AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF FOOD PROCESS ENGINEERING

M.Sc. IN FOOD PROCESSING, PRESERVATION
& BIOTECHNOLOGICAL PROCESSES – BIO-ECONOMY PRODUCTS DEVELOPMENT

Master's thesis

Production of poly (3-hydroxybutyrate) using post-consumer biobased packaging materials

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"Παραγωγή πολυ(3-υδροξυβουτυρικού) χρησιμοποιώντας βιοδιασπώμενα υλικά συσκευασίας"

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M.Sc: Food Processing, Preservation & Biotechnological Processes –Bio-economy Products Development
Department of Food Science & Human Nutrition
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Abstract

During the last decades, the massive production of plastics has caused a major environmental problem worldwide. Their deposition is one of the biggest problems, as due to their structure and origin, their degradation in the environment can't be achieved. The threat of depleting fossil fuels and the increasing environmental awareness of the public has turned the market to "greener" alternatives derived from renewable energy sources. Biobased materials have gained a great research interest due to their biodegradable properties, thus they can be produced from renewable resources.

Bioplastics such as PLA and PHAs have gained a high interest, as their chemical and thermal properties are similar to propylene, thus their abiotic degradation is an important property in plastic waste management. The circular and sustainable production of biopolymers necessitates the development of efficient chemical and biological recycling of post-consumer bioplastics.

In this study various synthetic organic acids derived from the degradation of biopolymers, such as PLA, PHB and PBS, were used as carbon source in shake flasks and bioreactor fermentation. The bacterial strains *Paraburkholderia sacchari* DSM 17165 and *Cupriavidus necator* DSM 428 (H16) were evaluated, regarding their ability to consume these organic acids and to produce new PHB. More specifically, the organic acids evaluated were: (1) lactic acid (LA), derived after the degradation of PLA; (2) succinic acid, derived after the degradation of PBS; (3) acetic acid, containing into cellulose acetate and (4) 3-hydroxybutiric acid (3HB) and crotonic acid (CA), derived after the degradation of PHB. The organic acids were tested alone and as a mixture. Furthermore, hydrolysates produced after the hydrothermal degradation of PLA and the alkaline degradation of PHB, were also used as carbon source in shake flasks and bioreactor fermentations. In shake flasks fermentations using the bacterial strain *Paraburkholderia sacchari* DSM 17165, the highest PHB accumulation (71%) was achieved when 20 g/L of lactic acid was used as carbon source, following by the mixture of lactic acid:3-hydoxybutiric acid (68%) and the 3-hydroxybutiric (62%). The efficiency of the fermentations conducted in shake flasks using the bacterial strain *Cupriavidus necator* DSM 428 (H16) was low in

all cases. Further studies should be conducted, with different compositions of fermentation mediums, regarding the ability of *C. necator* to consume the various organic acids and accumulate PHB. Fed-batch bioreactor fermentation using PLA hydrolysate as carbon source with the bacterial strain *P. sacchari*, resulted in 27.7 g/L total dry weight (TDW) with a PHB content of 71.63% and a yield of 0.25 g/g. A mixture of PLA and PHB hydrolysate was also used as carbon source in fed-batch bioreactor fermentation with the bacterial strain *C. necator* H16 and a TDW of 9.8 g/L with a PHB content of 38% was achieved. The yield of the fermentations was 0.25%.

Scientific Area: food engineering

Keywords: Poly-(3-hydroxybutyrate), polylactic acid, fermentations, Paraburkholderia sacchari DSM 17165, Cupriavidus necator DSM 428 (H16), cyclic economy

Παραγωγή πολυ(3-υδροξυβουτυρικού) με χρήση υλικών συσκευασίας μετά την κατανάλωση με βάση τη βιολογική βάση

ΠΜΣ Επεξεργασία, Συντήρηση & Βιοτεχνολογικές Διεργασίες Τροφίμων - Ανάπτυξη Προϊόντων Βιοοικονομίας
Τμήμα Επιστήμης Τροφίμων & Διατροφής του Ανθρώπου
Εργαστήριο Μηχανικής & Επεξεργασίας Τροφίμων

Περίληψη

Τις τελευταίες δεκαετίες, η μαζική παραγωγή πλαστικών έχει προκαλέσει μείζον περιβαλλοντικό πρόβλημα παγκοσμίως. Η εναπόθεσή τους αποτελεί ένα από τα μεγαλύτερα προβλήματα, καθώς λόγω της δομής και της προέλευσής τους, η αποικοδόμησή τους στο περιβάλλον δεν μπορεί να επιτευχθεί.

Ο κίνδυνος εξάντλησης των ορυκτών καυσίμων και η αυξανόμενη περιβαλλοντική ευαισθητοποίηση του κοινού έχουν στρέψει την αγορά σε "πιο πράσινες" εναλλακτικές λύσεις που προέρχονται από ανανεώσιμες πηγές ενέργειας. Τα βιολογικά υλικά έχουν αποκτήσει μεγάλο ερευνητικό ενδιαφέρον λόγω των βιοδιασπώμενων ιδιοτήτων τους, επομένως μπορούν να παραχθούν από ανανεώσιμες πηγές.

Τα βιοπλαστικά όπως το PLA και τα PHAs έχουν αποκτήσει μεγάλο ενδιαφέρον, καθώς οι χημικές και θερμικές τους ιδιότητες είναι παρόμοιες με του προπυλενίου, επομένως η αβιοτική τους αποικοδόμηση αποτελεί σημαντική ιδιότητα στη διαχείριση πλαστικών αποβλήτων. Η κυκλική και βιώσιμη παραγωγή βιοπολυμερών απαιτεί την ανάπτυξη αποτελεσματικής χημικής και βιολογικής ανακύκλωσης των βιοπλαστικών μετά την κατανάλωση.

Στην παρούσα μελέτη χρησιμοποιήθηκαν διάφορα συνθετικά οργανικά οξέα που προέρχονται από την αποικοδόμηση βιοπολυμερών, όπως PLA, PHB και PBS, ως πηγή άνθρακα σε κωνικές φιάλες και ζυμώσεις σε βιοαντιδραστήρα. Τα βακτηριακά στελέχη *Paraburkholderia sacchari* DSM 17165 και *Cupriavidus necator* DSM 428 (H16) αξιολογήθηκαν, όσον αφορά την ικανότητά τους να καταναλώνουν αυτά τα οργανικά οξέα και να παράγουν εκ νέου PHB. Πιο συγκεκριμένα, τα οργανικά οξέα που αξιολογήθηκαν ήταν: (1) γαλακτικό οξύ (LA), που προέρχεται μετά την αποικοδόμηση του PLA, (2) ηλεκτρικό οξύ, που προέρχεται μετά την αποικοδόμηση του PBS, (3) οξικό οξύ, το οποίο περιέχεται στην οξική κυτταρίνη και (4) 3-υδροξυβουτυρικό οξύ (3HB) και κροτονικό οξύ (CA), που προέρχονται μετά την αποικοδόμηση του PHB. Τα οργανικά οξέα δοκιμάστηκαν μόνα τους, αλλά και ως μείγμα. Επιπλέον, τα υδρολύματα που παράγονται μετά την υδροθερμική αποικοδόμηση του PLA

και την αλκαλική αποικοδόμηση του PHB, χρησιμοποιήθηκαν επίσης ως πηγή άνθρακα σε κωνικές φιάλες και ζυμώσεις σε βιοαντιδραστήρες.

Πραγματοποιήθηκαν ζυμώσεις σε κωνικές φιάλες με τη χρήση του βακτηριακού στελέχους *Paraburkholderia sacchari* DSM 17165 και η υψηλότερη συσσώρευση PHB (71%) επιτεύχθηκε όταν χρησιμοποιήθηκαν 20 g/L γαλακτικού οξέος ως πηγή άνθρακα, ακολουθούμενη από το μείγμα γαλακτικού οξέος:3-υδοξυβουτυρικό οξύ (68%) και του 3-υδροξυβουτυρικού οξέος (62%). Η απόδοση των ζυμώσεων που πραγματοποιήθηκαν σε κωνικές φιάλες με τη χρήση του βακτηριακού στελέχους *Cupriavidus necator* DSM 428 (H16) ήταν χαμηλή σε όλες τις περιπτώσεις. Θα πρέπει να διεξαχθούν περαιτέρω μελέτες, με διαφορετικές συνθέσεις μέσων ζύμωσης, με σκοπό να μελετηθεί η ικανότητα του *C. necator* να καταναλώνει τα διάφορα οργανικά οξέα και να συσσωρεύει PHB. Ημι-συνεχής ζύμωση σε βιοαντιδραστήρα με χρήση υδρολύματος PLA ως πηγή άνθρακα με το βακτηριακό στέλεχος *P. sacchari*, οδήγησε σε 27,7 g/L ολικού ξηρού βάρους (TDW) με περιεκτικότητα σε PHB 71,63% και απόδοση 0,25 g/g. Τέλος, μείγμα υδρολύματος PLA και PHB χρησιμοποιήθηκε ως πηγή άνθρακα σε ημι-συνεχής ζύμωση σε βιοαντιδραστήρα με το βακτηριακό στέλεχος *C. necator* H16 και επιτεύχθηκε TDW 9,8 g/L με περιεκτικότητα σε PHB 38%. Η απόδοση της ζύμωσης ήταν 0,25%.

Επιστημονική Περιοχή: Μηχανική Τροφίμων

Λέξεις κλειδιά: Πολύ-(3-υδροξυβουτυρικό οξύ), Πολυγαλακτικό οξύ, κυκλική οικονομία, ζυμώσεις, Paraburkholderia sacchari DSM 17165, Cupriavidus necator DSM 428 (H16)

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Abbreviations

3HB	3-hydroxybutiric acid
AA	Acetic acid
Bt	Billion tons
CA	Crotonic acid
DCW	Dry Cell Weight
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
LA	Lactic acid
Mt	Million tones
PBS	Poly(butylene succinate)
PBSA	Poly(butylene succinate-co-butylene adipate)
PET	Polyethylene terephthalate
PHA	Polyhydroxy acids
PHB	Poly(3-hydroxybutirate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl Chloride
SA	Succinic acid

1. Introduction

1.1. Plastic and environmental pollution

Plastics are a category of polymers that were firstly produced in 1907 from a Belgian chemist called Leo Baekeland, by using formaldehyde and phenol, under heat and pressure (Wright & Kelly, 2017). A few years later, polyvinyl chloride (PVC) was invented, causing the greatest increase in demand during the Second World War, as production increased by 300% in the USA. Up until now, it is one of the most common plastics in both domestic and industrial use. Other well-known synthetic polymers that are used widespread are polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET), which are globally used in packaging and generally in the food industry.

Polyethylene (PE) is mostly used for the production of plastic bags, bottles and toys, polypropylene (PP) is used in food containers, whereas polyethylene terephthalate's (PET) advantage is its ability to be recycled. Over the last century, the use of plastics has been rapidly spread globally, with a production of 8.3 billion tons (Bt) of which 5.7 Bt were waste and 4.9 Bt were discarded into landfills or the natural environment, such as oceans and waterways (Barnes et al., 2009). The use of plastics is rising constantly until today, with Asia holding the first place in the ranking of the highest production (Ritchie, 2018), (Plastic Production Worldwide 2021 | Statista, n.d.).

