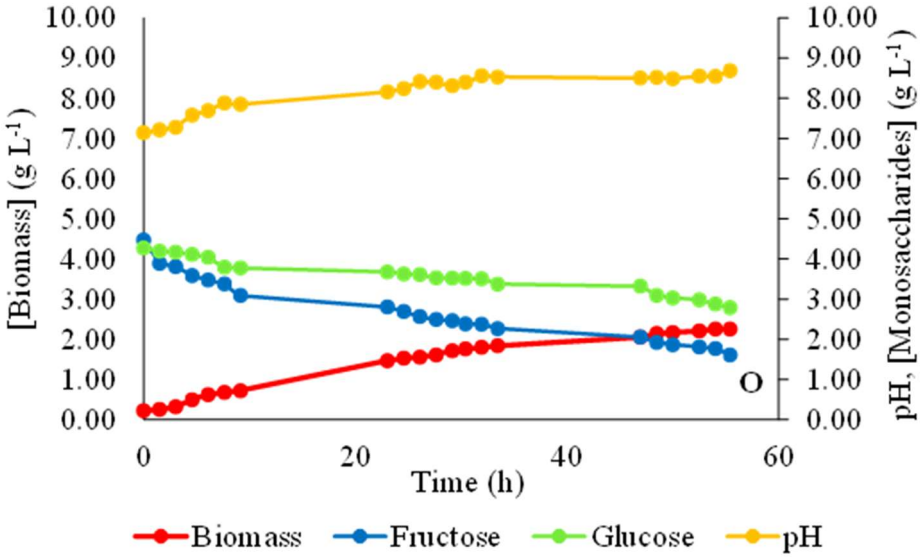


Figure 15 (continuation) - Evolution of pH and concentration of biomass and carbon sources of the assays for hydrolysate selection. K) HEH supplemented with LB medium. L) Urea as nitrogen source. M) Ammonia sulfate as nitrogen source. N) Ammonia chloride as a source of nitrogen. O) Yeast extract as nitrogen source.

The K assay, with LB medium, showed, as expected, the best bacterial growth. In this assay the [X]max achieved was 9,090 g L^{-1} with a specific growth rate of 0.077 h^{-1}.

In assay L with urea as nitrogen source, showed the best performance with MS medium with a [X]max of 6,369 g L^{-1} and a specific growth rate of 0.045 h^{-1}. The biomass concentration was quite similar to that reported in the literature in a similar study with the same bacteria but with a hydrolysate of brown algae as substrate, where [X]max was 6.40 g L^{-1} (Azizi et al., 2017).

The O assay with yeast extract as nitrogen source, had, contrary to what was expected, worse results of bacterial growth. In the study by Azizi et al., this nitrogen source produced bacterial growth similar to that of urea (Azizi et al., 2017). This may be due to the lower biomass concentration at the beginning of the test. Still, in this assay [X]max was 2,257 g L^{-1}.

Again, in the tests, there was no total consumption of carbon sources and in the K, L and N tests the values of biomass yields were higher than 1. Fructose consume rates were generally higher than glucose consumption rates, except for the N test where the opposite was found.

The quantification of PHA by gas chromatography was also performed in these assays and P(3HB) was produced in all assays. The N test (ammonia chloride as nitrogen source) was the one that showed a higher %PHA, 89.17% with a $Y_{PHA/S}$ of 0.854, while the assay L (Urea as nitrogen source) was the one that showed a lower %PHA, 86.97% with a $Y_{PHA/S}$ of 1.223. The %PHA obtained are much higher than in the study by Azizi et al. (44.93% to 50.32%). This may be due to the difference in the substrate used since Azizi et al. used a hydrolysate of brown algae. Nevertheless, in that study too, the highest percentage was for the ammonium test and the lowest percentage was for the urea test (Azizi et al. 2017).

Considering the data of Table 11, it was verified that the assays in which there was lower biomass growth presented higher %PHA, as expected. This points to the possibility that these assays have elapsed in nutrient deficit and priority has been given to polymer production to the detriment of biomass growth. In the analysis of PHA content, it was also verified that there was polymer production since the beginning of the assays, concluding that there may have been PHA production still in the inoculum phase.

Both %PHA and cell growth were affected by nitrogen source. Considering maximizing PHA production, ammonium chloride as nitrogen source would be the best choice for nitrogen rather than others. On the other hand, considering the bacterial growth, the best source of nitrogen is urea.

Phenolic compounds were analyzed throughout the tests and there was no consumption of these by *C.necator*. The figures for this analysis can be seen in the annex in section B, Figure 22. As in previous assays, tCOD and rCOD were determined for each of these assays. The evolution of tCOD and the evolution of rCOD was quite similar in all assays. However, the total rCOD consumed is higher than the total tCOD consumed, confirming the consumption of other carbons sources in HEH.

5. Bioreactor assays

After choosing hydrolysate and nitrogen source (urea) that led to the best performance of growth and PHA production, the process scale-up from flask to bioreactor was studied.

Figure 16 shows the results of R1 assay.

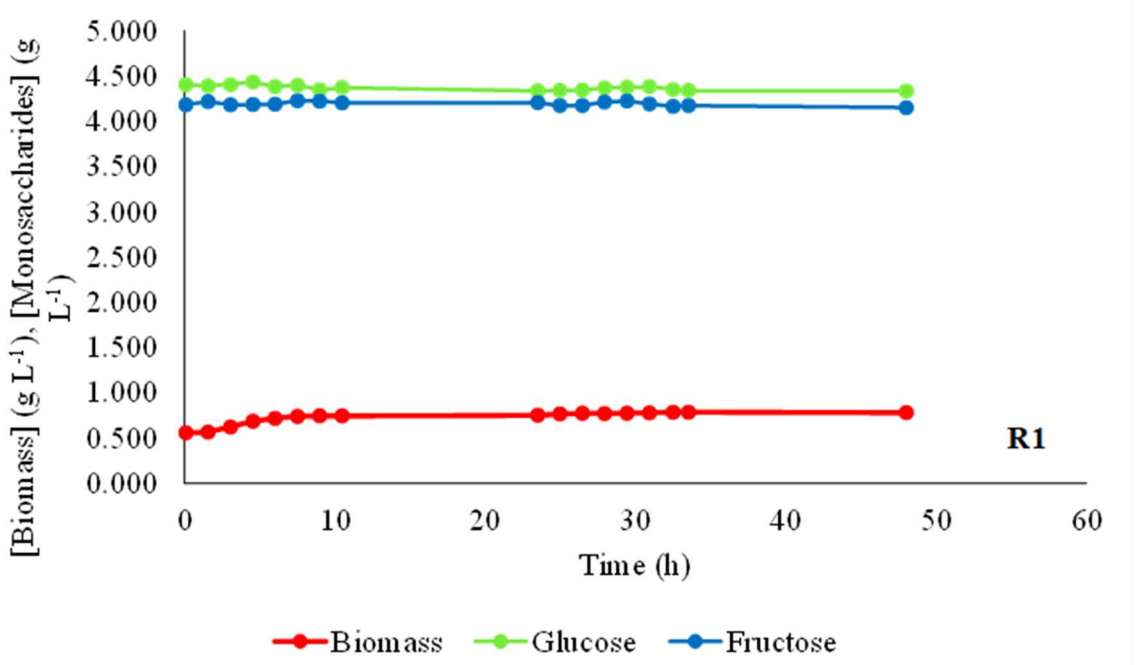


Figure 16 - Evolution of the concentration of biomass and carbon sources of the first reactor assay (R1).

In the first test, the control of the DO (about 30%) was done by automatically by adjusting the agitation. This control led to the test taking place with very low agitation (about 120 rpm). This stirring value did not allow bacterial growth. The [X]max in this assay was 0.779 g L^{-1} and the [X]initial was 0.559 g L^{-1}. Thus, in the R1 assay there was no growth of the microorganism and, consequently, there was also no consumption of the available sugars in the medium.

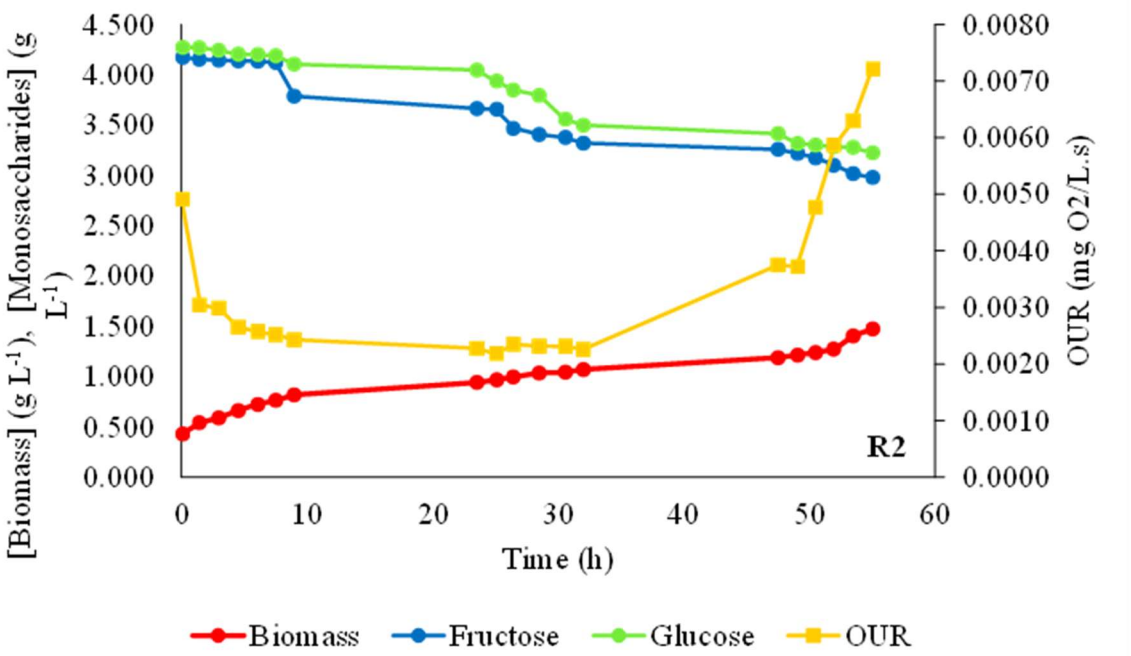


Figure 17 - Evolution of pH, concentration of biomass and carbon sources of the second bioreactor assay (R2).

Then R2 was performed after changing the following parameters of R1: agitation, DO control as well as OUR control.

The evolution of the concentration of biomass, carbon sources, and OUR along the operational time of the R2 assay is shown in Figure 17.

In the second reactor test the DO was left uncontrolled and, therefore, agitation was maintained at 250 rpm throughout the test. In this assay, the OUR was monitored.

Contrary to what happened in the R1 assay, in this assay there was some consumption of carbon sources as well as a higher microbial growth ([X]max was 1,474 g L^{-1}). However, as Figure 17 shows, the lag phase lasted about 52h and cannot be affirmed that after this time the exponential phase began. It was not possible to prolong the test beyond 55h and it was not possible to predict what would happen in relation to microbial growth and sugar consumption. However, as can be seen by the considerable increase in OUR, everything indicates that *C. necator* would has started its growth.

Phenolic compounds were analyzed throughout the tests and there was no consumption of these by *C. necator*. The graphs for this analysis can be seen in the annex in section B, Figure 22.

The enormous lag phase observed in R2 may explain the fact that the conditions under which the inoculum was carried out are different from the conditions of the reactor test. During the inoculum an orbital agitation was applied, and the aeration of the culture was done by means of the agitation itself, since the erlenmeyer was fully closed in order to maintain sterility. When transferred to the bioreactor, the culture was subjected to mechanical agitation and aeration made with air intake in the reactor. These changes probably led to the culture requiring a higher time of adaptation and consequently to a higher lag phase.

This test should have taken place in fed-batch and two pulses of 500 mL each were prepared. However, the pulses were not given since the culture remained in the lag phase until the test was finished.

The biomass collected in each sample of this assay was analyzed by gas chromatography. However, the presence of PHA was not verified, which would be expected since the culture did not leave the lag phase.

6. Overall performance of *C. necator* DSM 545 using GB hydrolysate

The consumption of carbon sources by *C. necator* in both hydrolysates was demonstrated. However, hydrolysate from hydrothermal extraction provided better bacterial growth. This may be due to the fact that acidic extraction generates a bigger number of inhibitors of microbial activity, such as phenolic compounds (Juarez et al. 2018).

Fructose was found to be the preferred carbon source since its consumption rates were always slightly higher than glucose consumption rates. This has been reported in several studies that concluded that *C. necator* is capable of metabolizing fructose as the dominant saccharide present in grape sugar extract, but its capacity to utilize glucose is limited, less productive and requires the adaptation to this sugar (Franz et al. 2012) (Volodina et al. 2016).

Kovalcik et al. conducted a study in which hydrolyzed grape pomace was used as raw material for the production of P(3HB) by *C. necator*. In this study, a %PHAmax of about 50% was achieved (Kovalcik et al. 2020).

Compared to the above-mentioned studies, the biomass concentration obtained in this study was quite similar, however, the %PHA obtained were much higher. These results showed the good perspectives of GB as a substrate for P(3HB) production by *C. necator*.

7. PHA extraction

Although the ideal conditions for the production of PHA (nutrient deficit and excess carbon) were not applied during the assays in shake flasks, P(3HB) was produced and extracted after mixing the biomass from assays M, N and O. From 0.553 g of lyophilized biomass, 0.310 g of PHB were extracted. Figure 18 shows the extracted polymer.