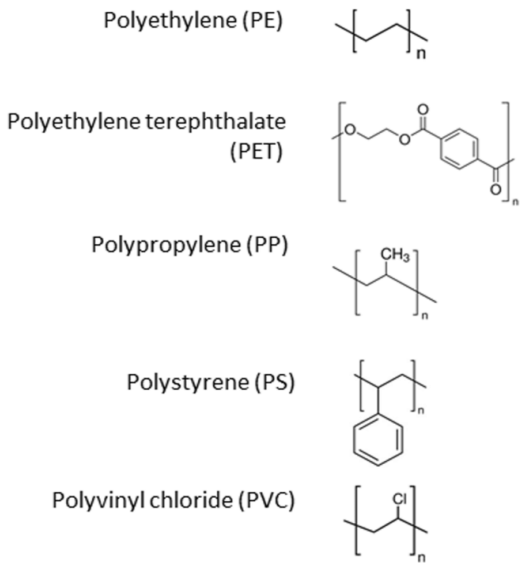


Figure 1 Chemical structure of different polymers

Most of these plastics are produced from petrochemical monomers and most of their use is in the packaging field. Plastics, and mainly in the packaging industry, are made for only one single use and for products that have a short shelf life (foods or cosmetics). Therefore, their production is continuous and large-scale and the waste of these packaging and plastics are discarded in landfills or the environment. The main concern is the life cycle of these plastics as most of them are not biodegradable

and it takes years or centuries to be degraded. Therefore, their life-ending and the recycling is unknown and not well studied, as most recycled plastics can only be recycled a specific number of times before being useless. Based on global data, 6300 million tons (Mt) of plastic garbage had been produced as of 2015 and only 9% of it had been recycled, while 12% had been burned, and 79% had ended up in the natural environment. In the case that these wastes end up in the environment, there is a huge contamination of the marine environment too. This affection raises awareness as the marine ecosystem is fully damaged by the high mortality of most of the species (Geyer et al., 2017).

As a result, an emergency degrading mechanism that overcomes the reported problems should be included in every piece of plastic that could end up in the environment (Tiso et al., 2022).

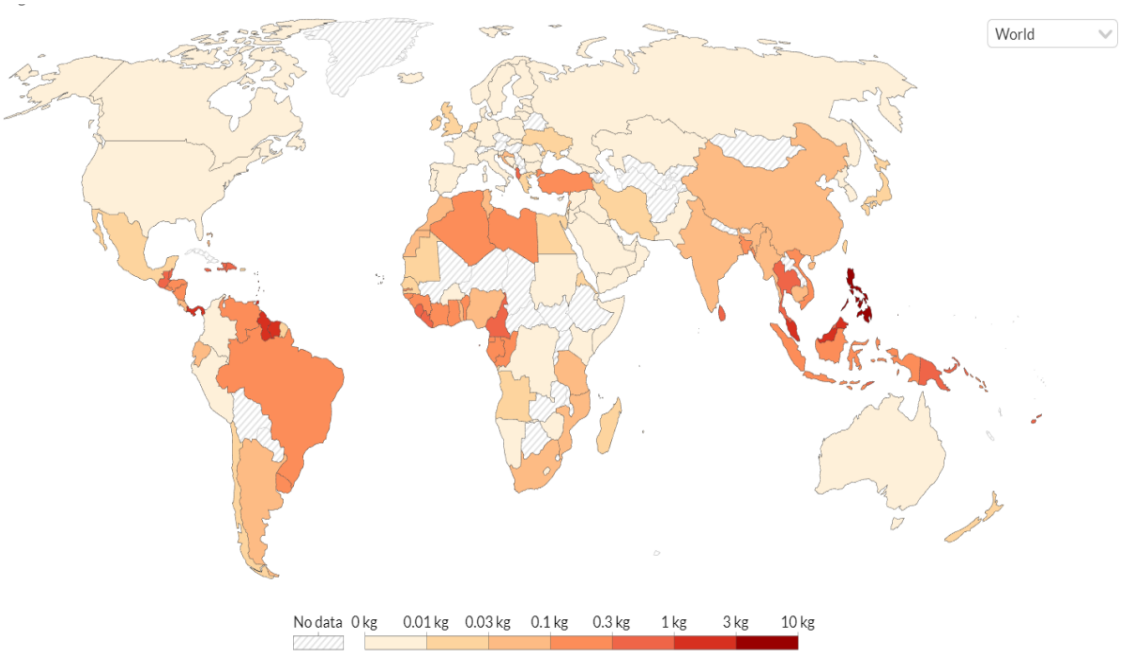


Figure 2 Plastic waste emitted to the ocean per capita, 2019 (Maijer et al., 2021)

The main awareness is that conventional plastics are not able to degrade through microbiological fermentation and the reason is that their short time of appearance has not updated the development of enzymatic science. A serious issue that is considered dangerous for the environment is that most of the plastics at the surface of the ocean have been converted to smaller pieces of plastics (< 1cm), known as microplastics. These microplastics can be easily absorbed by different kinds of species, including humans, causing serious health problems (Cózar et al., 2014).

More and more research studies are focusing on the possibility of human consumption of microplastics. Microplastics have been found in processed foods and drinks like sugar, beer, and salt, as well as in seafood. Moreover, this situation is expanded also in the agricultural field where it was found that synthetic fibers are present in byproducts remaining for up to 5 years after application (Wright & Kelly, 2017).

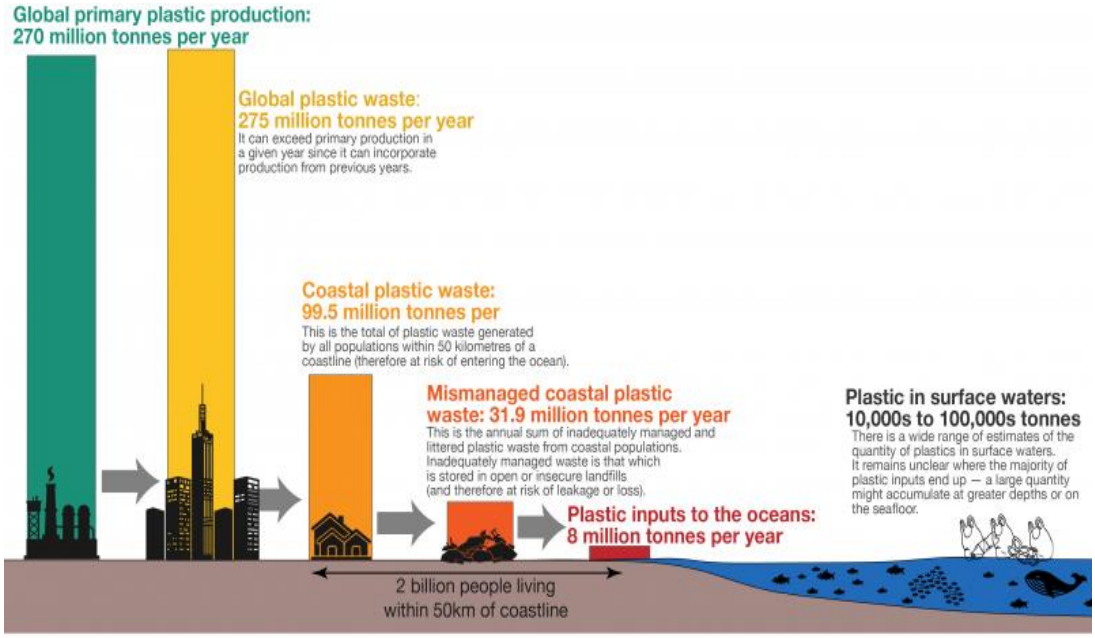


Figure 3 The pathway that the plastics enter the world's oceans (Jambeck et al., 2015; Eriksen et al., 2014)

1.2. Bioplastics

Nowadays, about 2/3 of the plastics that are used are petrochemicals such as polystyrene (PS), polypropylene (PP), polyethylene (PE) and terephthalate (PET). The need for alternative plastics arose in order to prevent the upcoming pollution and environmental disaster. As plastics are part of everyday life and moreover an important factor of the global market, including involvement of most of the industries, the idea of bioplastics arose. Bioplastics are materials fully biodegradable and sometimes biobased too, which are obtained mostly by renewable biomass sources, such as corn, sugar cane and waste fats and oils. Most of the renewable resources that are used as feedstock for the production of bioplastics are derived from the food industry or similar fields (such as agriculture byproducts). They are not all biodegradable, which is why they are usually divided into bio-based, biodegradable and non-biodegradable. Examples of bioplastics that are biodegradable and are trying to become biobased are PHAs and polylactic acid, as well as lignocellulosic substrates. The aim is to recycle them, either by hydrolysis or microbial decomposition. Whereas the BIO-plastics that have been created are derived from natural biomass sources and the emissions are greener and more environmentally friendly, since the structure is the same as conventional plastics, making them non-biodegradable. Examples of such plastics are bio-PET (Narancic et al., 2020).

During the 1990s more and more industries have switched their methods and processes based on green chemistry in order to achieve higher sustainability (Iles & Martin, 2013). These new processes have proved to be less expensive and more productive than the conventional methods. So, the main goal of the industry was to introduce to the business field green technology. As the market needs to decrease the use of fossil fuels, considering not only the pollution but moreover the ever-increasing

prices, the science of biotechnology was introduced. Even though the use of biomass to synthesize bioplastics is not widely used, a lot of research has been made in order to reduce the cost of the process and prove that these renewable sources can be effectively used for the production of common polymers (Carole et al., 2004).

The inability of some plastics to biodegrade means that their chemical makeup cannot be sufficiently altered by water, carbon dioxide, or methane found in the environment, or by naturally occurring biological methods such as fermentation. Biodegradable and bio-based plastics, on the other hand, are degraded materials either with natural methods through the time, or simple chemical and biological applications, leading to a greener cycle-end life compared to conventional plastics (Tiso et al., 2022).

The biodegradation of plastics is an issue that is prevalent today. Bioplastics, i.e. plastics produced biologically from natural sources, does not mean that they are biodegradable, because sometimes they have the same structure as conventional plastics. The ability of biodegradable plastics to be degraded is based on different factors such as hydrophilicity/hydrophobicity, the degree of crystallisation, the structure of the polymer, the monomers it is composed of, etc. These characteristics can indicate the durability of these bioplastics, as well as predict the time needed to compost or decompose them, and the most suitable conditions for the recycling processes (aerobic/anaerobic, pH, water, receptivity to specific microorganisms, etc.) (Narancic et al., 2020).

The most known bioplastics in the modern industry are polyhydroxyalkanoate (PHA), polylactic acid (PLA) and polybutylene succinate (PBS).

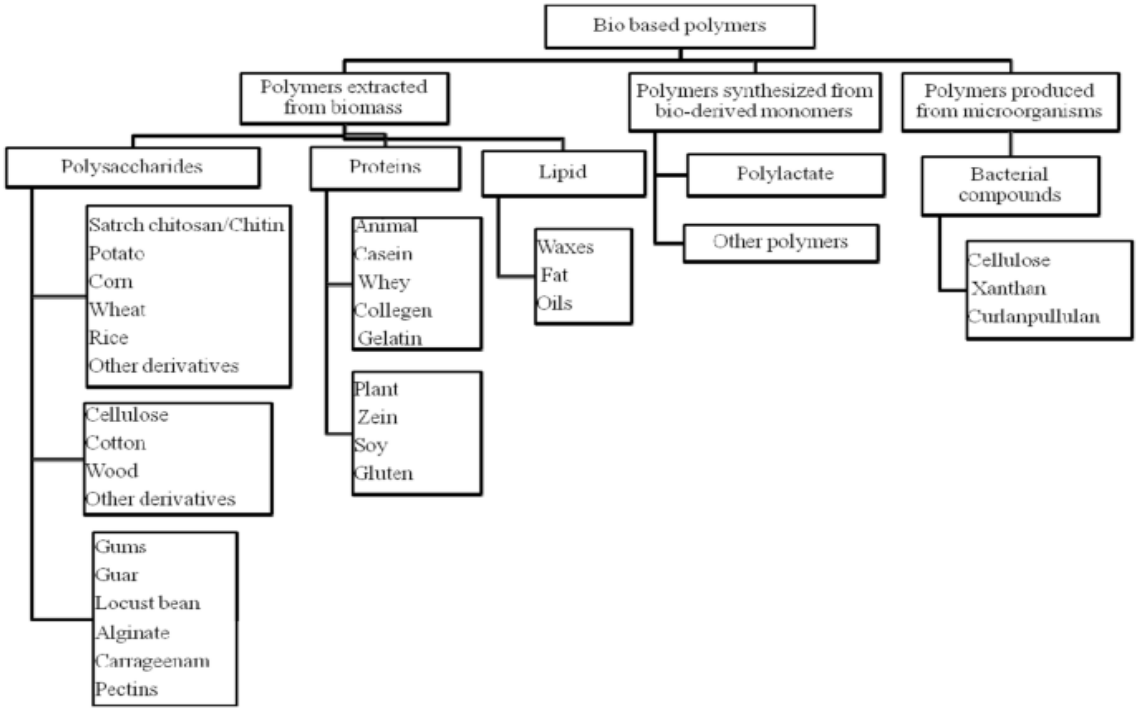


Figure 4 The different categories of bio-based polymers (Mangaraj et al., 2018)

1.3. Poly(hydoxyalkanoates)

Poly(hydroxyalkanoates) (PHAs) are a group of polyesters that can be produced by microorganisms and serve as carbon and energy storage. PHAs' production is possible by using renewable carbon sources through bacterial fermentation and is one of the most promising bioplastics in the industry. It is mainly produced by more than 100 bacteria, either gram-positive or gram-negative, which are aerobic or anaerobic (Tan et al., 2014). Usually, 600 to 35,000 (R)-hydroxy fatty acid monomer units compose a PHA molecule, constituting linear polyesters. Each monomer unit consists of a side chain R group, which is often a saturated alkyl group but it can also be unsaturated, straight, or branched chain and containing aliphatic, typical aromatic groups or halogenated, epoxidized and branched. (Singh et al., 2018). This is the most common type of structure, with the least exceptions, which is due to the stereospecificity of the enzymes responsible for their synthesis.