Figure 18 - PHA extracted from M, N and O assays.

The obtained polymer was characterized by Fourier-transform infrared spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and Thermogravimetric analysis (TGA).

7.1. PHA characterization by FTIR

The extracted polymer was characterized by FTIR, whose spectra are shown in Figure 19 as well the commercial P(3HB). For the identification of PHA by FTIR, the 3000-2800 and 1740-1700 cm^{-1} regions are often used (Simon-Colin et al. 2009)(Simon-Colin et al. 2008)(Sathiyanarayanan et al. 2017).

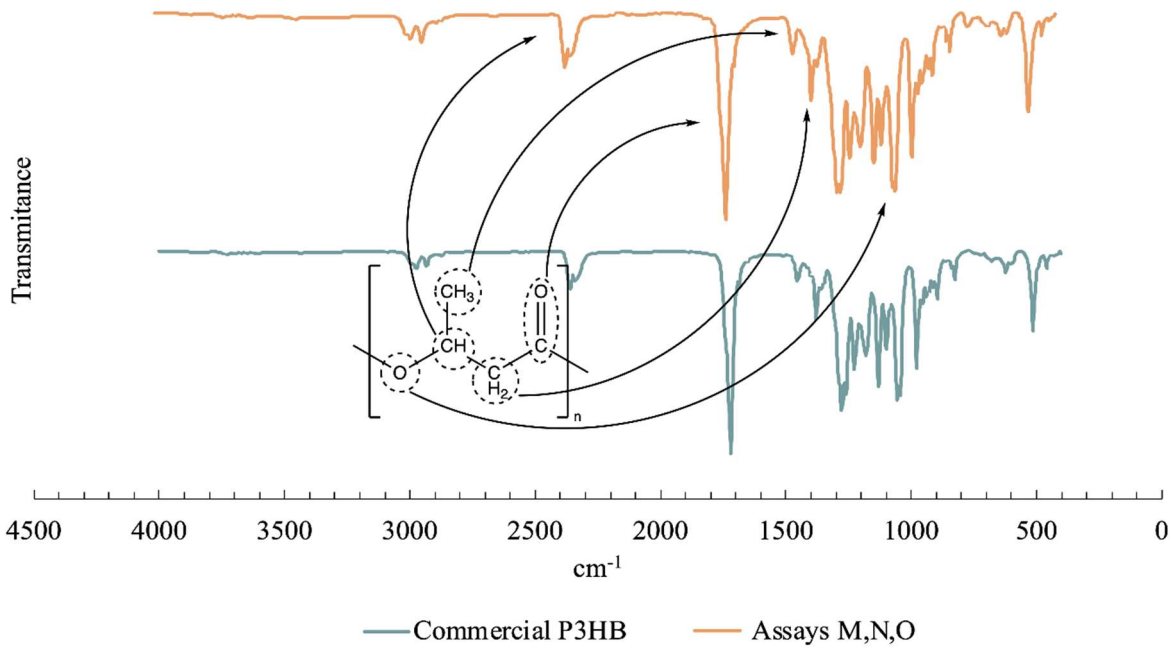


Figure 19 - FTIR spectra of the PHA extracted M, N and O assays in comparison with commercial P(3HB).

The peak in the 1740-1700 cm^{-1} region is present in the commercial P(3HB), as well as in the extracted polymer and corresponding to specific rotations around carbon atoms. This represents the ester carbonyl (C=O) stretching, confirming the presence of P(3HB) (Huang et al., 2016) (Sathiyanarayanan et al. 2017) (Christelle Simon-Colin et al. 2008). Several peaks in the region of 1500-1000 cm^{-1} were also detected and these represent the CH_3, CH_2 twist, as well as C-O stretches (Arun et al. 2009) (Sathiyanarayanan et al. 2017) Absorption bands in the region around 3000, (2934 and 2925 cm^{-1}) represent (C–H) stretching (Sindhu et al. 2013) (Huang et al., 2016).

Moreover, the transmittance minimums coincide in the same wave number (cm^{-1}) and the percentage of transmittance is very similar for both polymers analysed. Thus, that the

extracted polymer is P(3HB), which confirms de results of GC and it is accordance with the type of the carbon source used.

7.2. TGA and DSC analysis of extracted polymer

The thermogram obtained by TGA is shown in Figure 20 and shows that the polymer produced was degraded mainly in a one-step process. In this process, a considerable mass loss of 99.7% of the total mass of the polymer was observed at around 275-300 ºC. This temperature range is coincident with the thermal degradation that of P(3HB), which is known to be associated with random scission of the polymer chain leading to evolution of crotonic acid (Martino et al. 2014). The thermogram showed a minor mass reduction of 5.53% from 100 ºC to 275 ºC which was due to thermal degradation of the solvent such as water, lower molecular weight solvents present in the polymer or gas desorption taken place. Degradation temperature between 275 ºC and 300 ºC resulted in loss of low molecular weight compounds of the degradation products of biopolymer. Maximum mass loss of biopolymer was observed at 300 ºC associated with the ester cleavage of PHA component by elimination reaction (Martino et al. 2014).

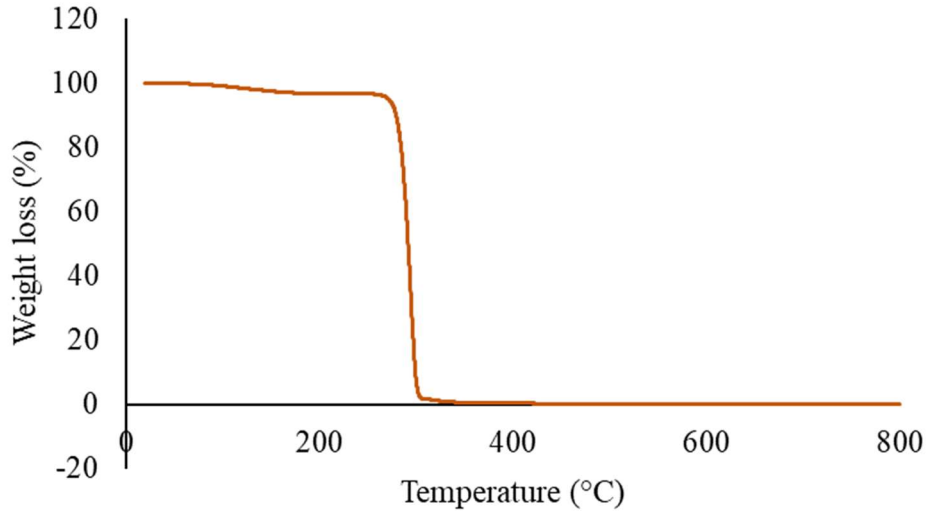


Figure 20 - Thermogravimetric (TGA) curve of P(3HB) produced and extracted from assays M, N and O.

Among all thermal properties, the thermal stability is mostly important as PHA with low melting temperature and increased thermal stability are desirable. The DSC analysis is a measure of assessing the molecular mobility of the polymer chains. The molecular mobility is manifested in terms of the glass transition temperature (Tg), which is an important characteristic of the polymer. Glass transition temperatures reported in literature for P(3HB) (obtained from different substrates) have wide variation. Altaee et al. (2016) have reported Tg of 2.79 ^{o}C for P(3HB) synthesized from crude palm kernel oil. Sandhya et al. (2013) have reported Tg of 10-15 ^{o}C for P(3HB) synthesized from fermentation of paddy straw. However, it was not possible to observer Tg in the chromatogram of the obtained polymer. The melting temperature determined for the polymer obtained was approximately 170.63 ^{o}C and the melting enthalpy was 33.7886 J/g. This temperature is in accordance with the melting temperatures reported in the literature for P(3HB) (Pradhan et al. 2017).The crystallinity degree of the biopolymer was calculated from the melting enthalpy and a value of 23.14% was obtained. Crystallinity affects rheological properties of the polymer, especially the melting behavior during processing. In general, polymers with low crystallinity degrees showed a wider processing window, whilst polymers with higher crystallization degrees typically showed a sharper melting range (Rosengart et al. 2015).

CHAPTER IV – CONCLUSIONS AND FUTURE WORK

1. Conclusions

The present work aimed to study the production of PHA by *C. necator* using the hydrolyzed grape bagasse as substrate

In a first phase, two distinct pretreatments were studied, hydrothermal hydrolysis and acidic hydrolysis, were performed during different times. Considering that for all times carbon sources were found in both hydrolysis, the time 2h was selected because it has the best ratio of sugar concentration and expenditure. Hydrolysates from both pretreatments were characterized in terms of potential carbon sources and microbial activity inhibitors, namely, phenolic compounds. Glucose (6.48 g L^{-1} in hydrothermal hydrolysis and 7.59 g L^{-1} in acid hydrolysis) and fructose (6.64 g L^{-1} in hydrothermal hydrolysis and 7.56 g L^{-1} in acid hydrolysis) were identified as the main carbon sources. Phenolic compounds had a concentration in the obtained hydrolysate 0.282 gGAE L^{-1} after hydrothermal hydrolysis and 0.448 gGAE L^{-1} after acidic hydrolysis.

Preliminary tests were performed to understand the capacity of *C. necator* in hydrolysate. In these first assays very long lag phases, 21-25h, were observed, so changes were made in the inoculum and pre-inoculum preparation to improve the duration of the lag phase (the incubation time of the pre-inocula was increased from 12h to 24h and the composition of the culture medium of the inoculum was changed to a composition similar to that of the assay to obtain a faster adaptation of microorganisms to hydrolysates). With these changes lag phase decreased to 6-8.5h. In these assays, it was also verified that the hydrolysate with higher potential for a better microbial growth was the hydrolysate from hydrothermal hydrolysis.

Two different supplementations (LB and MS media) were also tested. Despite promoting better microbial growth, LB medium was not economically viable, and MS was used for the following assays. In order to improve *C. necator* growth, several sources of nitrogen were tested with MS medium. The nitrogen source that promoted a higher growth of the microorganism was urea with [X]max was 6,369 g L^{-1} with a PHA content of 86% after 52.5h of cultivation. However, the nitrogen source that promoted the highest content in PHA was ammonia chloride, 87.17%. The polymer extracted from these assays was characterized and it was confirmed that it was P(3HB).

The reactor assays were carried out with the hydrolyzed and nitrogen source selected (urea) in the previous tests. In the first assay there was no microbial growth or consumption of carbon sources. This may be due to the low agitation that the assay occurred in the search to control the OD. This test took place for 48 hours and the biomass did not go beyond 0. 779 g L^{-1}. In the second assay it can be predicted that C. necator would be entering the exponential phase of growth after 52 h of lag phase. The test could not be prolonged further, and the biomass did not go beyond 1,474 g L^{-1}.

The possibility of producing PHA from waste from the wine industry was showed by the results of this work. This is a process that can be implemented in Portugal due to the strong wine industry.

Over the last century, much of industrial and technological development has been based on fossil resources. Abundance has led to waste and waste leads us to a crisis with little chance of return. In addition, the accumulation of plastic debris in the environment and its adverse effects are increasingly a concern. The paradigm must be changed and PHAs are undoubtedly part of that change.

2. Future work

In the present study, only two types of hydrolysis were tested to pre-treat GB. Moreover, despite testing several times of hydrolysis, all assays were performed at the same temperature. In future work it would be important, therefore, to test other pretreatments as well as other temperatures for acidic and hydrothermal.

A more detailed characterization of hydrolysate can also help to better understand the behavior of the bacteria and therefore optimize its growth and consequently the production of PHA.

The scale-up of the process must be optimized both at the level of air supply and at the level of agitation. Once the operating conditions of the test have been well defined, it is necessary to establish the number of pulses to be administered in a fed-batch process so that the C/N ratio in the reactor is favorable to maximizing PHA production.

CHAPTER V – REFERENCES

Abubakar, H. G., A. S. Abdulkareem, A. Jimoh, O. D. Agbajelola, J. O. Okafor, and E. A. Afolabi. 2016. "Optimization of Biodiesel Production from Waste Cooking Oil." *Energy Sources, Part A: Recovery, Utilization and Environmental Effects* 38 (16): 2355–61. https://doi.org/10.1080/15567036.2015.1040899.

Achkar, Jean H. El, Thomas Lendormi, Dominique Salameh, Nicolas Louka, Richard G. Maroun, Jean Louis Lanoisellé, and Zeina Hobaika. 2018. "Influence of Pretreatment Conditions on Lignocellulosic Fractions and Methane Production from Grape Pomace." *Bioresource Technology* 247: 881–89. https://doi.org/10.1016/j.biortech.2017.09.182.

Aguilar, Alfredo, Tomasz Twardowski, and Roland Wohlgemuth. 2019. "Bioeconomy for Sustainable Development." *Biotechnology Journal* 14 (8): 1–11. https://doi.org/10.1002/biot.201800638.

Aguilar, Alfredo, Roland Wohlgemuth, and Tomasz Twardowski. 2018. "Perspectives on Bioeconomy." *New Biotechnology* 40: 181–84. https://doi.org/10.1016/j.nbt.2017.06.012.

Ahammad, S. Z.; Gomes, J.; Sreekrishnan, T. R. 2008. "Wastewater Treatment ForproductionofH2S-Free Biogas." *Journal of Chemical Technology & Biotechnology* 83 (May): 1163–69. https://doi.org/10.1002/jctb.