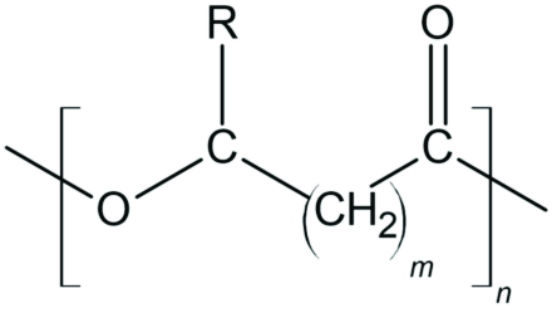


Figure 5 The general chemical structure of PHA's, with m taking rates from 1-3 and the number of monomers and R the alkyl group (Gomes Gradíssimo et al., 2020).

PHA is a family of different polymers with different numbers of monomers and more that 140 different kinds of them have been identified. Their production can be performed by different bacterias, either during the consumption of a carbon source until they reach the stationary phase or after the depletion of a nutrient such as oxygen, phosphorus and nitrate (Raza et al., 2018).

The PHA's, according to their molecular weight can be divided into three categories, the short-chain length with 3 to 5 C, the medium-chain length with 5 to 14 C, and the long-chain length with more than 14 C. The first category includes polymers such as the P(3HB), P(4HB), PHV and the copolymer P(3HB-co-3HV), the second one includes homopolymers such as P(3HHx), P(3HO) as well as copolymers of the 2 aforementioned P(3HHx-co-3HO) and the last category being the result of copolymerization of poly-hydroxy alkanoic esters of short and medium chain lengths, with a typical example being the copolymer [P(3HB-co-3HV-co- 3HHD-co-3HOD)] (Miranda De Sousa Dias et al., 2017).

These monomers are mostly known are the 3-hydroxybutyrate (3HB) and the 3-hydroxyvalerate (3HV), with the first one to synthesis the most used biopolymer of the group of PHAs, the poly-(3-hydroxybutyrate) (PHB). Another way to classify the various polymers of the PHA family is into homopolymers and heteropolymers. Homopolymers include bioplastics composed of one type of

polymer while heteropolymers are composed of different types of polymers. Homopolymers, compared to heteropolymers, have a higher fragility and crystallinity which makes them more difficult to process (Miranda De Sousa Dias et al., 2017).

The intracellular accumulation of PHA is stored in the form of granules. These microorganisms that are responsible for the production of PHA are also able to enable many different catabolic pathways combined with anabolic pathways, a characterization that leads to the production of PHA from many different carbon sources, such as sugars, acids (lactic acid, acetic acid, succinic acid etc), alcanes and gases. Although PHAs are primarily produced intracellularly, genetically modified organisms can deposit it extracellularly. In this case, after the metabolism of the entire carbon source, PHAs can be depolymerized and their by-products have the ability to be used both as a carbon and energy source (Możejko-Ciesielska & Kiewisz, 2016).

1.3.1 The metabolic pathways of PHAs

The carbon source is metabolized to create energy and precursor molecules through a variety of enzymatic processes, including glycolysis and the tricarboxylic acid (TCA) cycle. Through a series of enzymatic reactions, the precursor molecules, which are typically acetyl-CoA, are transformed into PHA monomers. Enzymes like PHA synthase and acetyl-CoA acetyltransferase are involved in this process. The PHA polymer is created following the polymerization of the PHA monomers. PHA synthase enzymes help to catalyze this polymerization reaction by joining the monomers together, usually with ester bonds.

To be more specific, depending on the type of microorganism and its properties, PHAs are produced by three different metabolic pathways. The first pathway involves bacteria such as *Necator* and *Paraburkholderia* and usually produces PHA's short-length chain. At the beginning, 2 molecules of acetyl-CoA, which comes from the TAC cycle and are converted to acetyl-acetyl-CoA using the β-ketothiolase co-enzyme. Then with the help of NADPHA-acetyl-acetyl-Coa, acetyl-acetyl-CoA is converted to 3-hydroxybutyril-CoA and then this to the final product, which is the result of ester bond formation to produce the final biopolymer (Sharma et al., 2021).

The second pathway produces mainly medium-length chain PHAs and is found in bacteria such as Aeromonas and Pseudomonas. This metabolic pathway involves lipid metabolism which is an important source for the production of PHAs. Therefore, it includes the beta-oxidation pathway, which uses different hydroxyalkanoate monomers. These monomers will be polymerized at the end with the help of the PHA synthase enzyme.

The third pathway also produces mainly PHAs medium-length chain, but using as carbon sources mainly sugars, such as glucose, fructose and sucrose or animal lipids. The pathway follows the conversion of (R)-hydroxy acyl to CoA, which is catalysed by the enzyme acyl-ACP-CoA transacetylase. Then again using PHA polymerase the polymers are synthesised (Gradíssimo et al., 2020).

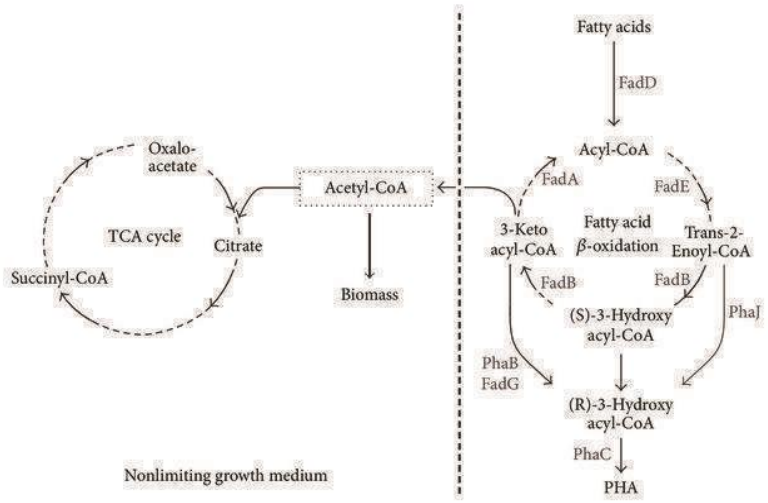


Figure 6 The metabolic pathway of producing PHA with the use of *Cupriavidus necator* (Martín-Bufájer et al., 2016)

1.3.2 Poly(3-hydroxybutyrate)

1.3.2.1 Structure of poly(3-hydroxybutyrate)

As mentioned above, PHB is included in the family of PHAs. PHB is a homopolymeric, biodegradable polymer with a high melting point, at 180 oC and can be used in a wide range of temperatures, from -30 to 130 oC. Like the rest of PHAs, The monomer of PHB consists of a hydroxyl group, a carboxyl group and an -R group. The -R group is a methyl group, where this gives it the characteristic of being non-aqueous soluble and it usually consists of 4-5 carbon atoms, which puts it in the short chain legth type of PHA's. PHB is produced intracellularly and stored in granule forms that are 0.2-0.5 μm in diameter. The percentage of grains can reach up to 90% of the biomass. The molecular weight of PHB varies between 10,000-300,000 g/mol and depends on various factors such as the fermentation time, the type of microorganism, the type of carbon source and its quantity, etc.

It is reported to be one of the first PHAs to achieve isolation, produced by almost all prokaryotic bacteria. Like the other biopolymers, PHB's crystallinity is an important property to be concerned, for its structure and use. In order to improve the properties of PHB in its use as a bioplastic, it is often chosen to be blended with other biopolymers, such as Poly-L-lactic acid (PLLA) (Jirage et al., 2013).

There are three ways to pick up PHB. The first one is the cycle-opening polymerization with β-butyrolactone as the first source and zinc and aluminum as possible catalysts. The second way is through natural sources and the third and the one most researched is through microbial fermentation, ecofriendly, trying the use of natural renewable resources. Over 50 different species of bacteria have been identified that achieve production. The main bacteria known for their ability to produce PHB are species of the genera *Paraburkholderia, Cupriavidus necator, A. Latus, Escherichia c., Pseudomonas* and *Protomonas*.

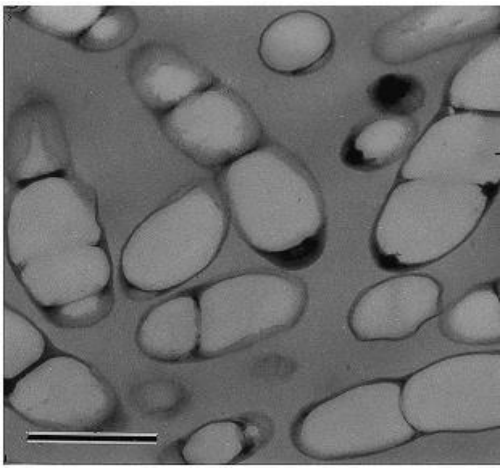


Figure 7 An image of granules of PHB (Sukan, 2015)

1.3.3 Polylactic acid (PLA)

Polylactic acid is one of the most prevalent biobased polymers in today's industry to replace common petrochemical plastics. It is a product of polymerization of lactic acid monomers.Its an aliphatic polyester, which is considered promising in the biotechnological field, since its monomer, lactic acid can be produced naturally, during fermentation of sugar, starch and other renewable resources in nature, extracted from different kinds of food and plants, characterizing in this way as biobased (Murariu & Dubois, 2016). For this reason, many other natural are considered prosperous carbon sources to be used for the production of PLA and for this reason many researches and new technologies are being developed. Apart from its biodegradability, it is important that during its production the emissions are very low and environmentally friendly, but it also requires very little energy (Bax & Müssig, 2008).

Its physicochemical properties are important in order to optimize its use on the modern biotechnology as a replacement for conventional plastics, taking into consideration its strength and mobility, which ensure the creation of biofilms (Yu et al., 2023; Farah et al., 2016). PLA is consist of 2 different monomers of lactic acid, the D- and the L-Lactic acid, with L-enantiomer (99.5%) being the most prevalent during the production of lactate by fermentation over D-enantiomer (0.5%). Nowadays, there are different methods of producing PLA, polycondensation directly and ring opening polymerization being the most popular. There are the polymers produced from lactic acid, which form PLA, and those produced from lactide, which are called polylactide. The main way to produce PLA with high molecular weight is by ring opening polymerization.

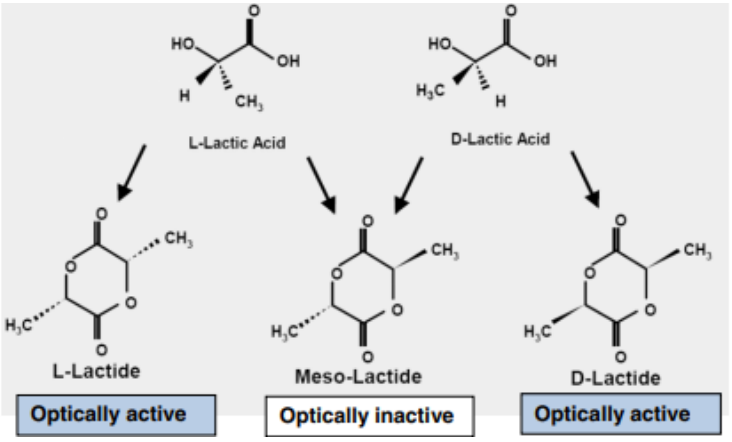


Figure 8 The different stereoisomers of lactic acid (Murariu & Dubois, 2016)

The past few years considerable research has been made because of the need of alterification of PLA's properties. The need is having controlled physicochemical characterizations in order to be used in different scientific fields.