Albuquerque, Priscilla B.S., and Carolina B. Malafaia. 2018. "Perspectives on the Production, Structural Characteristics and Potential Applications of Bioplastics Derived from Polyhydroxyalkanoates." *International Journal of Biological Macromolecules* 107 (PartA): 615–25. https://doi.org/10.1016/j.ijbiomac.2017.09.026.

Allison, Brittany J., and Christopher W. Simmons. 2018. "Obtaining Multiple Coproducts from Red Grape Pomace via Anthocyanin Extraction and Biogas Production." *Journal of Agricultural and Food Chemistry* 66 (30): 8045–53. https://doi.org/10.1021/acs.jafc.8b02250.

Alsafadi, Diya, and Othman Al-Mashaqbeh. 2017. "A One-Stage Cultivation Process for the Production of Poly-3-(Hydroxybutyrate-Co-Hydroxyvalerate) from Olive Mill Wastewater by Haloferax Mediterranei." *New Biotechnology* 34: 47–53. https://doi.org/10.1016/j.nbt.2016.05.003.

Álvarez-Chávez, Clara Rosalía, Sally Edwards, Rafael Moure-Eraso, and Kenneth Geiser. 2012. “Sustainability of Bio-Based Plastics: General Comparative Analysis and Recommendations for Improvement.” *Journal of Cleaner Production* 23 (1): 47–56. https://doi.org/10.1016/j.jclepro.2011.10.003.

Amini, Malihe, Hassan Yousefi-Massumabad, Habibollah Younesi, Hajar Abyar, and Nader Bahramifar. 2020. “Production of the Polyhydroxyalkanoate Biopolymer by Cupriavidus Necator Using Beer Brewery Wastewater Containing Maltose as a Primary Carbon Source.” *Journal of Environmental Chemical Engineering* 8 (1): 103588. https://doi.org/10.1016/j.jece.2019.103588.

Andler, R., V. Pino, F. Moya, E. Soto, C. Valdés, and C. Andreeßen. 2021. “ Synthesis of Poly-3-Hydroxybutyrate (PHB) by Bacillus Cereus Using Grape Residues as Sole Carbon Source .” *International Journal of Biobased Plastics* 3 (1): 98–111. https://doi.org/10.1080/24759651.2021.1882049.

Anjum, Anbreen, Mohammad Zuber, Khalid Mahmood Zia, Aqdas Noreen, Muhammad Naveed Anjum, and Shazia Tabasum. 2016. “Microbial Production of Polyhydroxyalkanoates (PHAs) and Its Copolymers: A Review of Recent Advancements.” *International Journal of Biological Macromolecules* 89: 161–74. https://doi.org/10.1016/j.ijbiomac.2016.04.069.

Aramvash, Asieh, Fatemeh Moazzeni Zavareh, and Narges Gholami Banadkuki. 2018. “Comparison of Different Solvents for Extraction of Polyhydroxybutyrate from Cupriavidus Necator.” *Engineering in Life Sciences* 18 (1): 20–28. https://doi.org/10.1002/elsc.201700102.

Arumugam, A., S. G. Senthamizhan, V. Ponnusami, and S. Sudalai. 2018. “Production and Optimization of Polyhydroxyalkanoates from Non-Edible Calophyllum Inophyllum Oil Using Cupriavidus Necator.” *International Journal of Biological Macromolecules* 112: 598–607. https://doi.org/10.1016/j.ijbiomac.2018.02.012.

Arun, A., R. Arthi, V. Shanmugabalaji, and M. Eyini. 2009. “Microbial Production of Poly-β-Hydroxybutyrate by Marine Microbes Isolated from Various Marine Environments.” *Bioresource Technology* 100 (7): 2320–23. https://doi.org/10.1016/j.biortech.2008.08.037.

Atlić, Aid, Martin Koller, Dietrich Scherzer, Christoph Kutschera, Elizabeth Grillo-Fernandes, Predrag Horvat, Emo Chiellini, and Gerhart Braunegg. 2011. “Continuous

Production of Poly([R]-3-Hydroxybutyrate) by Cupriavidus Necator in a Multistage Bioreactor Cascade." *Applied Microbiology and Biotechnology* 91 (2): 295–304. https://doi.org/10.1007/s00253-011-3260-0.

Azizi, Nahid, Ghasem Najafpour, and Habibollah Younesi. 2017. "Acid Pretreatment and Enzymatic Saccharification of Brown Seaweed for Polyhydroxybutyrate (PHB) Production Using Cupriavidus Necator." *International Journal of Biological Macromolecules* 101: 1029–40. https://doi.org/10.1016/j.ijbiomac.2017.03.184.

Bengtsson, Simon, Anton Karlsson, Tomas Alexandersson, Luca Quadri, Markus Hjort, Peter Johansson, Fernando Morgan-Sagastume, et al. 2017. "A Process for Polyhydroxyalkanoate (PHA) Production from Municipal Wastewater Treatment with Biological Carbon and Nitrogen Removal Demonstrated at Pilot-Scale." *New Biotechnology* 35: 42–53. https://doi.org/10.1016/j.nbt.2016.11.005.

Berezina, Nathalie, Bopha Yada, and Rodrigue Lefebvre. 2015. "From Organic Pollutants to Bioplastics: Insights into the Bioremediation of Aromatic Compounds by Cupriavidus Necator." *New Biotechnology* 32 (1): 47–53. https://doi.org/10.1016/j.nbt.2014.09.003.

Besi, Matteo De, and Kes McCormick. 2015. "Towards a Bioeconomy in Europe: National, Regional and Industrial Strategies." *Sustainability (Switzerland)* 7 (8): 10461–78. https://doi.org/10.3390/su70810461.

Bharathiraja, B., J. Iyyappan, J. Jayamuthunagai, R. Praveen Kumar, Ranjna Sirohi, Edgard Gnansounou, and Ashok Pandey. 2020. "Critical Review on Bioconversion of Winery Wastes into Value-Added Products." *Industrial Crops and Products* 158 (September): 112954. https://doi.org/10.1016/j.indcrop.2020.112954.

Bolonio, David, María Jesús García-Martínez, Marcelo F. Ortega, Magín Lapuerta, José Rodríguez-Fernández, and Laureano Canoira. 2019. "Fatty Acid Ethyl Esters (FAEEs) Obtained from Grapeseed Oil: A Fully Renewable Biofuel." *Renewable Energy* 132: 278–83. https://doi.org/10.1016/j.renene.2018.08.010.

Brodin, Malin, María Vallejos, Mihaela Tanase Opedal, María Cristina Area, and Gary Chinga-Carrasco. 2017. "Lignocellulosics as Sustainable Resources for Production of Bioplastics – A Review." *Journal of Cleaner Production* 162: 646–64. https://doi.org/10.1016/j.jclepro.2017.05.209.

Bustos, G., N. de la Torre, A. B. Moldes, J. M. Cruz, and J. M. Domínguez. 2007.

"Revalorization of Hemicellulosic Trimming Vine Shoots Hydrolyzates Trough Continuous Production of Lactic Acid and Biosurfactants by L. Pentosus." *Journal of Food Engineering* 78 (2): 405–12. https://doi.org/10.1016/j.jfoodeng.2005.10.008.

Butlin, Martin. 2002. "'Is This a Private War or Can Anyone Join in?': A Plea for a Broader Look at Blake's Color-Printing Techniques." *Blake - An Illustrated Quarterly* 36 (2): 45–49.

Cavalheiro, João M.B.T., M. Catarina M.D. de Almeida, Christian Grandfils, and M. M.R. da Fonseca. 2009. "Poly(3-Hydroxybutyrate) Production by Cupriavidus Necator Using Waste Glycerol." *Process Biochemistry* 44 (5): 509–15. https://doi.org/10.1016/j.procbio.2009.01.008.

Cavalheiro, João M.B.T., Rodrigo S. Raposo, M. Catarina M.D. de Almeida, M. Teresa Cesário, Chantal Sevrin, Christian Grandfils, and M. M.R. da Fonseca. 2012. "Effect of Cultivation Parameters on the Production of Poly(3-Hydroxybutyrate-Co-4-Hydroxybutyrate) and Poly(3-Hydroxybutyrate-4-Hydroxybutyrate-3-Hydroxyvalerate) by Cupriavidus Necator Using Waste Glycerol." *Bioresource Technology* 111: 391–97. https://doi.org/10.1016/j.biortech.2012.01.176.

Chen, Zhiqiang, Long Huang, Qinxue Wen, and Zirui Guo. 2015. "Efficient Polyhydroxyalkanoate (PHA) Accumulation by a New Continuous Feeding Mode in Three-Stage Mixed Microbial Culture (MMC) PHA Production Process." *Journal of Biotechnology* 209: 68–75. https://doi.org/10.1016/j.jbiotec.2015.06.382.

Christ, Katherine L., and Roger L. Burritt. 2013. "Critical Environmental Concerns in Wine Production: An Integrative Review." *Journal of Cleaner Production* 53: 232–42. https://doi.org/10.1016/j.jclepro.2013.04.007.

Colombo, Bianca, Tommy Pepè Sciarria, Maria Reis, Barbara Scaglia, and Fabrizio Adani. 2016. "Polyhydroxyalkanoates (PHAs) Production from Fermented Cheese Whey by Using a Mixed Microbial Culture." *Bioresource Technology* 218 (2016): 692–99. https://doi.org/10.1016/j.biortech.2016.07.024.

Conde, Teresa, and Solange I. Mussatto. 2016. "Isolation of Polyphenols from Spent Coffee Grounds and Silverskin by Mild Hydrothermal Pretreatment." *Preparative Biochemistry and Biotechnology* 46 (4): 406–9. https://doi.org/10.1080/10826068.2015.1084514.

Corbin, Kendall R., Yves S.Y. Hsieh, Natalie S. Betts, Caitlin S. Byrt, Marilyn Henderson,

Jozsef Stork, Seth DeBolt, Geoffrey B. Fincher, and Rachel A. Burton. 2015. “Grape Marc as a Source of Carbohydrates for Bioethanol: Chemical Composition, Pre-Treatment and Saccharification.” *Bioresource Technology* 193: 76–83. https://doi.org/10.1016/j.biortech.2015.06.030.

Cordano, Mark, R. Scott Marshall, and Murray Silverman. 2010. “How Do Small and Medium Enterprises Go ‘Green’? A Study of Environmental Management Programs in the U.S. Wine Industry.” *Journal of Business Ethics* 92 (3): 463–78. https://doi.org/10.1007/s10551-009-0168-z.

Cortés-Camargo, Stefani, Noelia Pérez-Rodríguez, Ricardo Pinheiro de Souza Oliveira, Blanca E.Barragán Huerta, and José Manuel Domínguez. 2016. “Production of Biosurfactants from Vine-Trimming Shoots Using the Halotolerant Strain Bacillus Tequilensis ZSB10.” *Industrial Crops and Products* 79: 258–66. https://doi.org/10.1016/j.indcrop.2015.11.003.

Cruz, Madalena V., Alexandre Paiva, Pedro Lisboa, Filomena Freitas, Vítor D. Alves, Pedro Simões, Susana Barreiros, and Maria A.M. Reis. 2014. “Production of Polyhydroxyalkanoates from Spent Coffee Grounds Oil Obtained by Supercritical Fluid Extraction Technology.” *Bioresource Technology* 157: 360–63. https://doi.org/10.1016/j.biortech.2014.02.013.

Cruz, Madalena V., Mafalda Cruz Sarraguça, Filomena Freitas, João Almeida Lopes, and Maria A.M. Reis. 2015. “Online Monitoring of P(3HB) Produced from Used Cooking Oil with near-Infrared Spectroscopy.” *Journal of Biotechnology* 194: 1–9. https://doi.org/10.1016/j.jbiotec.2014.11.022.

Dabbert, Stephan, Iris Lewandowski, Jochen Weiss, and Andreas Pyka. n.d. *Knowledge-Driven Developments in the Bioeconomy : Technological and Economic Perspectives*.

Devesa-Rey, R., X. Vecino, J. L. Varela-Alende, M. T. Barral, J. M. Cruz, and A. B. Moldes. 2011. “Valorization of Winery Waste vs. the Costs of Not Recycling.” *Waste Management* 31 (11): 2327–35. https://doi.org/10.1016/j.wasman.2011.06.001.

Dias, João M.L., Paulo C. Lemos, Luísa S. Serafim, Cristina Oliveira, Marta Eiroa, Maria G.E. Albuquerque, Ana M. Ramos, Rui Oliveira, and Maria A.M. Reis. 2006. “Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product.” *Macromolecular Bioscience* 6 (11): 885–906. https://doi.org/10.1002/mabi.200600112.

Díaz, Ana Belén, Ignacio De Ory, Ildefonso Caro, and Ana Blandino. 2012. "Enhance Hydrolytic Enzymes Production by Aspergillus Awamori on Supplemented Grape Pomace." *Food and Bioproducts Processing* 90 (1): 72–78. https://doi.org/10.1016/j.fbp.2010.12.003.

Dietrich, Karolin, Marie Josée Dumont, Luis F. Del Rio, and Valérie Orsat. 2017. "Producing PHAs in the Bioeconomy — Towards a Sustainable Bioplastic." *Sustainable Production and Consumption* 9 (September): 58–70. https://doi.org/10.1016/j.spc.2016.09.001.

Dwyer, Kyle, Farah Hosseinian, and Michel Rod. 2014. "The Market Potential of Grape Waste Alternatives." *Journal of Food Research* 3 (2): 91. https://doi.org/10.5539/jfr.v3n2p91.