All of PLAs properties (such as density, rheology, heat capacity etc.) are depending on its crystallization temperature. Crystallinity of PLA is considered to be an important property of the bioplastic, because it affects its mobility, its durability, its tensity and its velocity. PLA's characteristics could be controlled with the use of different catalysts.

Another important factor about PLA is its melting point (Tm), which is around 157-180°C, as well as Tg, 55 to 60°C. This is crucial for studying its capability of degradation either with enzymatic factor or hydrothermally. The density of PLA is calculated around 1248 g/L as the enthalpy of melting point is found in literature at about 93 J/g.

In many medical and industrial applications, PLA has surpassed traditional plastics as a leading biomaterial. Firstly, the PLA was used in medical science, with various applications such as implants with different applications such as implants, because the cost was much lower than conventional petrochemicals. In early research on PLA, it was used because of its low molecular weight. However, as the technology has evolved, the need to use higher molecular weights for various industrial uses has increased as the need to replace conventional plastics in everyday life has grown, with major industries interested in exploiting it being the packaging, food, automotive and electrical industries in general. The main reason for their high demand is both their biodegradability, but also their nontoxicity.

PLA is also considered hydrophobic, like PHAs, a property that can cause problems in contact with cells, leading to infection. PLA is not water soluble, as well as alcohols such as methanol and ethanol, but is soluble in a range of organic compounds such as dichloroacetic acid, methylene chloride, acetonitrile, 1,1,2-trichloroethane, dioxane and chlorofluorocarbons (Farah et al., 2016).

Another disadvantage in its properties is its long degradation time, a problem that may affect both medical applications and applications in the packaging and food industry, as their natural biodegradation will take years, resulting in their accumulation after deposition (Farah et al., 2016).

PLA's ability to biodegrade is the biggest feature that makes it attractive. Initial studies were carried out in the body of various animals in vivo, demonstrating that hydrolysis can take place naturally. The hydrolysis of PLA, however, takes longer and this is due to its structure. It is the alkyl group that gives PLA its hydrophobic character, which prevents water absorption. This time is also influenced by many factors such as temperature, molecular weight, pH, microorganisms, or catalysts that may be present.

The physicochemical properties of PLA polymers are largely dependent on the stereoisomers selected within the polymer chain during polymerization. It is very common to mix bioplastics in packaging in order to have better toughness and generally improve their properties. But this mixture can also significantly alter the properties of PLA.

Table 1 Mechanical and thermal properties of PLA

Molecular weight	65.000 g/mol
Density	1.252 g/cm^3
Tg	55^{o}C
Tm	165^{o}C
Specific heat	
55 $°C$	1590 $J/kg \times °C$
100 $°C$	1955 $J/kg \times °C$
190 $°C$	2060 $J/kg \times °C$
Thermal Conductivity	
55 $°C$	0.114 $W/m \times °C$
100 $°C$	0.197 $W/m \times °C$
190 $°C$	0.195 $W/m \times °C$

Concerning these properties of PLA, the low crystallization point is considered a disadvantage. On the other hand, Moreover, it is very common to blend polymers with PLA derived from different stereoisomers (L- and D-). This final biopolymer acquires an important property, as it increases by at least 55 oC the Tm, compared to other PLA homopolymers, making this heteropolymer an attractive candidate for the packaging industry. One of the key characteristics of PLA that is considered suitable for is its ability to produce films that can be easily processed and formed into all shapes and sizes. Blending PLA with cellulose or cellulose fibres provides stability and durability, especially as the percentage of cellulose increases. Therefore, this can be applied in the packaging sector, for the production of packaging useful in the food industry, offering properties that are lacking in packaging

made exclusively of PLA. Another way of optimising the properties of PLA is before and after the polymerisation stage. Often methods are chosen to extend the polymer chains to increase the M.W. of the polymer, giving it important advantages such as toughness, stability and ease of processing. (Narancic et al., 2020).

As regards the viability and costing of PLA, it is currently used to a small extent and in combination with conventional plastics. For this reason, the cost of its production and use in industry is particularly high. The aim of current research is to bring the price of PLA up to that of petrochemical plastics such as PET and nylon, so that soon all environmentally friendly bioplastics will be used more widely and frequently. Furthermore, a parallel objective is to achieve the exclusive use of PLA, without mixing it with petrochemicals (Dugan, 2001).

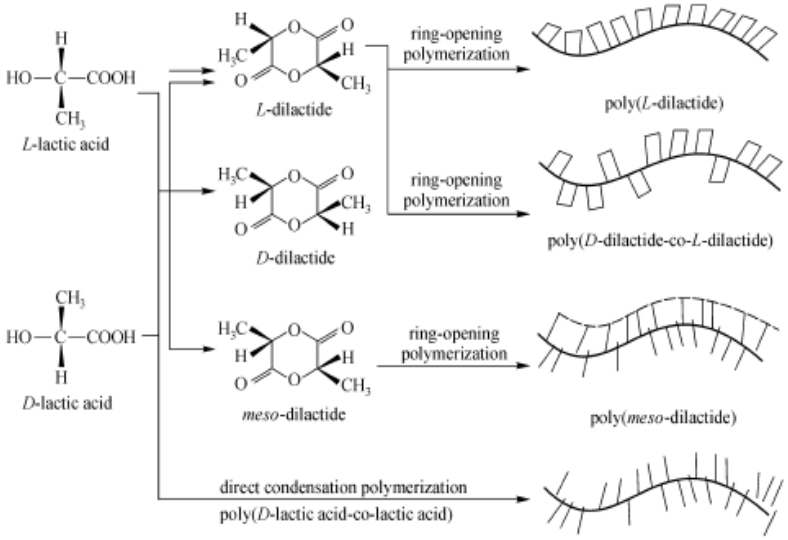
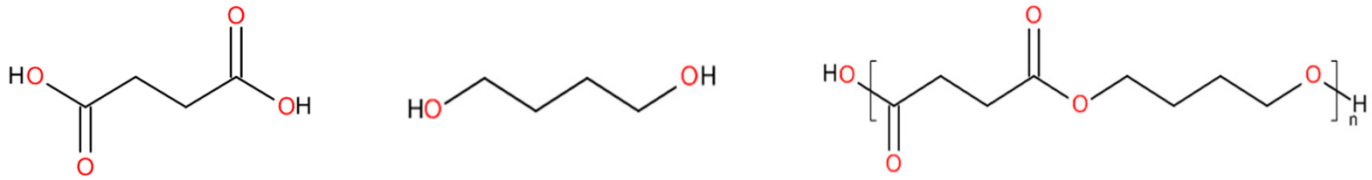


Figure 9 The polymerization of D- and L- Lactic (Cheng et al., 2009)

1.3.4 Poly(butylene succinate (PBS)

Poly(butylene succinate) or PBS is another well-known biobased plastic that is also belongs to the group of polyesters. Like the other biodegradable thermoplastics mentioned above, PBS exhibits significant physicochemical and mechanical properties, which make it particularly interesting in the new bioplastics and packaging market. More specifically, its ability to melt and biodegrade very easily is considered very important, as well as its great flexibility and significant thermal stability. Its stiffness as a material can sometimes prevent its use in certain applications. These properties can be differentiated and transformed by making changes in the co-polymerization process and molecular weight. PBS can be synthesized from the copolymerization of 1,4-butanediol and succinic acid. In the plastics and packaging industry, PBS is often used as a mix with other bioplastics such as PLA, in order to improve the mechanical properties of the final material and make the final product been more durable and flexible (Su et al., 2019).



(a) (b) (c)

Figure 10 Chemical structure of (i) succinate acid, (ii) 1,4-butanediol and (iii) poly(butylene succinate) (PBS) (Su et al., 2019)

PBS has been widely available on the bioplastics market since the 90's. Its price currently reaches 4 EUR/kg for PBS derived from petrochemical raw materials and 6 EUR/kg for PBS produced from biobased raw materials. Research on PBS over the last decade has been growing, with publications referring to it, increasing by over 110%. Succinic acid can be produced through microbial fermentations, using various bacterial strains. Various sugars has been studied as carbon source in such fermentations, as well as feedstocks derived from wastes streams. The main research objective at the moment is the exclusive production of succinic acid through microbial fermentation with the reuse of biological PBS stock, in order to reduce the use of petrochemicals and thus reduce the environmental footprint (Barletta et al., 2022).

1.4. Microorganisms that produce PHAs

The most common microorganisms used for the production of PHA's are *Pseudomonas putida, Cupriavidus necator, A. Lata, Azohydromonas, Burkholderia* etc. Both prokaryotic and eukaryotic cell types are able to produce PHAs. *Pseudomonas* strains, *P. oleovorans* and *P. putida* are known to produce medium-length chain's PHA, with the two last ones to be known for the production of (HAMCL)s copolymers that are produced from two to six different kinds of 3-hydroxy-n-alkanoates, while short-length chain PHAs are mostly produced from *Paraburkholderia* and *Rhodospirillum rubrum* (Raza et al., 2018).

PHA's can be divided into two categories, those that need a nutrient restriction for their production and those that do not. There are specific bacteria that are used for each case. For example,in order to produce PHAs by limiting the carbon source, bacterias such as *Spirillum sp*., *Hyphomicrobium sp*. and *Azospirillum brasiliense* are commonly used. Bacterial strains that are used for the production of PHAs under oxygen limitation are the Azotobacter *vinelandii*, *Azobacter beijerinckii* and *Rhizobium* ORS571, under phosphorus limitation *Rhodospirillum rubrum, Rhodobacter sphaeroidis, Caulobacter crescentus* and *Pseudomonas oleovorans* and under ammonia limitation, are the *Alcaligenes latus, Pseudomonas oleovorans, Pseudomonas cepacia* and *Ralstonia eutrophus.*

PHA's can be derived from the metabolism of different primary carbon sources. It can be produced from common sugars, such as glucose. Examples of microorganisms that can metabolize glucose to produce PHA's are of the genera *Escherichia, Ralstroni, Acinetobacter* etc. Also, alcohols can be used

as metabolites (such as methanol), in which case the bacteria *Methylobacterium* can be employed. Furthermore, lots of acids and ketones can be used as carbon source for the production of PHA's, using the bacterial strains *Azospirillum, Rhodobacter, Syntrophomonas* etc (Kim & Lenz, 2001).

The biggest challenge of PHA's production on a large scale is the high cost of, so the aim is to study how to scale up and assimilate them into the industry at the lowest possible cost and find the most suitable microorganism to achieve the highest production yield.

1.4.1. Bacterial strain Paraburkholderia sacchari DSM 17165

Paraburkholderia sacchari is an aerobic, gram-negative bacterium that was firstly obtained from sugarcane cultivation soil in Brazil. It is a mesophilic bacterium, and its growth is achieved at 28-30 ºC. Numerous genetic and bioprocess engineering studies have been conducted on this strain in different parts of the world with the goal of establishing the production of poly-hydroxyalkanoates) (PHA) from various substrates, particularly using agro-industrial byproducts. *Paraburkholderia sacchari* is a prospective industrial strain that can consume the most common sugars from lignocellulosic hydrolysates, mostly glucose and xylose, as well as other substrates like sugarcane and it can efficiently produce PHB intracellularly. (Oliveira-Filho et al., 2021)

A number of different experiments have been conducted in order to isolate bacteria capable of producing PHA's polymers. Almost 100 strains have been previously used, using sugar or propionic acid as carbon source. These studies showed that using propionic acid as a carbon source, resulted in a lower PHA's yield, compared to the corresponding experiments using different types of sugars. Most bacterial strains are found to perform high productivity in the case that carbon sources such as glucose, glycerol, sucrose or organic acids are used. The highest total dry weight (TDW) achieved is up to 70% while the intracellular production of PHB is estimated to be around 0.40 h^{-1}.