Elain, Anne, Adélaïde Le Grand, Yves Marie Corre, Magali Le Fellic, Nicolas Hachet, Véronique Le Tilly, Patrick Loulergue, Jean Luc Audic, and Stéphane Bruzaud. 2016. "Valorisation of Local Agro-Industrial Processing Waters as Growth Media for Polyhydroxyalkanoates (PHA) Production." *Industrial Crops and Products* 80: 1–5. https://doi.org/10.1016/j.indcrop.2015.10.052.

Ene, Simona Andreea, Carmen Teodosiu, Brindusa Robu, and Irina Volf. 2013. "Water Footprint Assessment in the Winemaking Industry: A Case Study for a Romanian Medium Size Production Plant." *Journal of Cleaner Production* 43: 122–35. https://doi.org/10.1016/j.jclepro.2012.11.051.

Farah, Nadia Omar, Abdul Rahman Norrsquo Aini, Saadiah Hafid Halimatun, Mumtaz Tabassum, Lai Yee Phang, and Ali Hassan Mohd. 2011. "Utilization of Kitchen Waste for the Production of Green Thermoplastic Polyhydroxybutyrate (PHB) by Cupriavidus Necator CCGUG 52238." *African Journal of Microbiology Research* 5 (19): 2873–79. https://doi.org/10.5897/ajmr11.156.

Fernández, Carmen María, María Jesús Ramos, Ángel Pérez, and Juan Francisco Rodríguez. 2010. "Production of Biodiesel from Winery Waste: Extraction, Refining and Transesterification of Grape Seed Oil." *Bioresource Technology* 101 (18): 7019–24. https://doi.org/10.1016/j.biortech.2010.04.014.

Fiorese, Mônica Lady, Filomena Freitas, Joana Pais, Ana Maria Ramos, Gláucia M.F. De Aragão, and Maria A.M. Reis. 2009. "Recovery of Polyhydroxybutyrate (PHB) from Cupriavidus Necator Biomass by Solvent Extraction with 1,2-Propylene Carbonate."

Engineering in Life Sciences 9 (6): 454–61. https://doi.org/10.1002/elsc.200900034.

Follonier, S., R. Riesen, and M. Zinn. 2015. "Pilot-Scale Production of Functionalized Mcl-PHA from Grape Pomace Supplemented with Fatty Acids." *Chemical and Biochemical Engineering Quarterly* 29 (2): 113–21. https://doi.org/10.15255/CABEQ.2014.2251.

Follonier, Stéphanie, Miriam S. Goyder, Anne Claire Silvestri, Simon Crelier, Franka Kalman, Roland Riesen, and Manfred Zinn. 2014. "Fruit Pomace and Waste Frying Oil as Sustainable Resources for the Bioproduction of Medium-Chain-Length Polyhydroxyalkanoates." *International Journal of Biological Macromolecules* 71: 42–52. https://doi.org/10.1016/j.ijbiomac.2014.05.061.

Franz, A., R. Rehner, A. Kienle, and H. Grammel. 2012. "Rapid Selection of Glucose-Utilizing Variants of the Polyhydroxyalkanoate Producer Ralstonia Eutropha H16 by Incubation with High Substrate Levels." *Letters in Applied Microbiology* 54 (1): 45–51. https://doi.org/10.1111/j.1472-765X.2011.03171.x.

Gahlawat, G., and S. Kumar Soni. 2019. "Study on Sustainable Recovery and Extraction of Polyhydroxyalkanoates (PHAs) Produced by Cupriavidus Necator Using Waste Glycerol for Medical Applications." *Chemical and Biochemical Engineering Quarterly* 33 (1): 99–110. https://doi.org/10.15255/CABEQ.2018.1471.

Gahlawat, Geeta. 2019. *Production Strategies for Commercialization of PHA*. https://doi.org/10.1007/978-3-030-33897-8_4.

Gahlawat, Geeta, and Sanjeev Kumar Soni. 2017. "Valorization of Waste Glycerol for the Production of Poly (3-Hydroxybutyrate) and Poly (3-Hydroxybutyrate-Co-3-Hydroxyvalerate) Copolymer by Cupriavidus Necator and Extraction in a Sustainable Manner." *Bioresource Technology* 243: 492–501. https://doi.org/10.1016/j.biortech.2017.06.139.

Gall, S. C., and R. C. Thompson. 2015. "The Impact of Debris on Marine Life." *Marine Pollution Bulletin* 92 (1–2): 170–79. https://doi.org/10.1016/j.marpolbul.2014.12.041.

Ganeshkumar, Vimalkumar, Suresh R. Subashchandrabose, Rajarathnam Dharmarajan, Kadiyala Venkateswarlu, Ravi Naidu, and Mallavarapu Megharaj. 2018. "Use of Mixed Wastewaters from Piggery and Winery for Nutrient Removal and Lipid Production by Chlorella Sp. MM3." *Bioresource Technology* 256: 254–58. https://doi.org/10.1016/j.biortech.2018.02.025.

García, I. L., J. A. López, M. P. Dorado, N. Kopsahelis, M. Alexandri, S. Papanikolaou, M. A. Villar, and A. A. Koutinas. 2013. "Evaluation of By-Products from the Biodiesel Industry as Fermentation Feedstock for Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) Production by Cupriavidus Necator." *Bioresource Technology* 130: 16–22. https://doi.org/10.1016/j.biortech.2012.11.088.

Guzman Lagunes, F., and J. B. Winterburn. 2016. "Effect of Limonene on the Heterotrophic Growth and Polyhydroxybutyrate Production by Cupriavidus Necator H16." *Bioresource Technology* 221: 336–43. https://doi.org/10.1016/j.biortech.2016.09.045.

Huang, M. 2016. *Metabolic Engineering for Biofuels*. *Science*.

Ienczak, Jaciane Lutz, Willibaldo Schmidell, and Gláucia Maria Falcão De Aragão. 2013. "High-Cell-Density Culture Strategies for Polyhydroxyalkanoate Production: A Review." *Journal of Industrial Microbiology and Biotechnology* 40 (3–4): 275–86. https://doi.org/10.1007/s10295-013-1236-z.

Iwata, Tadahisa, Yoshihiro Aoyagi, Masahiro Fujita, Hideki Yamane, Yoshiharu Doi, Yoshio Suzuki, Akihisa Takeuchi, and Kentaro Uesugi. 2004. "Processing of a Strong Biodegradable Poly[(R)-3-Hydroxybutyrate] Fiber and a New Fiber Structure Revealed by Micro-Beam x-Ray Diffraction with Synchrotron Radiation." *Macromolecular Rapid Communications* 25 (11): 1100–1104. https://doi.org/10.1002/marc.200400110.

Jambeck, Jenna R., Q. Ji, Y.-G. Zhang, D. Liu, D. M. Grossnickle, and Z.-X. Luo. 2015. "Plastic Waste Inputs from Land into the Ocean." *Science* 347 (6223): 764–68. http://www.sciencemag.org/cgi/doi/10.1126/science.1260879.

Jin, Qing, Andrew P. Neilson, Amanda C. Stewart, Sean F. O'Keefe, Young Teck Kim, Megan McGuire, Geoffrey Wilder, and Haibo Huang. 2018. "Integrated Approach for the Valorization of Red Grape Pomace: Production of Oil, Polyphenols, and Acetone-Butanol-Ethanol." *ACS Sustainable Chemistry and Engineering* 6 (12): 16279–86. https://doi.org/10.1021/acssuschemeng.8b03136.

Juarez, Guenover Florette Y., Kurt Branden C. Pabiloña, Kirck Brandon L. Manlangit, and Alchris Woo Go. 2018. "Direct Dilute Acid Hydrolysis of Spent Coffee Grounds: A New Approach in Sugar and Lipid Recovery." *Waste and Biomass Valorization* 9 (2): 235–46. https://doi.org/10.1007/s12649-016-9813-9.

Kalia, Vipin Chandra, Subhasree Ray, Sanjay K.S. Patel, Mamtesh Singh, and Gajendra Pratap Singh. 2019. “The Dawn of Novel Biotechnological Applications of Polyhydroxyalkanoates.” *Biotechnological Applications of Polyhydroxyalkanoates*, 1–11. https://doi.org/10.1007/978-981-13-3759-8_1.

Kammerer, Dietmar, Achim Claus, Reinhold Carle, and Andreas Schieber. 2004. “Polyphenol Screening of Pomace from Red and White Grape Varieties (Vitis Vinifera L.) by HPLC-DAD-MS/MS.” *Journal of Agricultural and Food Chemistry* 52 (14): 4360–67. https://doi.org/10.1021/jf049613b.

Kang, Shimin, Jinxia Fu, and Gang Zhang. 2018. “From Lignocellulosic Biomass to Levulinic Acid: A Review on Acid-Catalyzed Hydrolysis.” *Renewable and Sustainable Energy Reviews* 94 (March 2017): 340–62. https://doi.org/10.1016/j.rser.2018.06.016.

Karandinos, George, and Philippe Bourgois. 2019. “The Structural Violence of Hyperincarceration — A 44-Year-Old Man with Back Pain.” *New England Journal of Medicine* 380 (3): 205–9. https://doi.org/10.1056/nejmp1811542.

Kataria, Rashmi, Trevor Woods, William Casey, Federico Cerrone, Reeta Davis, Kevin O’Connor, Rohit Ruhal, and Ramesh Babu. 2018. “Surfactant-Mediated Hydrothermal Pretreatment of Ryegrass Followed by Enzymatic Saccharification for Polyhydroxyalkanoate Production.” *Industrial Crops and Products* 111 (November 2017): 625–32. https://doi.org/10.1016/j.indcrop.2017.11.029.

Kaur, G., and I. Roy. 2015. “Lipopolystrategies for Large-Scale Production of Polyhydroxyalkanoates.” *Chemical and Biochemical Engineering Quarterly* 29 (2): 157–72. https://doi.org/10.15255/CABEQ.2014.2255.

Keshavarz, Tajalli, and Ipsita Roy. 2010. “Polyhydroxyalkanoates: Bioplastics with a Green Agenda.” *Current Opinion in Microbiology* 13 (3): 321–26. https://doi.org/10.1016/j.mib.2010.02.006.

Khanna, Shilpi, and Ashok K. Srivastava. 2005. “Recent Advances in Microbial Polyhydroxyalkanoates.” *Process Biochemistry* 40 (2): 607–19. https://doi.org/10.1016/j.procbio.2004.01.053.

Khosravi-Darani, Kianoush, Zahra Beigom Mokhtari, Tomohito Amai, and Kenji Tanaka. 2013. “Microbial Production of Poly(Hydroxybutyrate) from C1 Carbon Sources.” *Applied Microbiology and Biotechnology* 97 (4): 1407–24.

https://doi.org/10.1007/s00253-012-4649-0.

Kim, Seo Yeong, Yung Hun Yang, and Kwon Young Choi. 2020. "Bioconversion of Plant Hydrolysate Biomass into Biofuels Using an Engineered Bacillus Subtilis and Escherichia Coli Mixed-Whole Cell Biotransformation." *Biotechnology and Bioprocess Engineering* 25 (3): 477–84. https://doi.org/10.1007/s12257-019-0487-6.

Koller, Martin, R. Bona, C. Hermann, P. Horvat, J. Martinz, J. Neto, L. Pereira, P. Varila, and G. Braunegg. 2005. "Biotechnological Production of Poly(3-Hydroxybutyrate) with Wautersia Eutropha by Application of Green Grass Juice and Silage Juice as Additional Complex Substrates." *Biocatalysis and Biotransformation* 23 (5): 329–37. https://doi.org/10.1080/10242420500292252.

Koller, Martin, and Gerhart Braunegg. 2015. "Potential and Prospects of Continuous Polyhydroxyalkanoate (PHA) Production." *Bioengineering* 2 (2): 94–121. https://doi.org/10.3390/bioengineering2020094.

———. 2018. "Advanced Approaches to Produce Polyhydroxyalkanoate (PHA) Biopolyesters in a Sustainable and Economic Fashion." *The EuroBiotech Journal* 2 (2): 89–103. https://doi.org/10.2478/ebtj-2018-0013.

Koller, Martin, Ilona Gasser, Florian Schmid, and Gabriele Berg. 2011. "Linking Ecology with Economy: Insights into Polyhydroxyalkanoate-Producing Microorganisms." *Engineering in Life Sciences* 11 (3): 222–37. https://doi.org/10.1002/elsc.201000190.

Koller, Martin, Lukáš Maršálek, Miguel Miranda de Sousa Dias, and Gerhart Braunegg. 2017. "Producing Microbial Polyhydroxyalkanoate (PHA) Biopolyesters in a Sustainable Manner." *New Biotechnology* 37: 24–38. https://doi.org/10.1016/j.nbt.2016.05.001.

Kontogiannopoulos, Konstantinos N., Sotiris I. Patsios, and Anastasios J. Karabelas. 2016. "Tartaric Acid Recovery from Winery Lees Using Cation Exchange Resin: Optimization by Response Surface Methodology." *Separation and Purification Technology* 165: 32–41. https://doi.org/10.1016/j.seppur.2016.03.040.

Kosseva, Maria R., and Edy Rusbandi. 2018. "Trends in the Biomanufacture of Polyhydroxyalkanoates with Focus on Downstream Processing." *International Journal of Biological Macromolecules* 107 (PartA): 762–78. https://doi.org/10.1016/j.ijbiomac.2017.09.054.