Paraburkholderia sacchari is a bacteria known for its ability to degrade and metabolize complex carbon compounds, making it potentially useful in bioremediation processes. The bacterial cells are rod-shaped, 0.5-0.8 μm wide and 1.5-3 μm long and it has been observed to be susceptible to certain antibiotics, such as ampicillin, chloramphenicol, kanamycin and tetracycline (Brämer et al., 2001) Furthermore, *P. sacchari*, like other similar bacteria, is known for its ability to fix atmospheric nitrogen. This means that the enzyme nitrogenase, which converts atmospheric nitrogen gas (N_2) into ammonia (NH_3). So, in common fermentation processes, it is capable of reducing the need for external nitrogen source and improving the efficiency of nitrogen utilization. *P. sacchari* has been observed to have the ability to produce different copolymers when a variety of sugars is used as a carbon source. Through experiments, it was obtained that the ratio between the produced 3HB and the 3HHx polymers produced in parallel is around 1.6%, with the maximum ratio that can be achieved being around 2.4%, a ratio achieved after the addition of a mixture of glucose and hexanoic acid as a carbon source during fed-batch fermentation. (Oliveira-Filho et al., 2021) Additionally, the FDA organization has justified the use of poly (4- hydroxybutyrate) (P4HB), as a medication, as research for the production of

different drugs relating to diseases, have been carried out for some decades. So, this emphasizes the need of achieving highest yields in the production of co-polymers of PHB. Another positive impact of producing these co-polymers are the different properties in comparison with P(3HB) as the crystallization and the flexibility, making this bioproduct easier to be used massively at the industry. (Raposo et al., 2017)

In addition, the phenomenon of catabolic suppression was observed in many of the experiments carried out. For example, in sugar mixing, glucose consumption was preferred while xylose consumption was not performed. This fact has been observed in other cases of addition of two or three carbon sources, eventually affecting the percentage of total dry mass and intracellularly produced PHB (Lopes et al., 2009).

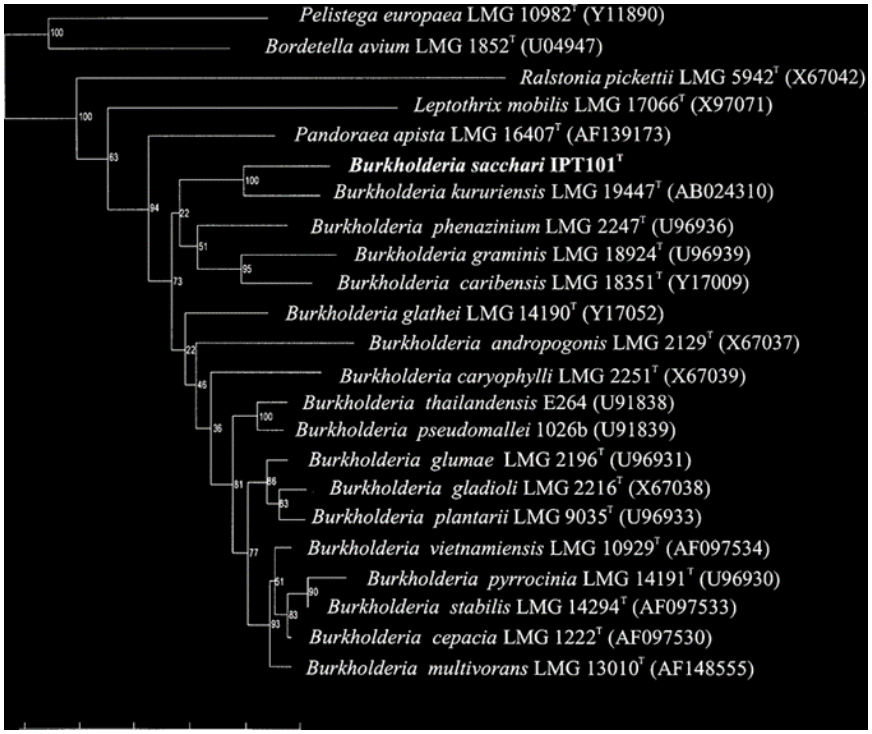


Figure 11 Based on comparisons of 16S rRNA sequences, a phylogenetic tree of *paraburkholderia sacchari* and related bacteria was created. (Brämer et al., 2001)

1.4.2. Bacterial strain Cupriavidus necator DSM 428 (H16)

The bacterial strain *Cupriavidus necator* H16 or *Ralstonia eutropha* H16 or *Alcaligenes eutrophus* as it was previously named, is a gram-negative, rod-shaped, aerobic bacterium. The temperature that has been observed as optimal for bacterial growth is 28-30°C and the desired pH is around 7.0 (Leiva-Candia et al., 2015). Its physicochemical properties make it a highly versatile bacterium in metabolic pathways. It is particularly well known for its ability to reproduce itself using CO_2 as a carbon source, H_2 as an electron source and O_2 as an electron acceptor. At the same time, it can consume many different carbon sources, such as sugars, acids, alcohols, etc. In terms of its industrial capabilities, it

can produce various biopolymers of the PHA family in large quantities as well as the ability to store intracellular PHAs. According to studies, the percentage of copolymers of PHA that it can store can reach up to 90% (Jawed et al., 2022). Compared to other bacteria that can produce PHA, it can't consume glycerol and glucose, but it can consume fructose efficiently. The bacterial strain can achieve high PHB production intracellularly, under phosphorus or oxygen limitation (Azubuike et al., 2020).

1.5. PHA recovery

PHA can be recovered by separating the biomass from the fermentation media when the biomass growth has reached a plateau, i.e. there is no increase over time. This is achieved either by stopping the air or by raising the temperature to around 80-100 oC. Separation from the fermentation liquid is then carried out most commonly by centrifugation followed by cleaning with water. (Koller, 2020). Successful uptake depends mainly on the handling of the biomass and careful practices for obtaining the intracellular product. Due to this sensitivity, pretreatment methods are applied to achieve the recovery of the highest percentage of PHA in the simplest way. The main pretreatment applied is high temperature, and this is because it has the potential to destabilize the outer membrane. Particular attention is however drawn to the range of values used depending on the type of microorganisms, as increased temperature is shown to lead to the activation of the enzyme depolymerase, capable of degrading the produced PHA (Madkour et al., 2013). Another way of drying biomass as pretreatment, which has proven to be more successful in terms of the risk of PHA degradation is freeze drying. However, elevated temperature treatment is the most economical way, since freeze drying requires the purchase of specific equipment (Koller et al., 2013).

For the recovery of PHA's intracellular cells there are 3 steps: the breaking of the cell, the extraction of the PHA capsules and the purification of the PHA capsules. Techniques such as high pressure, ultra sonic, bread mills, osmosis, chemical solvents, or specific lytic enzymes are usually used to break down the cells (Chen & Jiang, 2017). Currently, the recovery of PHA capsules is carried out by two methods: either with solvents or without solvents (Salerno et al., 2012).

One of the first methods of separating PHA was using organic solvents. Lemoigne was the first to achieve isolation using ethanol, followed by purification with chloroform and diethyl ether. Most of the methods that have been used are based on the basic property of the biopolymers of the PHA family not to dissolve in water, but mainly in organic solvents. The most common solvents that have been used and have been observed to be satisfactory are 1,2-chloroethane, chloropropane, chloroform or maybe mixtures of chloroform and methanol or other alcohols and cyclic or non-carbonate esters (Madkour et al., 2013). With the use of these organic solvents, the removal of lipids is achieved, leaving the desired PHA. In addition, the main concerns are the environmental impact and safety, as the solvents are considered toxic not only to the environment but to human health, causing for example carcinogenesis as well as respiratory, mucous membrane and eye irritation. (Choi & Lee, 1999).

Regarding enzymatic hydrolysis, a method with a mixture of enzymes has been studied for the recovery of PHB granules from *C. necator*. These enzymes are lytic and include for example lysozyme, nucleases, or phospholipases. This method starts with high heat to initiate destruction of the cell membrane, followed by enzyme catalysis and total decomposition of the excess biomass using anionic membranes (Koller et al., 2013).

In terms of chemical processes, recovery of PHA has been carried out with NaClO under high pH conditions. In this process, the majority of the biomass reacts with the NaClO solution and is converted into water-soluble. Despite its effectiveness, this method has two major disadvantages, as during the reaction: 1) a large percentage of the desired grains may be lost and 2) it can be toxic and dangerous, as the oxidation of NaClO has the potential to produce halogenated compounds. In more recent investigations carried out for medical purposes, it proved to be a satisfactory method as with the use of NaClO, the cellular biomass reacted completely. This was followed by centrifugation and purification with various polar solvents, dissolution in chlorophore and obtaining the desired granules of PHA in high purity. Subsequently, it proves to be a very promising method for industrial application.

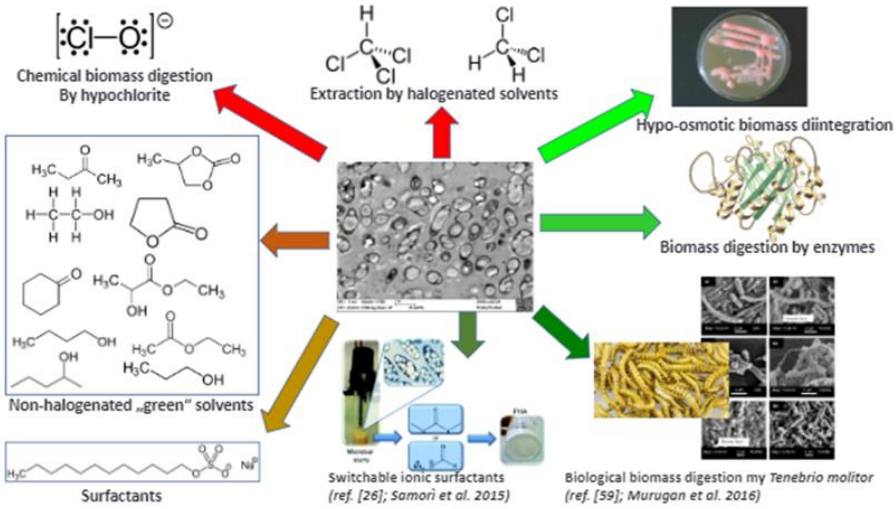


Figure 12 Several methods of PHA Recovery (Koller, 2020)

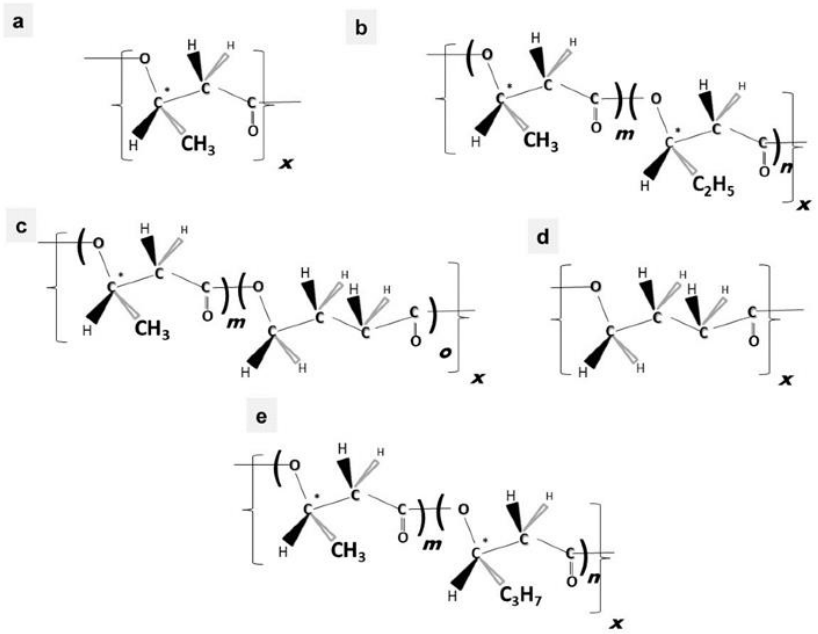


Figure 13 Chemical structure of a. P(3HB); b. P(3HB-co-3HV); c. P(3HB-co-4HB); d. P(4HB); e. P(3HB-co-3HHx) (Koller & Mukherjee, 2022).