Kourmentza, C., and M. Kornaros. 2016. "Biotransformation of Volatile Fatty Acids to

Polyhydroxyalkanoates by Employing Mixed Microbial Consortia: The Effect of PH and Carbon Source." *Bioresource Technology* 222: 388–98. https://doi.org/10.1016/j.biortech.2016.10.014.

Kourmentza, Constantina, Jersson Plácido, Nikolaos Venetsaneas, Anna Burniol-Figols, Cristiano Varrone, Hariklia N. Gavala, and Maria A.M. Reis. 2017. "Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production." *Bioengineering* 4 (2): 1–43. https://doi.org/10.3390/bioengineering4020055.

Kovalcik, Adriana, Iva Pernicova, Stanislav Obruca, Martin Szotkowski, Vojtech Enev, Michal Kalina, and Ivana Marova. 2020. "Grape Winery Waste as a Promising Feedstock for the Production of Polyhydroxyalkanoates and Other Value-Added Products." *Food and Bioproducts Processing* 124: 1–10. https://doi.org/10.1016/j.fbp.2020.08.003.

Kumar, Manish, Rashmi Rathour, Rashmi Singh, Yuqing Sun, Ashok Pandey, Edgard Gnansounou, Kun Yi Andrew Lin, Daniel C.W. Tsang, and Indu Shekhar Thakur. 2020. "Bacterial Polyhydroxyalkanoates: Opportunities, Challenges, and Prospects." *Journal of Cleaner Production* 263: 121500. https://doi.org/10.1016/j.jclepro.2020.121500.

Kyzas, George Z., Maria P. Symeonidou, and Kostas A. Matis. 2016. "Technologies of Winery Wastewater Treatment: A Critical Approach." *Desalination and Water Treatment* 57 (8): 3372–86. https://doi.org/10.1080/19443994.2014.986535.

la Rosa, Laura A. de, Jesús Omar Moreno-Escamilla, Joaquín Rodrigo-García, and Emilio Alvarez-Parrilla. 2018. *Phenolic Compounds*. *Postharvest Physiology and Biochemistry of Fruits and Vegetables*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-813278-4.00012-9.

Laycock, Bronwyn, Peter Halley, Steven Pratt, Alan Werker, and Paul Lant. 2013. "The Chemomechanical Properties of Microbial Polyhydroxyalkanoates." *Progress in Polymer Science* 38 (3–4): 536–83. https://doi.org/10.1016/j.progpolymsci.2012.06.003.

Lebreton, Laurent C.M., Joost Van Der Zwet, Jan Willem Damsteeg, Boyan Slat, Anthony Andrady, and Julia Reisser. 2017. "River Plastic Emissions to the World's Oceans." *Nature Communications* 8: 1–10. https://doi.org/10.1038/ncomms15611.

Lenihan, P., A. Orozco, E. O'Neill, M. N.M. Ahmad, D. W. Rooney, and G. M. Walker. 2010. "Dilute Acid Hydrolysis of Lignocellulosic Biomass." *Chemical Engineering Journal* 156 (2): 395–403. https://doi.org/10.1016/j.cej.2009.10.061.

Li, Mengxing, Nuwan K. Wijewardane, Yufeng Ge, Zheng Xu, and Mark R. Wilkins. 2020. "Visible/near Infrared Spectroscopy and Machine Learning for Predicting Polyhydroxybutyrate Production Cultured on Alkaline Pretreated Liquor from Corn Stover." *Bioresource Technology Reports* 9 (January): 100386. https://doi.org/10.1016/j.biteb.2020.100386.

Li, Mengxing, and Mark R. Wilkins. 2020. "Recent Advances in Polyhydroxyalkanoate Production: Feedstocks, Strains and Process Developments." *International Journal of Biological Macromolecules* 156: 691–703. https://doi.org/10.1016/j.ijbiomac.2020.04.082.

Li, Min, and Alvin C.K. Lai. 2015. "Review of Analytical Models for Heat Transfer by Vertical Ground Heat Exchangers (GHEs): A Perspective of Time and Space Scales." *Applied Energy* 151: 178–91. https://doi.org/10.1016/j.apenergy.2015.04.070.

Lokko, Yvonne, Marc Heijde, Karl Schebesta, Philippe Scholtès, Marc Van Montagu, and Mauro Giacca. 2018. "Biotechnology and the Bioeconomy—Towards Inclusive and Sustainable Industrial Development." *New Biotechnology* 40: 5–10. https://doi.org/10.1016/j.nbt.2017.06.005.

López, Nancy I., M. Julia Pettinari, Pablo I. Nikel, and Beatriz S. Méndez. 2015. "Polyhydroxyalkanoates: Much More than Biodegradable Plastics." *Advances in Applied Microbiology* 93: 73–106. https://doi.org/10.1016/bs.aambs.2015.06.001.

Lucas, Marco S., José A. Peres, Bing Yan Lan, and Gianluca Li Puma. 2009. "Ozonation Kinetics of Winery Wastewater in a Pilot-Scale Bubble Column Reactor." *Water Research* 43 (6): 1523–32. https://doi.org/10.1016/j.watres.2008.12.036.

Luzi, Francesca, Luigi Torre, José Maria Kenny, and Debora Puglia. 2019. "Bio- and Fossil-Based Polymeric Blends and Nanocomposites for Packaging: Structure-Property Relationship." *Materials* 12 (3). https://doi.org/10.3390/ma12030471.

Mak, Tiffany M.W., Xinni Xiong, Daniel C.W. Tsang, Iris K.M. Yu, and Chi Sun Poon. 2020. "Sustainable Food Waste Management towards Circular Bioeconomy: Policy Review, Limitations and Opportunities." *Bioresource Technology* 297: 122497. https://doi.org/10.1016/j.biortech.2019.122497.

Marichelvam, M. K., Mohammad Jawaid, and Mohammad Asim. 2019. “Corn and Rice Starch-Based Bio-Plastics as Alternative Packaging Materials.” *Fibers* 7 (4): 1–14. https://doi.org/10.3390/fib7040032.

Martinez, Gonzalo A., Stefano Rebecchi, Deborha Decorti, Joana M.B. Domingos, Andrea Natolino, Daniele Del Rio, Lorenzo Bertin, Carla Da Porto, and Fabio Fava. 2016. “Towards Multi-Purpose Biorefinery Platforms for the Valorisation of Red Grape Pomace: Production of Polyphenols, Volatile Fatty Acids, Polyhydroxyalkanoates and Biogas.” *Green Chemistry* 18 (1): 261–70. https://doi.org/10.1039/c5gc01558h.

Martino, Lucrezia, Madalena V. Cruz, Alberto Scoma, Filomena Freitas, Lorenzo Bertin, Mariastella Scandola, and Maria A.M. Reis. 2014. “Recovery of Amorphous Polyhydroxybutyrate Granules from Cupriavidus Necator Cells Grown on Used Cooking Oil.” *International Journal of Biological Macromolecules* 71: 117–23. https://doi.org/10.1016/j.ijbiomac.2014.04.016.

Mendes, Joana A.S., Ana M.R.B. Xavier, Dmitry V. Evtuguin, and Luísa P.C. Lopes. 2013. “Integrated Utilization of Grape Skins from White Grape Pomaces.” *Industrial Crops and Products* 49: 286–91. https://doi.org/10.1016/j.indcrop.2013.05.003.

Mohd Yatim, Ain Farhana, Ishak Muhammad Syafiq, Kai Hee Huong, Al Ashraf Abdullah Amirul, Abdul Wahid Mohd Effendy, and Kesaven Bhubalan. 2017. “Bioconversion of Novel and Renewable Agro-Industry by-Products into a Biodegradable Poly(3-Hydroxybutyrate) by Marine Bacillus Megaterium UMTKB-1 Strain.” *Biotechnologia* 98 (2): 141–51. https://doi.org/10.5114/bta.2017.68313.

Moita, R., and P. C. Lemos. 2012. “Biopolymers Production from Mixed Cultures and Pyrolysis By-Products.” *Journal of Biotechnology* 157 (4): 578–83. https://doi.org/10.1016/j.jbiotec.2011.09.021.

Morais, Cristiana, Filomena Freitas, Madalena V. Cruz, Alexandre Paiva, Madalena Dionísio, and Maria A.M. Reis. 2014. “Conversion of Fat-Containing Waste from the Margarine Manufacturing Process into Bacterial Polyhydroxyalkanoates.” *International Journal of Biological Macromolecules* 71: 68–73. https://doi.org/10.1016/j.ijbiomac.2014.04.044.

Mothes, G., C. Schnorpfeil, and J. U. Ackermann. 2007. “Production of PHB from Crude Glycerol.” *Engineering in Life Sciences* 7 (5): 475–79. https://doi.org/10.1002/elsc.200620210.

Mozejko-Ciesielska, Justyna, Karolina Szacherska, and Paulina Marciniak. 2019. "Pseudomonas Species as Producers of Eco-Friendly Polyhydroxyalkanoates." *Journal of Polymers and the Environment* 0 (0): 0. https://doi.org/10.1007/s10924-019-01422-1.

Nguyen, Quynh Anh, Eun Jin Cho, Dae Seok Lee, and Hyeun Jong Bae. 2019. "Development of an Advanced Integrative Process to Create Valuable Biosugars Including Manno-Oligosaccharides and Mannose from Spent Coffee Grounds." *Bioresource Technology* 272: 209–16. https://doi.org/10.1016/j.biortech.2018.10.018.

Obruca, S., P. Benesova, L. Marsalek, and I. Marova. 2015. "Use of Lignocellulosic Materials for PHA Production." *Chemical and Biochemical Engineering Quarterly* 29 (2): 135–44. https://doi.org/10.15255/CABEQ.2014.2253.

Obruca, Stanislav, Ivana Marova, Ondrej Snajdar, Ludmila Mravcova, and Zdenek Svoboda. 2010. "Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) by Cupriavidus Necator from Waste Rapeseed Oil Using Propanol as a Precursor of 3-Hydroxyvalerate." *Biotechnology Letters* 32 (12): 1925–32. https://doi.org/10.1007/s10529-010-0376-8.

Obruca, Stanislav, Sinisa Petrik, Pavla Benesova, Zdenek Svoboda, Libor Eremka, and Ivana Marova. 2014. "Utilization of Oil Extracted from Spent Coffee Grounds for Sustainable Production of Polyhydroxyalkanoates." *Applied Microbiology and Biotechnology* 98 (13): 5883–90. https://doi.org/10.1007/s00253-014-5653-3.

OECD. 2011. "Industrial Biotechnology and Climate Change." *Oecd*, no. January: 1–38. www.oecd.org/sti/biotechnology%0Ahttps://www.oecd.org/sti/biotech/49024032.pdf.

Østergaard, Poul Alberg. 2012. "Comparing Electricity, Heat and Biogas Storages' Impacts on Renewable Energy Integration." *Energy* 37 (1): 255–62. https://doi.org/10.1016/j.energy.2011.11.039.

Papadaki, Eugenia, and Fani Th Mantzouridou. 2019. "Citric Acid Production from the Integration of Spanish-Style Green Olive Processing Wastewaters with White Grape Pomace by Aspergillus Niger." *Bioresource Technology* 280 (November 2018): 59–69. https://doi.org/10.1016/j.biortech.2019.01.139.

Philip, S.; Keshavarz, T.; Roy, I. 2007. "Polyhydroxyalkanoates: Biodegradable Polymers with a Range of Applications." *Journal of Chemical Technology & Biotechnology* 82 (May): 233–47. https://doi.org/10.1002/jctb.

Philp, Jim C., Alexandre Bartsev, Rachael J. Ritchie, Marie Ange Baucher, and K. Guy. 2013. “Bioplastics Science from a Policy Vantage Point.” *New Biotechnology* 30 (6): 635–46. https://doi.org/10.1016/j.nbt.2012.11.021.

Pittmann, T., and H. Steinmetz. 2016. “Potential for Polyhydroxyalkanoate Production on German or European Municipal Waste Water Treatment Plants.” *Bioresource Technology* 214: 9–15. https://doi.org/10.1016/j.biortech.2016.04.074.

Poli, Annarita, Paola Di Donato, Gennaro Roberto Abbamondi, and Barbara Nicolaus. 2011. “Synthesis, Production, and Biotechnological Applications of Exopolysaccharides and Polyhydroxyalkanoates by Archaea.” *Archaea* 2011. https://doi.org/10.1155/2011/693253.

Pradhan, Sushobhan, Arup Jyoti Borah, Maneesh Kumar Poddar, Pritam Kumar Dikshit, Lilendar Rohidas, and Vijayanand S. Moholkar. 2017. “Microbial Production, Ultrasound-Assisted Extraction and Characterization of Biopolymer Polyhydroxybutyrate (PHB) from Terrestrial (P. Hysterophorus) and Aquatic (E. Crassipes) Invasive Weeds.” *Bioresource Technology* 242: 304–10. https://doi.org/10.1016/j.biortech.2017.03.117.

Pullman, Madeleine E., Michael J. Maloni, and Jesse Dillard. 2010. “Sustainability Practices in Food Supply Chains: How Is Wine Different?” *Journal of Wine Research* 21 (1): 35–56. https://doi.org/10.1080/09571264.2010.495853.

Rai, R., T. Keshavarz, J. A. Roether, A. R. Boccaccini, and I. Roy. 2011. “Medium Chain Length Polyhydroxyalkanoates, Promising New Biomedical Materials for the Future.” *Materials Science and Engineering R: Reports* 72 (3): 29–47. https://doi.org/10.1016/j.mser.2010.11.002.

Ravindran, Rajeev, and Amit K. Jaiswal. 2016. “Exploitation of Food Industry Waste for High-Value Products.” *Trends in Biotechnology* 34 (1): 58–69. https://doi.org/10.1016/j.tibtech.2015.10.008.

Ray, Subhasree, and Vipin Chandra Kalia. 2017. “Biomedical Applications of Polyhydroxyalkanoates.” *Indian Journal of Microbiology* 57 (3): 261–69. https://doi.org/10.1007/s12088-017-0651-7.

Raza, Zulfiqar Ali, Sharjeel Abid, and Ibrahim M. Banat. 2018. “Polyhydroxyalkanoates: Characteristics, Production, Recent Developments and Applications.” *International Biodeterioration and Biodegradation* 126 (January 2017): 45–56.

https://doi.org/10.1016/j.ibiod.2017.10.001.

Raza, Zulfiqar Ali, Muhammad Rizwan Tariq, Muhammad Irfan Majeed, and Ibrahim M. Banat. 2019. "Recent Developments in Bioreactor Scale Production of Bacterial Polyhydroxyalkanoates." *Bioprocess and Biosystems Engineering* 42 (6): 901–19. https://doi.org/10.1007/s00449-019-02093-x.

Reddy, C. S.K., R. Ghai, Rashmi, and V. C. Kalia. 2003. "Polyhydroxyalkanoates: An Overview." *Bioresource Technology* 87 (2): 137–46. https://doi.org/10.1016/S0960-8524(02)00212-2.

Riedel, Sebastian L., Stefan Jahns, Steven Koenig, Martina C.E. Bock, Christopher J. Brigham, Johannes Bader, and Ulf Stahl. 2015. "Polyhydroxyalkanoates Production with Ralstonia Eutropha from Low Quality Waste Animal Fats." *Journal of Biotechnology* 214: 119–27. https://doi.org/10.1016/j.jbiotec.2015.09.002.

Rivera, Oscar Manuel Portilla, Ana Belén Moldes, Ana María Torrado, and José Manuel Domínguez. 2007. "Lactic Acid and Biosurfactants Production from Hydrolyzed Distilled Grape Marc." *Process Biochemistry* 42 (6): 1010–20. https://doi.org/10.1016/j.procbio.2007.03.011.

Rodríguez-Pazo, Noelia, José Manuel Salgado, Sandra Cortés-Diéguez, and José Manuel Domínguez. 2013. "Biotechnological Production of Phenyllactic Acid and Biosurfactants from Trimming Vine Shoot Hydrolyzates by Microbial Coculture Fermentation." *Applied Biochemistry and Biotechnology* 169 (7): 2175–88. https://doi.org/10.1007/s12010-013-0126-1.

Rončević, Zorana, Jovana Grahovac, Siniša Dodić, Damjan Vučurović, and Jelena Dodić. 2019. "Utilisation of Winery Wastewater for Xanthan Production in Stirred Tank Bioreactor: Bioprocess Modelling and Optimisation." *Food and Bioproducts Processing* 117: 113–25. https://doi.org/10.1016/j.fbp.2019.06.019.

Ros, C. Da, C. Cavinato, D. Bolzonella, and P. Pavan. 2016. "Renewable Energy from Thermophilic Anaerobic Digestion of Winery Residue: Preliminary Evidence from Batch and Continuous Lab-Scale Trials." *Biomass and Bioenergy* 91: 150–59. https://doi.org/10.1016/j.biombioe.2016.05.017.

Ros, C. Da, C. Cavinato, F. Cecchi, and D. Bolzonella. 2014. "Anaerobic Co-Digestion of Winery Waste and Waste Activated Sludge: Assessment of Process Feasibility." *Water Science and Technology* 69 (2): 269–77. https://doi.org/10.2166/wst.2013.692.

Rosengart, Alessandro, M. Teresa Cesário, M. Catarina M.D. de Almeida, Rodrigo S. Raposo, Ana Espert, Elena Díaz de Apodaca, and M. Manuela R. da Fonseca. 2015. "Efficient P(3HB) Extraction from Burkholderia Sacchari Cells Using Non-Chlorinated Solvents." *Biochemical Engineering Journal* 103: 39–46. https://doi.org/10.1016/j.bej.2015.06.013.

Ruggieri, Luz, Erasmo Cadena, Julia Martínez-Blanco, Carles M. Gasol, Joan Rieradevall, Xavier Gabarrell, Teresa Gea, Xavier Sort, and Antoni Sánchez. 2009. "Recovery of Organic Wastes in the Spanish Wine Industry. Technical, Economic and Environmental Analyses of the Composting Process." *Journal of Cleaner Production* 17 (9): 830–38. https://doi.org/10.1016/j.jclepro.2008.12.005.

Rusendi, D., and John D. Sheppard. 1995. "Hydrolysis of Potato Processing Waste for the Production of Poly-β-Hydroxybutyrate." *Bioresource Technology* 54 (2): 191–96. https://doi.org/10.1016/0960-8524(95)00124-7.

Sabapathy, Poorna Chandrika, Sabarinathan Devaraj, Katharina Meixner, Parthiban Anburajan, Preethi Kathirvel, Yuvaraj Ravikumar, Hossain M. Zabed, and Xianghui Qi. 2020. "Recent Developments in Polyhydroxyalkanoates (PHAs) Production – A Review." *Bioresource Technology* 306 (February): 123132. https://doi.org/10.1016/j.biortech.2020.123132.

Salgado, José Manuel, Luís Abrunhosa, Armando Venâncio, José Manuel Domínguez, and Isabel Belo. 2016. "Combined Bioremediation and Enzyme Production by Aspergillus Sp. in Olive Mill and Winery Wastewaters." *International Biodeterioration and Biodegradation* 110: 16–23. https://doi.org/10.1016/j.ibiod.2015.12.011.

Salvachúa, Davinia, Eric M. Karp, Claire T. Nimlos, Derek R. Vardon, and Gregg T. Beckham. 2015. "Towards Lignin Consolidated Bioprocessing: Simultaneous Lignin Depolymerization and Product Generation by Bacteria." *Green Chemistry* 17 (11): 4951–67. https://doi.org/10.1039/c5gc01165e.

Sathiyanarayanan, Ganesan, Shashi Kant Bhatia, Hun Suk Song, Jong Min Jeon, Junyoung Kim, Yoo Kyung Lee, Yun Gon Kim, and Yung Hun Yang. 2017. "Production and Characterization of Medium-Chain-Length Polyhydroxyalkanoate Copolymer from Arctic Psychrotrophic Bacterium Pseudomonas Sp. PAMC 28620." *International Journal of Biological Macromolecules* 97: 710–20. https://doi.org/10.1016/j.ijbiomac.2017.01.053.

Sato, Shunsuke, Tetsuya Fujiki, and Keiji Matsumoto. 2013. “Construction of a Stable Plasmid Vector for Industrial Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyhexanoate) by a Recombinant Cupriavidusnecator H16 Strain.” *Journal of Bioscience and Bioengineering* 116 (6): 677–81. https://doi.org/10.1016/j.jbiosc.2013.05.026.

Simon-Colin, C., K. Alain, G. Raguénès, S. Schmitt, N. Kervarec, C. Gouin, P. Crassous, B. Costa, and J. G. Guezennec. 2009. “Biosynthesis of Medium Chain Length Poly(3-Hydroxyalkanoates) (Mcl PHAs) from Cosmetic Co-Products by Pseudomonas Raguenesii Sp. Nov., Isolated from Tetiaroa, French Polynesia.” *Bioresource Technology* 100 (23): 6033–39. https://doi.org/10.1016/j.biortech.2009.06.075.

Simon-Colin, Christelle, Gérard Raguénès, Bernard Costa, and Jean Guezennec. 2008. “Biosynthesis of Medium Chain Length Poly-3-Hydroxyalkanoates by Pseudomonas Guezennei from Various Carbon Sources.” *Reactive and Functional Polymers* 68 (11): 1534–41. https://doi.org/10.1016/j.reactfunctpolym.2008.08.005.

Sindhu, Raveendran, Nagapoosam Silviya, Parameswaran Binod, and Ashok Pandey. 2013. “Pentose-Rich Hydrolysate from Acid Pretreated Rice Straw as a Carbon Source for the Production of Poly-3-Hydroxybutyrate.” *Biochemical Engineering Journal* 78: 67–72. https://doi.org/10.1016/j.bej.2012.12.015.

Sirohi, Ranjna, Ayon Tarafdar, Shikhangi Singh, Taru Negi, Vivek Kumar Gaur, Edgard Gnansounou, and B. Bharathiraja. 2020. “Green Processing and Biotechnological Potential of Grape Pomace: Current Trends and Opportunities for Sustainable Biorefinery.” *Bioresource Technology* 314 (May): 123771. https://doi.org/10.1016/j.biortech.2020.123771.

Solaiman, Daniel K.Y., Richard D. Ashby, Thomas A. Foglia, and William N. Marmer. 2006. “Conversion of Agricultural Feedstock and Coproducts into Poly(Hydroxyalkanoates).” *Applied Microbiology and Biotechnology* 71 (6): 783–89. https://doi.org/10.1007/s00253-006-0451-1.

Sousa, Eldina Castro, Ana Maria Athayde Uchôa-Thomaz, José Osvaldo Beserra Carioca, Selene Maia de Morais, Alessandro de Lima, Clécio Galvão Martins, Cristiane Duarte Alexandrino, et al. 2014. “Chemical Composition and Bioactive Compounds of Grape Pomace (Vitis Vinifera L.), Benitaka Variety, Grown in the Semiarid Region of Northeast Brazil.” *Food Science and Technology* 34 (1): 135–42.

https://doi.org/10.1590/S0101-20612014000100020.

Standard Methods For the Examination of Water and Wastewater. 2018. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association. https://doi.org/doi:10.2105/SMWW.2882.

Sukruansuwan, Vibhavee, and Suchada Chanprateep Napathorn. 2018. "Use of Agro-Industrial Residue from the Canned Pineapple Industry for Polyhydroxybutyrate Production by Cupriavidus Necator Strain A-04." *Biotechnology for Biofuels* 11 (1): 1–15. https://doi.org/10.1186/s13068-018-1207-8.

Sun, Xiao, Hasan K. Atiyeh, Mengxing Li, and Yan Chen. 2020. "Biochar Facilitated Bioprocessing and Biorefinery for Productions of Biofuel and Chemicals: A Review." *Bioresource Technology* 295 (October 2019): 122252. https://doi.org/10.1016/j.biortech.2019.122252.

Taherzadeh, Mohammad J., and Keikhosro Karimi. 2008. *Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review*. *International Journal of Molecular Sciences*. Vol. 9. https://doi.org/10.3390/ijms9091621.

Tan, Giin Yu Amy, Chia Lung Chen, Ling Li, Liya Ge, Lin Wang, Indah Mutiara Ningtyas Razaad, Yanhong Li, Lei Zhao, Yu Mo, and Jing Yuan Wang. 2014. "Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review." *Polymers* 6 (3): 706–54. https://doi.org/10.3390/polym6030706.

Tufail, Saiqa, Sajida Munir, and Nazia Jamil. 2017. "Variation Analysis of Bacterial Polyhydroxyalkanoates Production Using Saturated and Unsaturated Hydrocarbons." *Brazilian Journal of Microbiology* 48 (4): 629–36. https://doi.org/10.1016/j.bjm.2017.02.008.

Varsha, Y. M., and R. Savitha. 2011. "Overview on Polyhydroxyalkanoates: A Promising Biopol." *Journal of Microbial and Biochemical Technology* 3 (5): 99–105. https://doi.org/10.4172/1948-5948.1000059.

Verlinden, Rob A.J., David J. Hill, Melvin A. Kenward, Craig D. Williams, Zofia Piotrowska-Seget, and Iza K. Radecka. 2011. "Production of Polyhydroxyalkanoates from Waste Frying Oil by Cupriavidus Necator." *AMB Express* 1 (1): 1–8. https://doi.org/10.1186/2191-0855-1-11.

Volodina, Elena, Matthias Raberg, and Alexander Steinbüchel. 2016. "Engineering the Heterotrophic Carbon Sources Utilization Range of Ralstonia Eutropha H16 for

Applications in Biotechnology." *Critical Reviews in Biotechnology* 36 (6): 978–91. https://doi.org/10.3109/07388551.2015.1079698.

Wang, Shen Yu, Zhen Wang, Ming Ming Liu, Yu Xu, Xiao Jun Zhang, and Guo Qiang Chen. 2010. "Properties of a New Gasoline Oxygenate Blend Component: 3-Hydroxybutyrate Methyl Ester Produced from Bacterial Poly-3-Hydroxybutyrate." *Biomass and Bioenergy* 34 (8): 1216–22. https://doi.org/10.1016/j.biombioe.2010.03.020.

Wang, Wei, Shihui Yang, Glendon B. Hunsinger, Philip T. Pienkos, and David K. Johnson. 2014. "Connecting Lignin-Degradation Pathway with Pre-Treatment Inhibitor Sensitivity of Cupriavidus Necator." *Frontiers in Microbiology* 5 (MAY): 1–11. https://doi.org/10.3389/fmicb.2014.00247.

Wang, Ying, Jin Yin, and Guo Qiang Chen. 2014. "Polyhydroxyalkanoates, Challenges and Opportunities." *Current Opinion in Biotechnology* 30: 59–65. https://doi.org/10.1016/j.copbio.2014.06.001.