1.6. Properties of PHA's

PHA's are a family of biopolymers with each coming from a different source and the number of carbons and monomers they are composed of varying. Therefore, each will display different chemical properties. There are some common properties that characterize all polymers of this group (Bugnicourt et al., 2014): are not water-soluble and therefore have resistance to hydrolysis,
flexible and adaptability, display resistance to UV radiation, non-resistant to acids and bases, dissolved in chlorinated carbons, biodegradable and adequate for various medical uses, are submerged in water, non-hazardous, are not as adhesive when liquefied compared to other polymers.

Key differences in the properties of PHA's compared to conventional plastics are the high melting point (Tm), ranging between 160-175°C, glass transition temperature which values are -5 to 5°C (Tg) and the ultimate strength (σ) that reaches up to 40 MPa, as well as the morphology. Other important thermochemical properties are the crystallinity that is valued around ~65%, It also appears to be more fragility than other polymers, with the ductility to range from 4-16% (ε) (Koller & Mukherjee, 2022). The main reason of this factor is that during the second crystallization of the amorphous phase is happening when being kept in room temperature whereas the temperature of glass transition is also close to room temperature. Therefore, chemical bonds are easily broken. It is observed that the kinetics of crystallization is considerably slow, a property that significantly affects the bonds between crystals and the amorphous chain. At the same time, they have properties identical to petrochemical plastics, i.e., the ability to resist moisture as well as perfume permeability (Bugnicourt et al., 2014).

A main advantage of the PHA's is the ability to biodegrade even with in compost, marine particles, or ground, depending on different factors, such the percentage of the oxygen, the microorganism's existence, the temperatures, the MW of the polymer and the water activity (Stal, 1992). According to most research, the ability of this biodegradation depends mainly on the amount of O_2 available and the percentage of CO_2 produced (Suzuki et al., 2020). Under aerobic conditions, PHA decomposes into CO_2 and H_2O, while under anaerobic conditions, PHA degrades into CO_2 and CH_4 (Ebnesajjad, 2012).

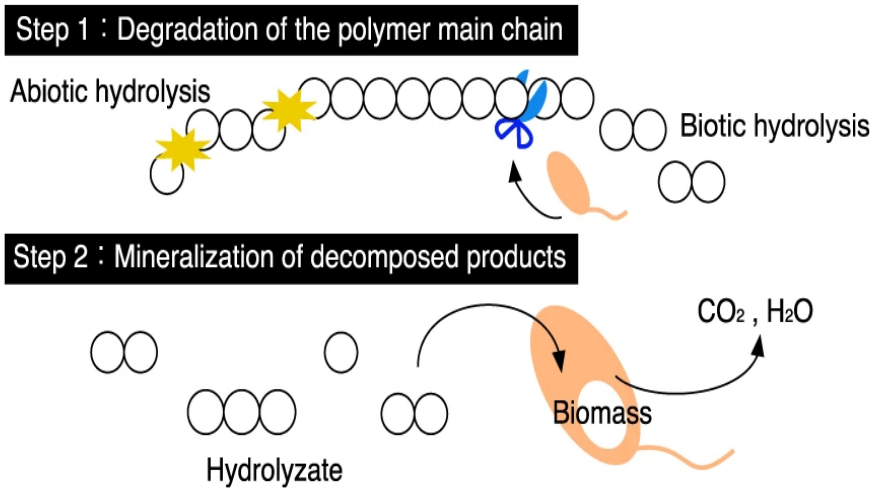


Figure 14 Steps of biodegradation of PHA (Suzuki et al., 2020).

1.7.Biodegradation of PHA's

Degradation in general is the reaction of polymers to obtain the monomers, usually either by enzymatic, microbial or hydrothermal hydrolysis. Mostly in PHA's it is divided into two categories: intracellular and extracellular. Intracellular degradation can take place after carbon source limitation during bacterial fermentation, leading to take the monomers and it has been observed that in terms of in-vivo degradation that it yields the maximum rate of hydrolysis. If the PHA is composed of only one type of monomer (for example only 3HB acid) it is classified as homopolymer, whereas when it is composed of more than one type of monomer it is heteropolymer. The enzymes found to degrade PHAs extracellularly, hydrolyze partially crystallized PHBs. In this case, it is noticed that the heteropolymer polymers are being hydrolyzed easier and better and homopolymer polymers and the main reason is the high crystallinity of homopolymer polymers in comparison with the heteropolymer PHAs that are consisted with more amorphous areas (Ong et al., 2017).

1.8. Applications of bioplastics

Nowadays, packaging is a main and highly important step to the supply chain of food industry and in general an important factor to the global market. Conventional plastics have been established throughout the years, making the process easiest and less costly, so their replacement from bioplastics

it’s not easy, but not infeasible either. According to recent research, there are bioplastics such as PLA and PHAs that are prosperous for the packaging industry. Considering the PLA, that is mainly used in packaging industry, it is a biopolymer that can be easily produced commercially through the polymerization of lactic acid. On the other hand, PHB is mainly produced through bacteria fermentations, and it is commonly found in combination with other bioplastics in packaging materials. This happens due to the high cost of the material, thus the improvement of the shape and the resistance of the final product.

The production of biobased packaging is based on the techniques used for regular packaging (Jariyasakoolroj et al., 2019). The highest amount of plastics globally are used from in packaging industries (2/5 of the total plastic production), making their replacement with bioplastics mandatory. The 60% of the plastics produced the last 5 years are destined for packaging, with a total production of 2 million tons. The main problems of bioplastics is the high production cost compared to conventional plastics, as well as their performance and characteristics which need further improvement. By replacing plastics with bioplastics, emissions will be greatly reduced and the packaging will cease to end up in landfills or in the environment, but there will be a green life cycle that will be recycled or reused in another form (processing and use of the main source of carbon, carbon dioxide, etc.).

Bioplastics has many applications in various sectors, with a lot of interest gaining the application in medicine. Bioplastics can be used for the production of pharmaceuticals, as they are good transporters to the appropriate cells, as well as for medical therapeutic devices, such as implants and tissue bones. Moreover, their use in medicine is successful, apart from their activity in a nanomolecular context, and due to their non-toxicity, which allows them to be used in vivo, without the risk of damage and negative impact on the immune system or the occurrence of other problems or diseases (Narancic et al., 2020).

1.8.1. Applications of PHAs

PHA's have been very attractive biopolymers in the industry in recent years due to their properties mentioned above and mainly for their biodegradability, showing that they are considered highly capable of replacing the petrochemical biopolymers commonly used in various sectors. The main sectors that seem to be able and willing to use bioplastic PHA's are the agricultural economy, the packaging industry, the food industry as well as the medical and cosmetics industry.

As far as medicine is concerned, the applications are various, for example implants, as a drug delivery tool (it is suitable for in vivo delivery of drugs that are hydrophobic and target specific cancer cells), technical valves for the heart, in transplant and tissue engineering but also candidates as therapeutic drugs to treat diseases such as Alzheimer's, osteoporosis or chondropathy (Zhang et al., 2018).

As far as agriculture is concerned, the applications are equally numerous. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] is a co-biopolymer of the group of PHA's that is used for the production of biodegradable films. These films have the ability to protect the various crops and therefore enhancing their efficiency. Agricultural nets are also used, which tend to replace nets made of petrochemical materials in nets usually made of biopolymer blends such as P(4HB) or P(3HB)/PLA. The main advantage of their use is non-toxicity and direct deposition and biodegradation in the soil (Kalia, 2019).

As far as the industry of packaging is concerned the use of various PHA's but more so PHB, is becoming more and more common. In particular, it is used as packaging in food products, but also as a food coating. Apart from their biodegradability and their flexibility as packaging, it has been proven through research that have been started since 2002, both their durability and serious benefits for longer shelf life of food, since it prevents contamination by a plethora of toxic food-related microorganisms such as the genera Listeria and Salmonella (Koller, 2014).

2. Purpose of this work

The aim of this research work is the chemical and biological recycling of biopolymers and the utilization of their degradation monomers as a carbon source for the production of poly(3-hydroxybutyrate). Two different bacterial strains were evaluated, regarding their ability of consuming the monomers of polylactic acid (PLA), polybutylene succinate (PBS), poly(3-hydroxybutyrate) (PHB) and cellulose acetate using either synthetic organic acids or hydrolysate derived after PLA and PHB degradation.

More specifically, the production of (PHB) was studied using the bacterial strains *Paraburkholderia sacchari* DSM 17165 and *Cupriavidus necator* H16, as well as the capability of the microorganisms to produce and accumulate PHB using the degradation monomers of PLA, PBS and PHB as carbon source.

Batch fermentations were carried out in shake flasks in liquid medium and the two bacterial strains were evaluated regarding their ability to consume different synthetic organic acids separately or in combination (lactic acid, succinic acid, acetic acid, crotonic acid, 3-hydroxybutyric acid). The chemical and hydrothermal degradation of PLA and PHB occurred. The produced PLA hydrolysate and combination of PLA and PHB hydrolysate were used as carbon source in bioreactor fermentation, using the two bacterial strains to produce PHB.

3. Materials and Methods

3.1. Microorganisms

The bacterial strains *Parabulchorderia sacchari* DSM 17165 and *Cupriavidus necator* H16, were used in bacterial fermentations, for the production of poly(3-hydroxybutyrate). Both bacterial strains were provided from the german microorganism and cell collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zelktulturen GmbH – German Collection of Microorganisms and Cell Cultures, Leibinz Institute, Germany). The preservation of the bacterial cells occurred in pure glycerol at -80°C.

3.2. Chemicals

Various organic acids were used as carbon sources in shake flasks fermentations. Acetic acid, crotonic acid, 3-hydroxybutiric acid, lactic acid and succinic acid were purchased from Sigma-Aldrich. Concentrated solutions were prepared in deionized water (dH_2O) at pH 6.8 using 5M NaOH solution.

3.3. Chemical and hydrothermal degradation of PHB and PLA

Hydrothermal hydrolysis of polylactic acid (PLA) was carried out. Duran bottles of 250ml, with 100ml active volume of distilled water were used to conduct these experiments. PLA was weighed at concentrations of 5%,10% and 20% PLA/water ratio. The experiments were carried out in autoclave at 140°C for 4h and subsequently for 3h.

Alkaline hydrolysis of PHB was carried out, using NaOH solution. For this degradation, 20 g of PHB in powder form was added to in 1 L aqueous solution of NaOH 0.6 M. The hydrolysis of PHB was performed in Duran glass bottles at the temperature of 70ºC. Samples were taken at regular intervals and analysed for soluble degradation products. The solution was filtered with a 0.2 μm membrane filter and the filtrate kept at 4ºC for later HPLC analysis. 2M HCl was added in the solutions to adjust the pH at 5-7.

3.4. Inoculum preparation

The preculture media for *P.sacchari* contained (per liter): 1g, $(NH_4)_2SO_4$, 4.5g $Na_2HPO_4 \times 2H_2O$, 1.5g KH_2PO_4, 0.2 g $MgSO_4 \times 7H_2O$, 1g yeast extract, 1mL trace elements and 20 g of the carbon source, which in this case was glucose. The trace elements contained (per liter): 10g $FeSO_4 \times 7H_2O$, 2.25g $ZnSO_4 \times 2H_2O$, 1g $CuCO_4 \times 5H_2O$, 0.5g $MnSO_4 \times H_2O$, 2g $CaCl_2 \times 2H_2O$, 0.23g $NaB_4O_7 \times 10H_2O$, 0.1g $(NH_4)_6MO_70_{24}$ and 35% HCl. For the preculture media for *C. necator* H16 the composition was the following (per liter): 10g meat extract, 10g peptone and 2g yeast extract. The pH of the medium of *P. sacchari* was adjusted to 6.8 and for the medium of *C. necator* was adjusted to 7, with the addition of 2M HCl solution. The inoculum for both bacterial strains was incubated in 500

mL baffled flasks, with 100 mL active volume, at 30ºC and 250 rpm for 14-16h for the *P. sacchari* and 12-14h for *C. necator*. The optical density was measured at 600 nm and when the value was 8-10, 10% of the inoculum was transferred in shake flasks and bioreactor fermentations.