Wittenauer, Judith, Sonja MäcKle, Daniela Sußmann, Ute Schweiggert-Weisz, and Reinhold Carle. 2015. "Inhibitory Effects of Polyphenols from Grape Pomace Extract on Collagenase and Elastase Activity." *Fitoterapia* 101: 179–87. https://doi.org/10.1016/j.fitote.2015.01.005.

Xiang, Qian, Yong Y. Lee, and Robert W. Torget. 2004. "Kinetics of Glucose Decomposition during Dilute-Acid Hydrolysis of Lignocellulosic Biomass." *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology* 115 (1–3): 1127–38. https://doi.org/10.1385/ABAB:115:1-3:1127.

Xu, Y., R. H. Wang, A. A. Koutinas, and C. Webb. 2010. "Microbial Biodegradable Plastic Production from a Wheat-Based Biorefining Strategy." *Process Biochemistry* 45 (2): 153–63. https://doi.org/10.1016/j.procbio.2009.09.001.

Yoon, S. Y., S. H. Han, and S. J. Shin. 2014. "The Effect of Hemicelluloses and Lignin on Acid Hydrolysis of Cellulose." *Energy* 77: 19–24. https://doi.org/10.1016/j.energy.2014.01.104.

Yousuf, R.G. 2017. "Novel Polyhydroxybutyrate (PHB) Production Using a Waste Date Seed Feedstock." *PhD Thesis*, 265. https://www.escholar.manchester.ac.uk/jrul/item/?pid=uk-ac-man-scw:313698.

Yu, Jian, and Heiko Stahl. 2008. "Microbial Utilization and Biopolyester Synthesis of

Bagasse Hydrolysates." *Bioresource Technology* 99 (17): 8042–48. https://doi.org/10.1016/j.biortech.2008.03.071.

Zacharof, Myrto Panagiota. 2017. "Grape Winery Waste as Feedstock for Bioconversions: Applying the Biorefinery Concept." *Waste and Biomass Valorization* 8 (4): 1011–25. https://doi.org/10.1007/s12649-016-9674-2.

Zhang, Lu, and Xiangyang Sun. 2016. "Improving Green Waste Composting by Addition of Sugarcane Bagasse and Exhausted Grape Marc." *Bioresource Technology* 218: 335–43. https://doi.org/10.1016/j.biortech.2016.06.097.

Zhang, Yi, Apiredan Wusiman, Xiang Liu, Chunli Wan, Duu Jong Lee, and Joo Hwa Tay. 2018. "Polyhydroxyalkanoates (PHA) Production from Phenol in an Acclimated Consortium: Batch Study and Impacts of Operational Conditions." *Journal of Biotechnology* 267 (December 2017): 36–44. https://doi.org/10.1016/j.jbiotec.2018.01.001.

Zheng, Yi, Christopher Lee, Chaowei Yu, Yu Shen Cheng, Christopher W. Simmons, Ruihong Zhang, Bryan M. Jenkins, and Jean S. Vandergheynst. 2012. "Ensilage and Bioconversion of Grape Pomace into Fuel Ethanol." *Journal of Agricultural and Food Chemistry* 60 (44): 11128–34. https://doi.org/10.1021/jf303509v.

CHAPTER VI – APPENDIX

A - Calibration curves for determination of cell dry weight

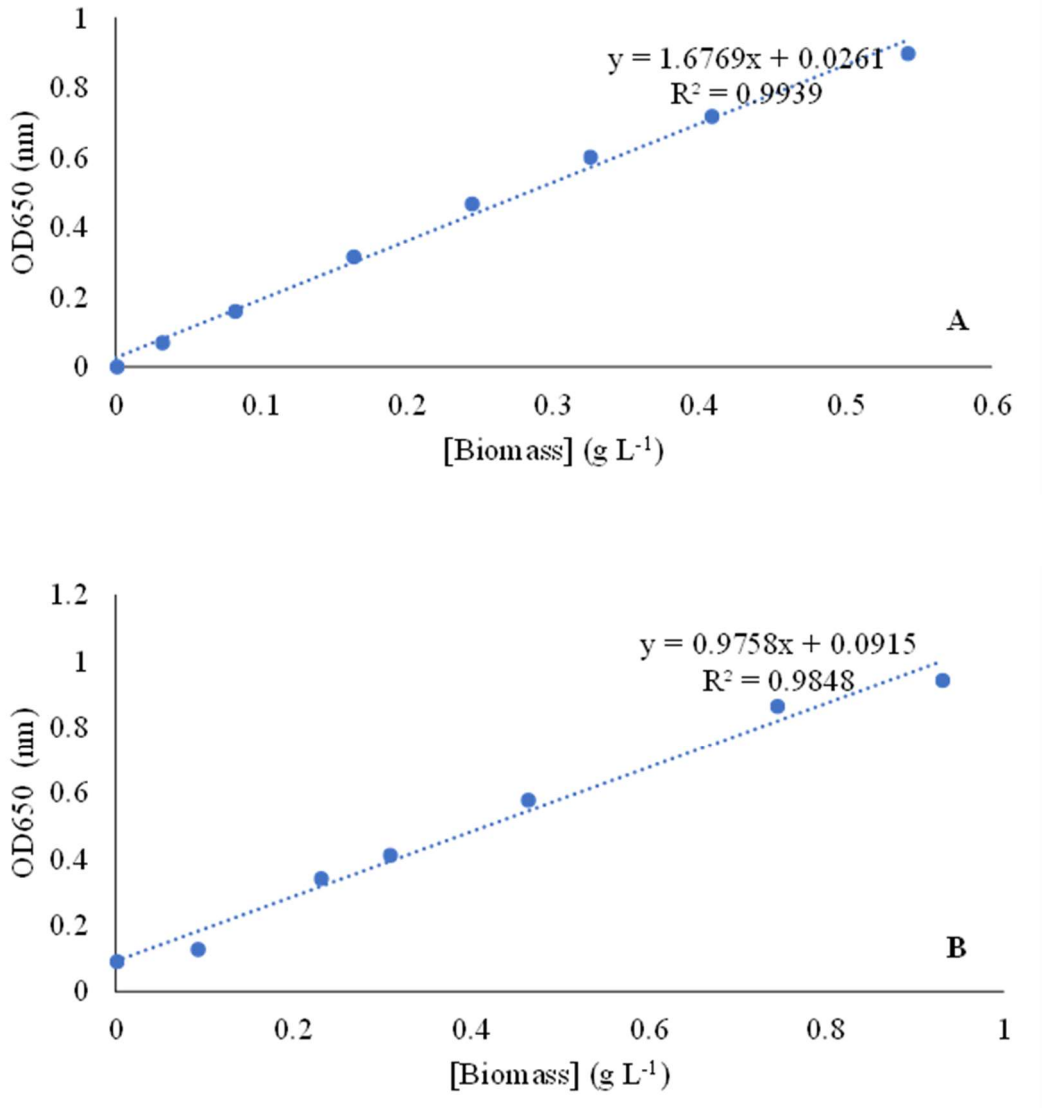


Figure 21 - Calibration curve of biomass concentration versus OD650 for C. necator in synthetic medium (A) and in hydrolysate (B).

B – Evolution of Phenolic Compounds through of the assays G, H, I, J, K, L, M, N, O, R1 and R2

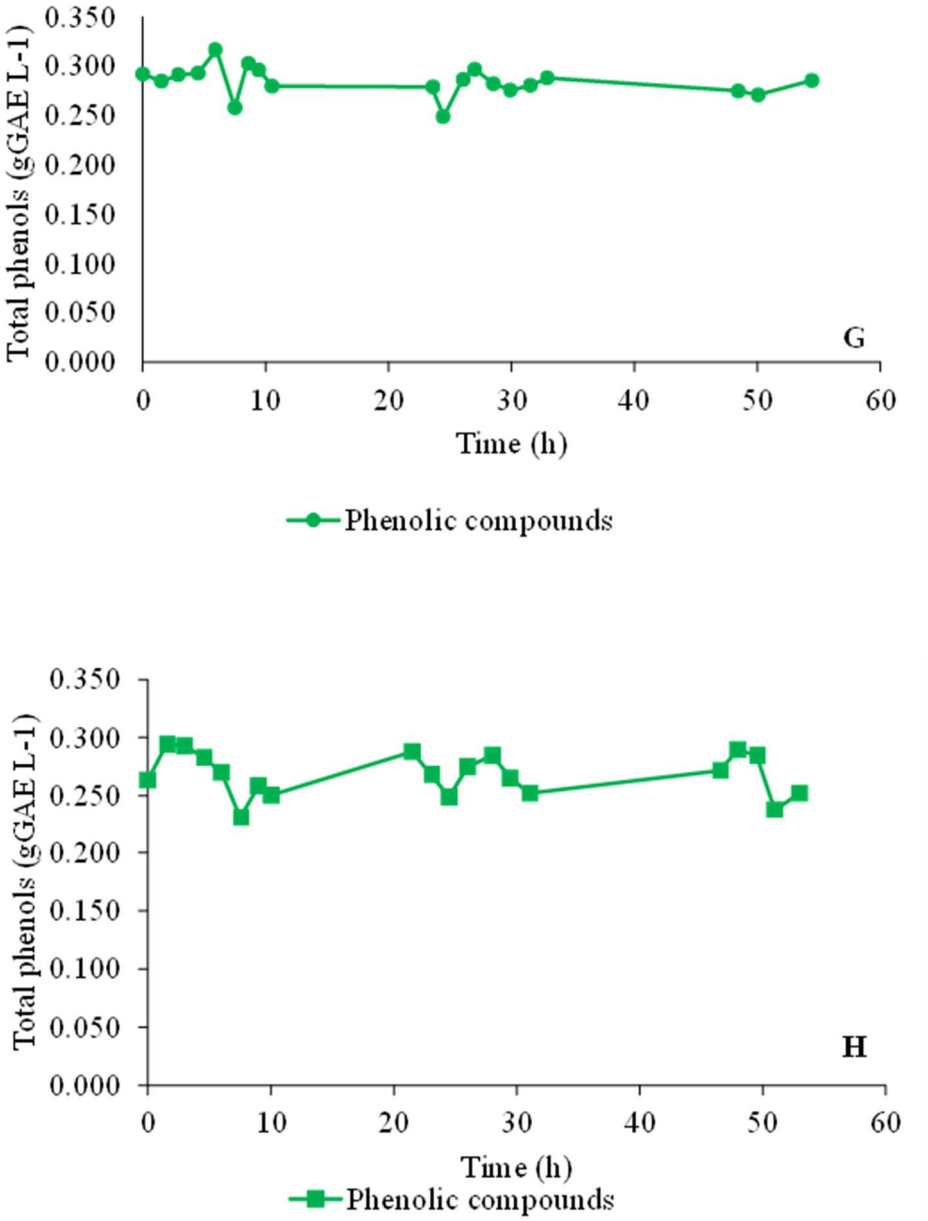


Figure 22 - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2.

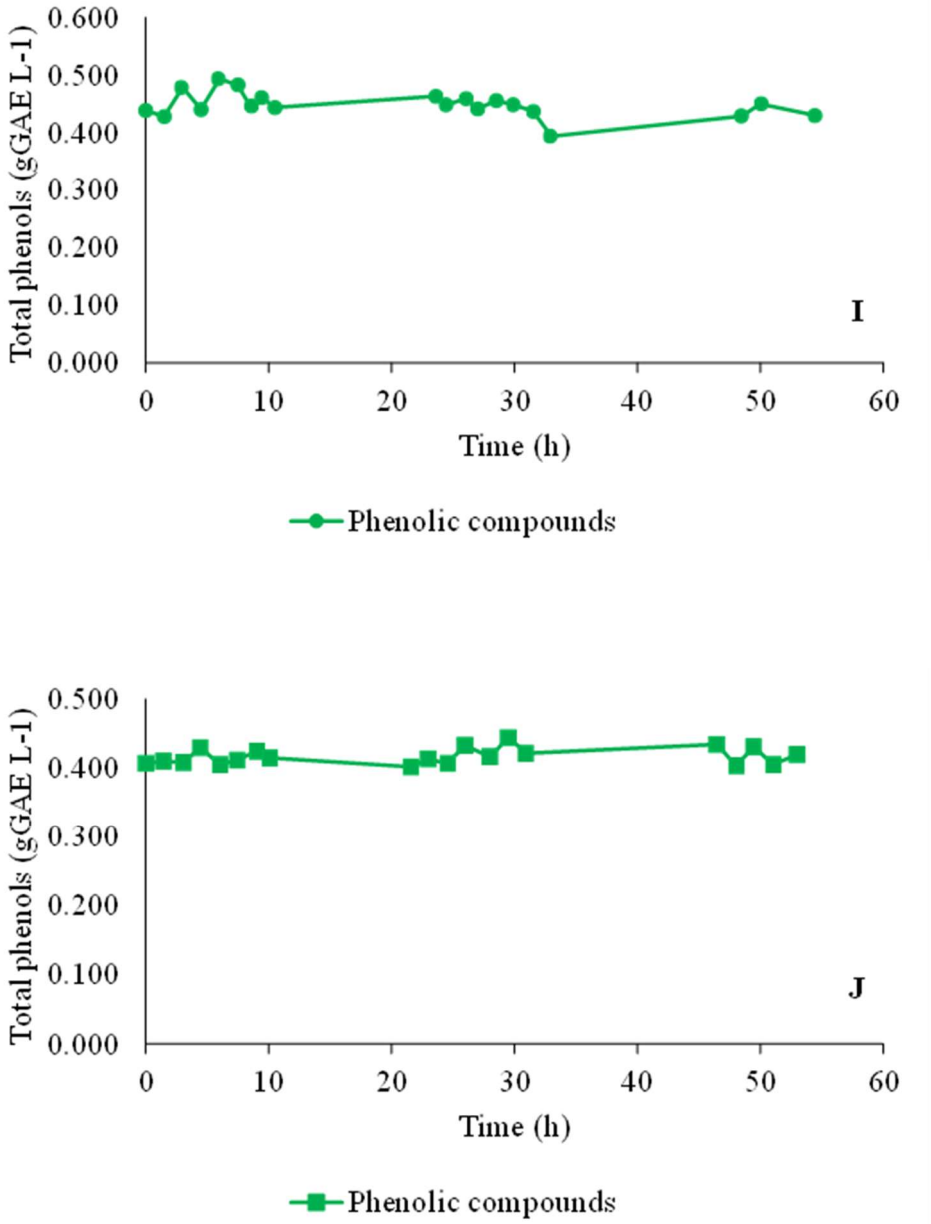
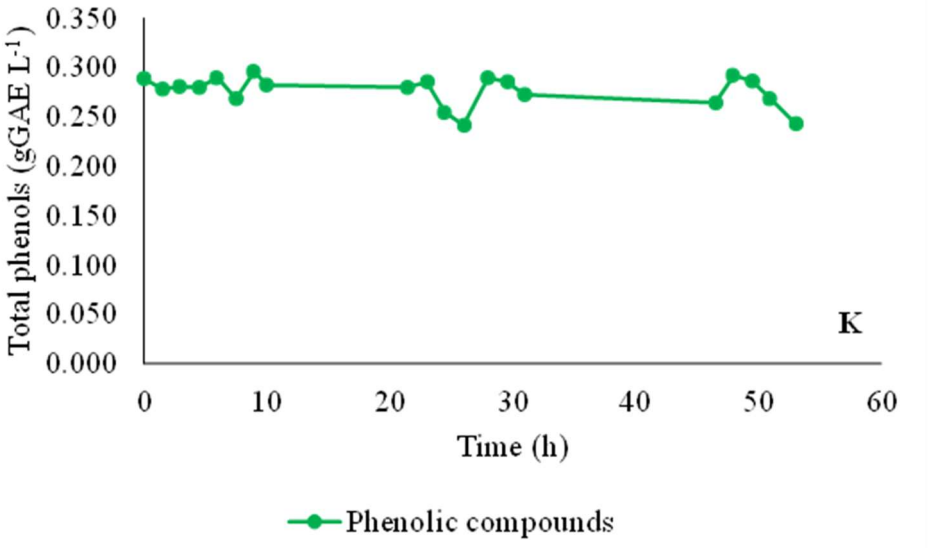


Figure 22 (continuation) - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2



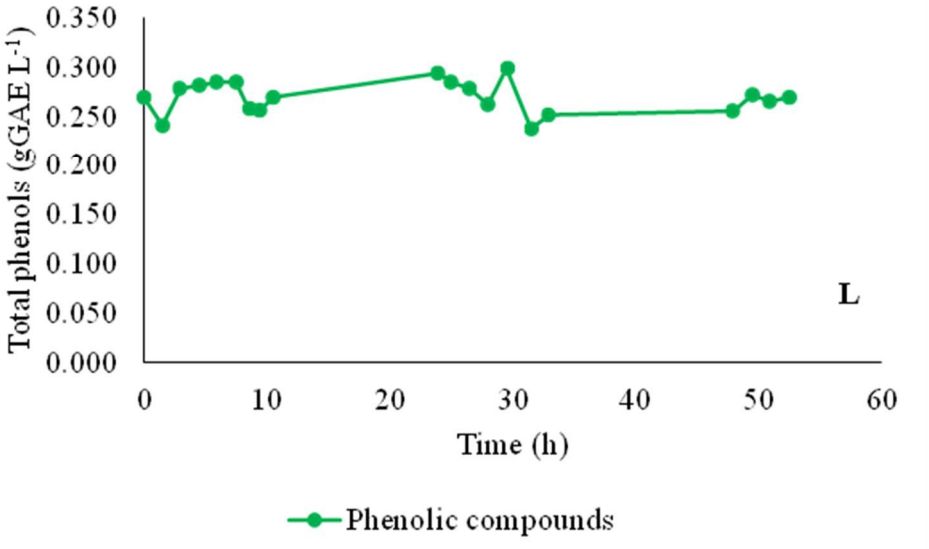


Figure 22 (continuation) - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2

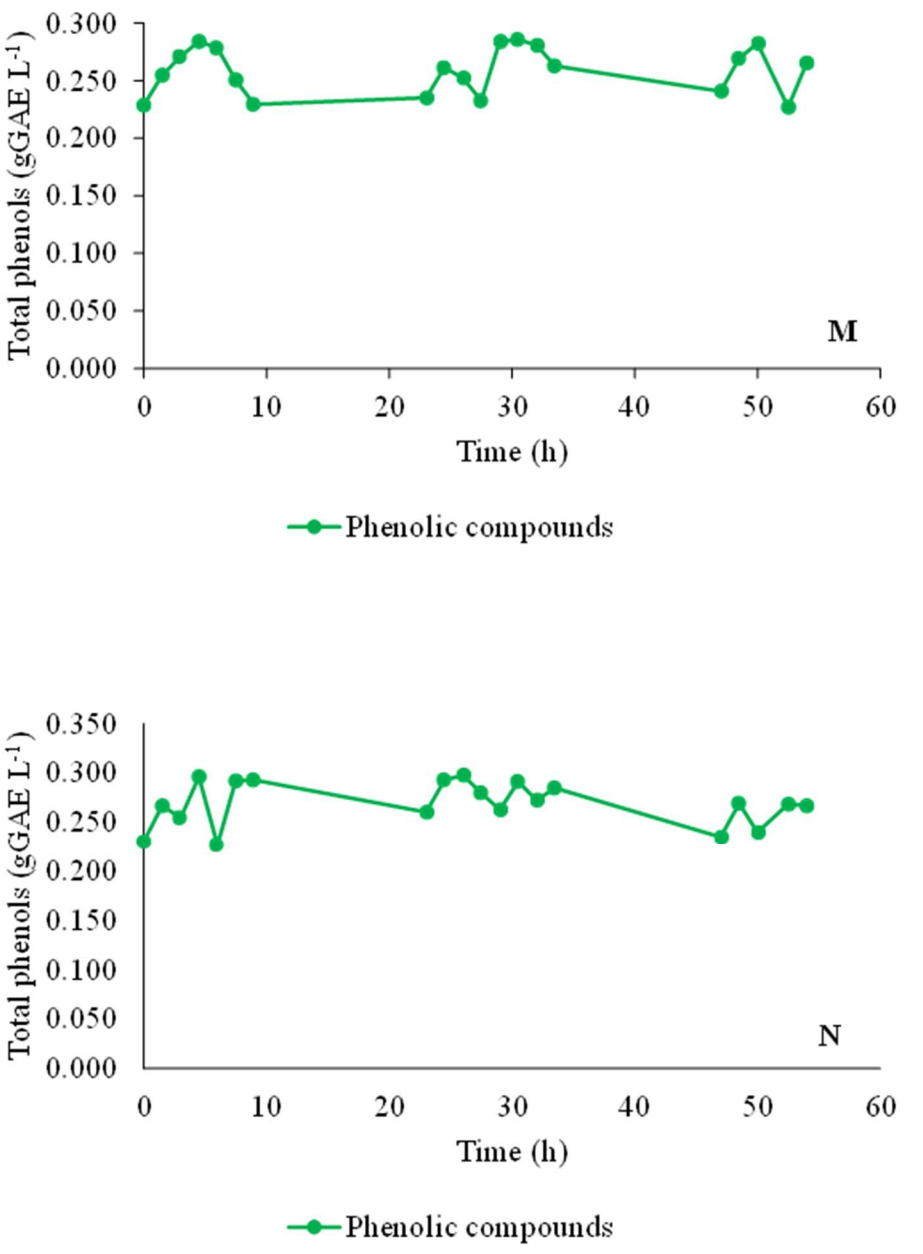
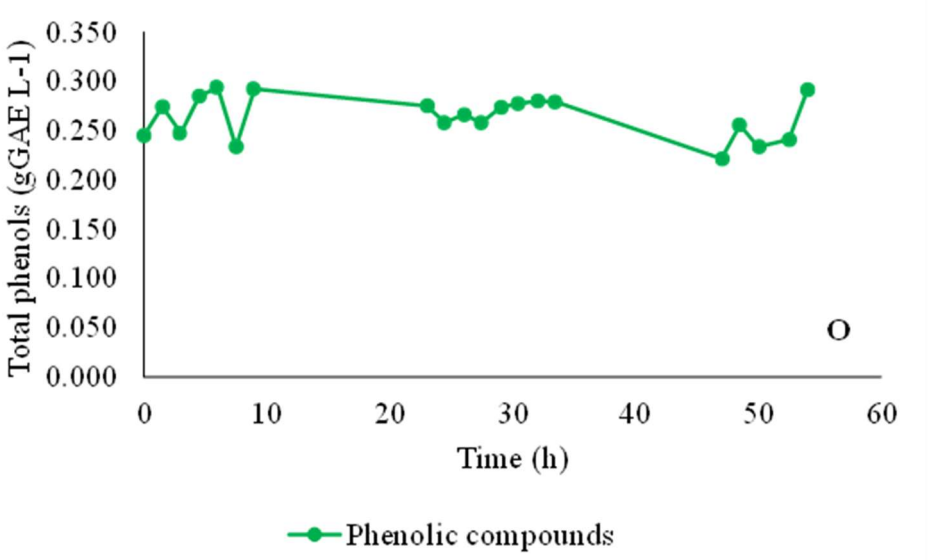


Figure 22 (continuation) - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2



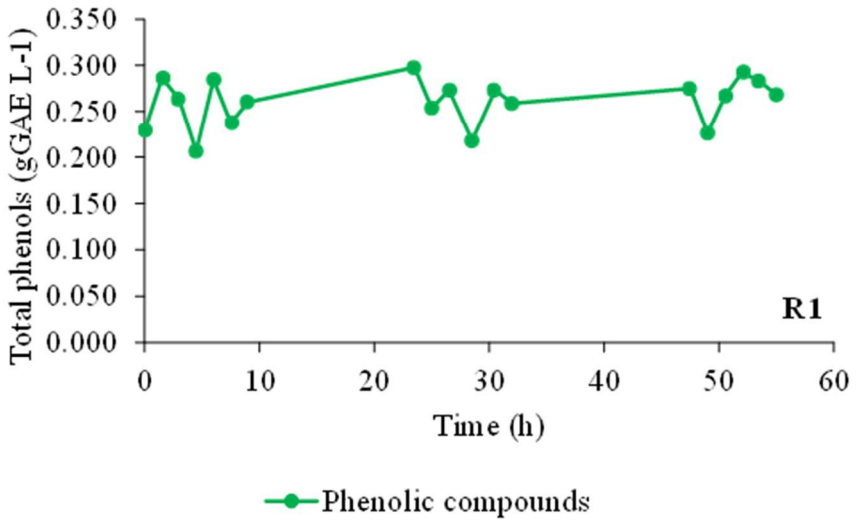


Figure 22 (continuation) - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2

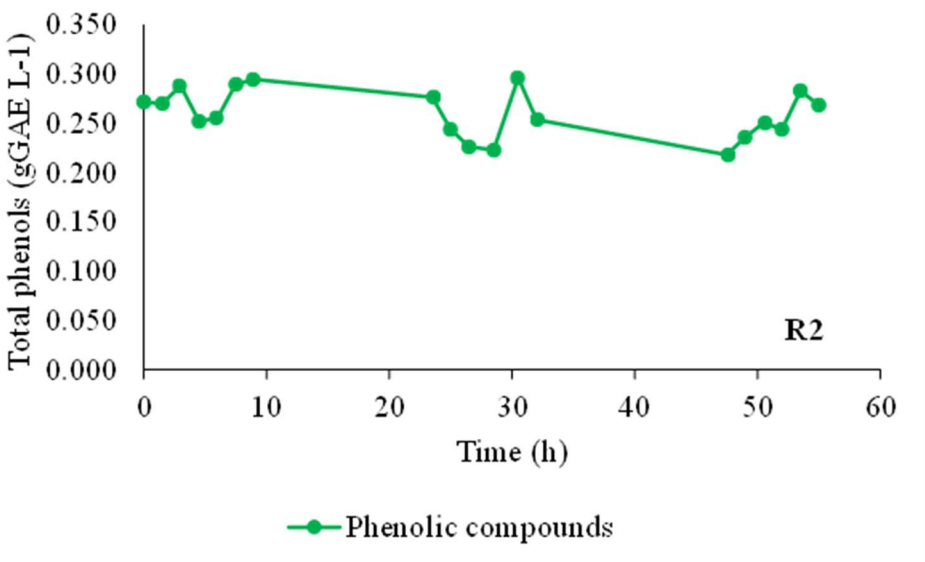


Figure 22 (continuation) - Evolution of Phenolic Compounds through of the trials G, H, I, J, K, L, M, N, O, R1 and R2