3.5. Shake flasks fermentations for PHB production

Batch fermentations were carried out in 500 mL baffled flasks containing 100 mL medium Initially, different organic acids in different concentrations and combinations of them were used as carbon source for the production of PHB, using the bacterial strains, *P. sacchari* DSM 17165 and *C. necator H16.* More specifically the following concentrations were used for the *P.sacchari* cultivations: 8.2 g/L and 16.16 g/L succinic acid (SA), 9.67 g/L and 20.51 g/L lactic acid (LA), 3.4 g/L and 7.67 g/L acetic acid (AA), 4.64 g/L and 9.69 g/L 3-Hydroxybutiric acid (3HBA), 7.85 g/L and 20.66 g/L PLA hydrolysate, as well as mixtures of organic acids such as 8.57 g/L 3HBA and Crotonic acid (CA), 9.62 g/L 3HBA and LA, CA 8.9 g/L LA, 3HBA and SA, 10.63 g/L 3HBA and CA, 15.77 g/L LA and SA, 9.5 g/L LA and CA and 9.8 g/L SA and CA. The concentrations used for *C. necator* H16 cultivations were: 9.16 g/L SA, 10.34 g/L LA, 7.72 g/L CA, 8.47 g/L 3HBA:CA:LA and 11.07 g/L 3HBA: CA.

The fermentation experiments were carried out at a temperature of 30°C under a stirring rate of 180 rpm. The pH was adjusted to 6.8 by adding 1M HCl or 1 M NaOH. The final volume of nutrient medium was 100 mL in 500 mL shake flasks for *P. sacchari* and 50mL in 250 ml shake flasks for *C. necator*. To monitor the fermentations, samples were taken in regular period of times under aseptic conditions to monitor the sugars consumed and determine the biomass and final product that was produced.

3.6. Bioreactor fermentation for PHB production

Batch and fed-batch fermentations were carried in a 2L bioreactor (Eppendorf BioFlo 120), with a working volume of 700 mL, using both the bacterial strains *P.sacchari* and *C.necator* H16. The composition of fermentation media was (per liter): 4g $(NH_4)_2SO_4$, 3g KH_2PO_4, 1.7g citric acid, 40mg EDTA and 10mL trace elements.

In batch and fed-batch fermentations, 10 g/L and 20 g/L lactic acid was used as a carbon source respectively, with the bacterial strain *P.sacchari*. In the fed-batch fermentation a concentrated solution of 460.39 g/L lactic acid was added as feeding solution during the fermentation process. The pH was adjusted to 6.8-7.0 using a solution of 2M NH_4OH and 5M HCl. The aeration rate used was 2.5 vvm and the agitation was kept constant at 900 rpm.

A fed-batch fermentation with pulses feeding strategy was carried with the bacterial strain *C. necator* H16, using a mixture of PLA hydrolysate after hydrothermal degradation and PHB hydrolysate after alkaline hydrolysis using NaOH solution. The initial concentration of the mixture was 10 g/L (70% LA, 20% 3HB and 10% CA). The composition of fermentation media was (per liter): 5g $Na_2H_2PO_4 \times 2H_2O$, 11.6g $Na_2PO_4 \times 12H_2O$, 0.54g urea, 0.06g $CaCl_2 \times 2H_2O$, 0.39g $MgSO_4 \times 7H_2O$,

1g peptone, 1g meat extract and 0.4g yeast extract. The pH was adjusted to 7.0 using a solution of 2M NaOH and 5M H_3PO_4. The aeration rate used was 1.5 vvm and the agitation was kept constant at 800 rpm. When required, antifoam was added to the bioreactor (Loan et al., 2022).

Sterilization of the media, instruments and consumable products used in the microbial fermentations was carried out in an autoclave at 121°C for 20 minutes. Commercial sugar and acids were sterilized separately from the other growth medium after being dissolved in a small amount of deionized water to avoid a Maillard reaction between the sugars and the nitrogen content in the growth medium. After sterilization, they were mixed under aseptic conditions with the respective growth medium in a vertical filament flow chamber to avoid contamination.

3.7. Analytical methods

3.7.1 Quantification of dry cell weight

The measurement of the dry cell weight (DCW) was used as the basis for calculating the biomass concentration. To separate the cells from the fermentation liquid, the samples were centrifuged in a Heraeus - Biofuge Pico centrifuge for 8 min at 10,000 rpm. The biomass was rinsed with deionized water, centrifuged again, rinsed again with acetone, and transferred to McCartney vials that had previously been dried and weighed. The supernatant was then collected for additional analyses. The entire thing was kept in a drying oven for about a day at a constant temperature of 50°C until the weight of the vials was constant. The vials were then put in a desiccator to stabilize them at room temperature before being weighed on an analytical balance. From the difference between the weight and the gross weight of the vial - biomass, the net weight of the dry cell mass was obtained and by extrapolating the measurement obtained, the final dry cell mass concentration value was calculated in g/L.

3.7.2 Determination of the concentration of organic acids

The organic acid monomers were analyzed by liquid chromatography (HPLC) using the SHIMADZU UFLC XR system, equipped with a Rezex ROA-organic acid H+ column (300 mm length x 7.8 mm internal diameter, Phenomenex), with a mobile phase of 10 mM H2SO4 at 0.6 mL/min flow rate and 65°C. 10 μL of sample was injected and the duration of the analytical method was 24 minutes (for lactic acid, succinic acid and acetic acid) and 40 minutes (for 3HB acid and crotonic acid).

3.7.3 Quantification of intracellular PHB

The concentration of PHB was determined based on the production of 3-hydroxybutyric acid to propyl ester (Riis & Mai, 1988). Specifically, the dried biomass of each sample was treated as follows: Firstly, 2 mL of 1,2-dichloroethane was added to each sample. Subsequently, 2 mL of acidified propanol solution (HCl:n-propanol 1:4) was transferred to the suspension with subsequent addition of 200 μL of benzoic acid, which was used, as an internal standard for the calibration curve of the method.

After the addition of each reagent, 20 seconds of stirring in vortex was followed and then the samples were placed in a water bath at 100°C for 2 hours. After this time, the samples were removed from the water bath and left to cool until reaching room temperature. Phase separation was achieved by adding 4 mL of deionized water, stirring for another 20 seconds and left them to stabilize.

The organic phase was collected and transferred to small vials with sodium anhydrous sulphate (Na_2SO_4) in order to avoid any moisture of the sample. Then, gas chromatography was used to calculate the PHB concentration.

Gas chromatography (GC) was used for the calculation of intracellular PHB. The gas chromatograph (Shimadzu, Nexis GC 2030) that was used has an automatic sampler (AOC-20i plus), a Flame Ionization Detector (FID) and a Mega-Wax column (30 m × 0.25 mm, film thickness 0.25 μm, MEGA S.r.l.). Helium was used as carrier gas at a flow rate of 1 mL/min. The initial oven temperature was 100°C for one minute. Then at a rate of 25 °C/min it was raised to 160°C, where it remained constant for another 1 min, and then at a rate of 10°C/min it was increased to 188°C. Immediately afterwards, at a temperature rate of 25°C/min the temperature rose to 250°C where it remained for 5 min. Benzoic acid was used as a standard solution at a concentration of 200 mg/L and the standard curves in the gas chromatography was determined with the use of commercial monomers.

4. Results and Discussion

4.1. Degradation of PLA and PHB

The degradation of polylactic acid (PLA) was carried out by hydrothermal treatment in the autoclave for 3h and 4h at 140°C. The PLA degradation occurred in Duran bottles of 250 mL, with a working volume of 100mL. In the case of 3h treatment the concentration of PLA solution was 50 g/L, while in the case of 4h treatment different concentrations were tested (50 g/L, 100 g/L, 150 g/L, 200 g/L). In all cases the complete degradation of PLA was achieved (100% PLA recovery).

The degradation of PHB was carried out by using 0.6M NaOH solution at 70 ºC. for 24h. The total PHB degradation achieved was 100% and the composition of the produced hydrolysate was 60% 3-hydroxybutyric acid and 40% crotonic acid.

Piemonte and Gironi (2012), have tested the degradation of PLA at 160°C and 180°C, with different initial solids concentration (50 g/L and 100 g/L) and studied the kinetic degradation of PLA during the whole degradation process. In the case of 50 g/L PLA solution, the degradation of PLA at 160°C after 2h and 3h was higher than 90% in both cases (94.02% and 98.97% respectively). At the same temperature but with an initial solid concentration of 100 g/L, the degradation of PLA was 97% at around 105 min. At a higher temperature of 180°C, with an PLA solution of 50 g/L and 100 g/L, the degradation after 1h and 15 min was around 97%. A theoretical model was used in order to predict the degradation of PLA at lower temperatures, and it seems that PLA solutions with a concentration of 100 g/L and 200 g/L can be completely degraded at 140°C for 5h treatment. Elsawy et al. (2017), studied the hydrolysis of D- and L- oligomers of lactic acid by testing different pH values (e.g., 1.5, 4.5, and 7.4) at 65°C. The results showed that the rate of decomposition was faster at the extreme pH values, i.e., 1.5 and 7.4, than at the milder pH values (4.5).

Yu et al., (2005) studied the chemical hydrolysis of PHB using alkaline solutions at 70°C and the produced hydrolysate contained the 3HB and crotonic acid monomers. Different concentrations of sodium hydroxide solutions were used in order to study the kinetic of the degradation in each case. The results showed that at a concentration of 0.1M NaOH for 4h, the degradation of PHB was less than 5%, while at a concentration of 4M NaOH for 4h, more than 70% of PHB had been degraded.

4.2. Shake flasks fermentations using Paraburkholderia sacchari DSM 17165 for PHB production

Batch fermentations in shake flasks were carried out, using the bacterial strain *P. sacchari* DSM 17165. Various organic acids, as well as the degradation monomers derived after the hydrothermal and chemical degradation of PLA and PHB respectively, were evaluated as carbon sources. These experiments were conducted in order to evaluate the ability of *P. sacchari* to efficiently consume the degradation monomers of different biopolymers (i.e PHB, PLA) as well as accumulate intracellular PHB. The carbon source was efficiently consumed in all cases in parallel with bacterial growth and

PHB accumulation, while the mixtures LA:3HB:CA and SA:3HB:CA were not fully consumed by the bacterial strain.

Table 2 shows the carbon source consumption, TDW, PHB concentration, PHB content and yield achieved in shake flask cultures of *P.sacchari* with succinic acid, lactic acid, acetic acid, 3HB and mixtures of monomers such as 3HB:LA, 3HB:CA, LA:SA, LA:CA, SA:CA, LA:3HB:CA, SA:3HB:CA, as well as PLA and PHB hydrolysate. Commonly, biopolymers are used as a blend for packaging purposes in order to achieve better thermal and chemical properties of the final material. These experiments were carried out to study the ability of the bacteria to consume mixtures of biopolymers' monomers as well as produce new PHB in order to achieve a full biological recycling of biopolymers, producing new PHB. The concentration of the carbon source in all fermentations was 5-20 g/L. Concentrations higher than 20 g/L have been observed that can be inhibitory for bacterial growth.

Table 2 PHB production efficiency using various organic acids as carbon sources in shake flask cultures of *P. sacchari*

Carbon source	Consumed carbon source (g/L) [1]	TDW (g/L)	PHB (g/L)	PHB content (%)	Yield (g/g)
Succinic acid	8.2	5.6	2.07	37.06	0.25
Succinic acid	16.16	7.03	3.65	51.93	0.23
Acetic acid	3.40	1.50	0.63	42.09	0.18
Acetic acid	7.67	3.5	1.35	38.70	0.16
3HBA	4.64	2.1	1.00	47.60	0.22
3HBA	9.69	4.5	2.62	62.41	0.27
3HBA+CA	8.57	3.80	2.06	54.30	0.24
Lactic acid	9.67	5.7	2.59	45.46	0.27
Lactic acid	20.51	7.80	5.55	71.18	0.27
LA+CA	9.5	4.2	1.17	36.76	0.17
SA+CA	9.8	4.1	0.78	18.6	0.07
LA+SA	15.77	8.25	4.28	51.83	0.27
LA+3HBA	9.62	4.5	3.1	68.54	0.32
Hydrolysate PLA	7.85	3.8	1.59	41.8	0.20
Hydrolysate PLA	20.66	9.58	5.04	52.65	0.24

LA+3HBA+CA	NOT CONSUMED
SA+3HBA+CA	NOT CONSUMED

Shake flasks fermentations were carried out, using succinic acid solution at different initial concentrations (8.2 g/L and 16.16 g/L) as carbon source. In all cases the bacteria's growth was successful, and the succinic acid has been efficiently consumed. The carbon source in both cases was completely consumed until 8h and 15h respectively. The total dry weight for each concentration was around 2.07 g/L and 3.65 g/L, while the highest PHB content was achieved in the case of 16.16 g/L initial succinic acid concentration, reaching up to 50%, while the PHB content in the case that lower initial concentration of succinic acid was used, the PHB accumulation was around 37%. The yield of the fermentations was 0.25 g/g and 0.23 g/g respectively.

In the case of acetic acid, two batch fermentations were conducted using 3.40 g/L and 7.67 g/L initial acetic acid solution. The fermentation using 3.40 g/L carbon source, resulted in 1.50 g/L total dry weight, with a PHB content of 42.09%. In the case of 7.67 g/L initial carbon source concentration, 3.5 g/L of total dry biomass were produced containing 38.70% PHB. The yield of both fermentations were 0.18 g/g and 0.16 g/g respectively.

Commercial 3-hydrocybutyric acid, which is one of the monomers derived after the degradation process of PHB, was also used as carbon source for the production of PHB. The initial carbon source concentrations that were used in shake flasks fermentations were 4.64 g/L and 9.69 g/L of 3HB. In the case of 4.64 g/L the produced dry biomass was around 2.1 g/L, with a PHB content of 47.60% and the yield of fermentation was 0.22 g/g. In the case that 9.96 g/L of 3HB were used as initial carbon source, the resulted total dry weight was 4.5 g/L, with a PHB content of 62.41% and the yield of the fermentation was 0.27 g/g.

Lactic acid was used as carbon source in shake flasks fermentations, as it constitutes the monomer of PLA, at a concentration of 10 g/L and 20 g/L. When a concentration of 10 g/L was used, the carbon source was completely consumed at around 9h, but in the case higher LA concentration (20 g/L) the bacteria couldn't completely consume it until 25h. The total dry weight in both fermentations was around 5.7 g/L and 7.8 g/L, while the PHB content was 45.46% and 71.18% respectively. The yield in both cases was 0.27 g/g.

Batch fermentations were also carried out using as carbon source a mixture of the different monomers, in order to study the ability of the bacterial strain to consume them simultaneously. It is common in packaging applications, blends of biopolymers to be used in order to achieve better propertied of the final product. For example, blends of PHB and PLA are often used as packaging material, in order to enhance the stability, flexibility and durability of the final packaging.

In fermentations that only crotonic acid was used as carbon source, the bacterial strain couldn't efficiently consume it and accumulate intracellular PHB. When CA was used as carbon source combined with 3HB and/or LA and SA, it was completely consumed in most cases. In all the fermentations that CA was added as carbon source, the consumption of carbon sources was slower than the other fermentations and the complete consumption was achieved after 25h. Initially a mixture of 3HB:CA was tested as carbon source in shake flasks fermentations. These two monomers can be produced after the alkaline degradation of PHB, using sodium hydroxide solutions. The initial concentration of the mixture was 10 g/L, containing 60% 3HB and 40% CA. Both organic acids were consumed simultaneously during the fermentation. An initial concentration of 8.57 g/L of the mixture was used which was totally consumed until 28h of fermentation. The accumulation of PHB (54.3%) was lower than the case that 3HB was used alone as a carbon source and the yield of the fermentation was around 0.24 g/g. CA was also used as carbon source in combination with LA with a total initial concentration of 9.5 g/L. The fermentation resulted in 4.2 g/L total dry weight with a 36.76% PHB accumulation and the yield was 0.17 g/g. Finally, a mixture of SA and CA (9.8 g/L) were used as carbon, resulting in 4.1 g/L total biomass production with a very low PHB content (18.6%).

The next fermentation was conducted using a mixture of 3HB and LA as carbon source at a concentration of 9.53 g/L (50% 3HB and 50% LA). The consumption of LA was much slower when 3HB was present in the fermentation and the mixture was totally consumed at around 25h. The total dry weight was 5.02 g/L, achieving a very high PHB content of 53.08%. The yield of the fermentation was 0.29 g/g. A mixture of 50% SA and 50% LA with a concentration of 15.77 g/L was also used as carbon source. The mixture was completely consumed after 25h, and the total dry weight was 8.25 g/L with a PHB content of 51.83%.

Lactic acid derived from the hydrothermal degradation of the PLA was also used as carbon source in shake flasks fermentations. The hydrolysate concentrations that were used were 7.85 g/L and 20.66 g/L. The consumption rate seems to be similar to the fermentations where commercial lactic acid was used. The total dry weight was measured around 3.8 g/L and 9.58 g/L and the PHB content was 41.8% and 52.65% respectively. The yield of the fermentations was 0.20 and 0.24 g/g.

Finally, fermentations using mixtures of commercial 3HB, CA and LA as well as 3HB, CA and SA were also carried out. In both cases, the initial carbon concentration was around 10 g/L, containing 33% of each organic acid. The results showed that these combinations of organic acid couldn't be consumed and they inhibit the growth of the bacteria.

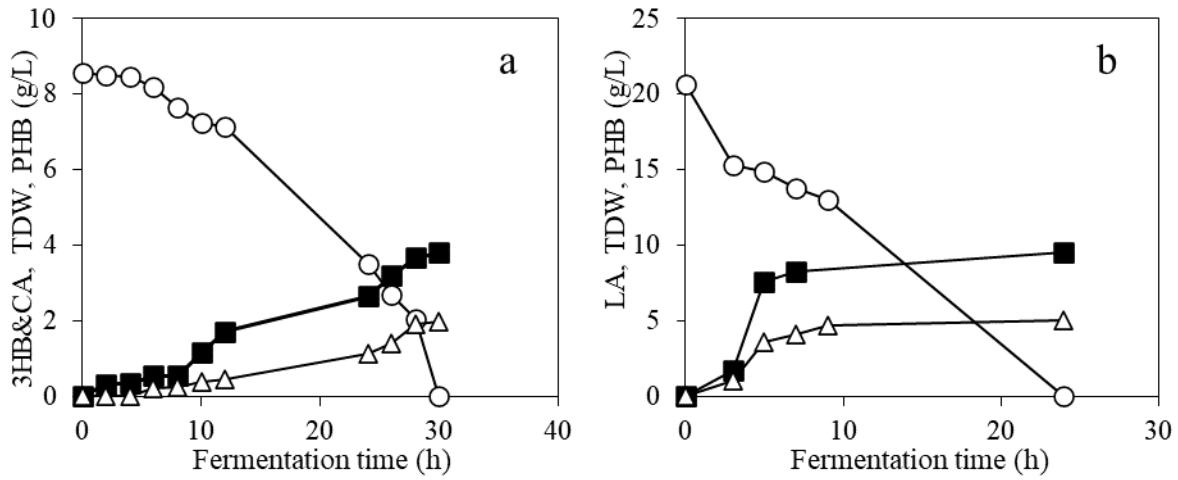


Figure 15 Shake flask fermentation for PHB production using (a) a mixture of 3HB:CA as carbon source with the bacterial strain *P. sacchari* and (b) PLA hydrolysate as carbon source with the bacterial strain *C. necator H16*

Table 3 shows results reported on literature-cited publications about PHB production using various carbon sources and bacterial strains. More specifically, Al-Battashi et al. (2019) used wastepaper as carbon source in batch shake flasks fermentations with *P. sacchari*. The initial concentration of the consumed hydrolysate was around 10.5 g/L. The dry biomass produced, 3.63 g/L, with a PHB content of 44.2% and a yield of 0.15 g/g. Zoghbi et al. (2023), conducted fed-batch fermentations, by adding glucose first and then crotonic acid as carbon source at a concentration of 11.1 g/L. CA was successfully consumed and the produced total dry biomass was 4.1 g/L. PHB content was 41.6% and the yield of the fermentation was 0.22 g/g. Fed-batch fermentations with different combinations of monomers were also carried out using the bacterial strain *P. sacchari*. Initially, fermentations were performed using a mixture of glucose (7.3 g/L) and 3HB acid (4.3 g/L), with subsequent addition of additional 3HB acid (6 g/L). The total carbon source consumed was 16.1 g/L, with the total dry weight reaching 7.9 g/L , with a PHB content of 45%. In another fermentation mixture of glucose and 3HMB were used as carbon source, followed by the addition of more 3HMB. The total consumed carbon source was 12.3 g/L. The total dry biomass was measured 5.7 g/L, with a PHB content of 31.6% and a yield of 0.15 g/g. According to the literature, various sugars have been tested alone or in combination in fermentations (glucose, sucrose, fructose, xylose, propionate, arabinose), as well as many industrial wastes such as softwood hemicellulose. The highest PHB contents achieved using sugars as carbon source was, 68.4% with sucrose and 71% with xylose (Gomez et al., 1996; Lopes et al., 2009; Guaman et al, 2018). When mixtures of sugars were used as carbon source, the highest PHB contents achieved were 74.6% when glucose and fructose were used, 65.9% with sucrose and propionate and 72.6% with γ-butyrolactone and sucrose. However, when softwood hemicellulosic hydrolysate was used as carbon source in order to produce PHB, a content of 80.5% was achieved (Dietrich et al., 2017; Oliveira-Filho et al., 2021).

Table 3 PHB fermentation using various carbon sources and bacterial strains reported in literature-cited reports.

Carbon source	Microorganism	Consumed carbon source (g/L) [1]	TDW (g/L)	PHB (g/L)	PHB content (%)	Yield (g/g)	Reference
Hydrolysate waste paper	*P. sacchari*	10.5	3.63	1.61	44.5	0.15	Al-Battashi et al. (2019)
Glucose + Crotonic acid	*P. sacchari*	11.1	4.1	1.71	41.6	0.22	Zoghbi et al. (2023)
Glucose + 3HB acid	*P. sacchari*	16.1	7.9	3.55	45	0.22	Zoghbi et al. (2023)
Glucose + 3HMB	*P. sacchari*	12.3	5.7	1.8	31.6	0.15	Zoghbi et al. (2023)
Softwood hemicellulosic hydrolysate	*P. sacchari*	36.32	7.1	5.72	80.5	0.16	Dietrich et al., 2017
Sucrose	*P. sacchari*	14.14	6.1	4.1	68.4	0.29	Gomez et al., 1996
Xylose	*P. sacchari*	15	8.02	5.7	71	0.39	Guamán et al., 2018
Glucose + Fructose	*P. sacchari*	15	5.5	4.1	74.6	0.29	Guamán et al., 2018
Molasses	*C. necator* ATCC *25207*	34.7	15.28	0.17	52.89	0.44	Ertan et al., 2021
Wheat straw	*C. necator* DSM *545*	-	15.1	0.017	80.1	-	Soto et al., 2019
Wheat bran	*C. necator* NCIMB 11599	46.46	24.5	14.82	62.5	0.319	Annamalai and Sivakumar, (2016)
Lactic Acid	*C. necator* H16	20	9.35	5.8	62	0.29	Linko et al., 1993

4.3. Shake flasks fermentations using Cupriavidus necator H16 for PHB production

Batch fermentations in shake flasks fermentation for PHB production were also carried out employing the bacterial strain *Cupriavidus necator* DSM 428 (H16). Commercial organic acids, such as succinic, acid, lactic acid, crotonic acid, and combinations of 3HB:CA, and LA:3HB:CA were studied as potential carbon sources in microbial fermentations. The initial concentration of the organic acids and the mixtures was around 10 g/L in all cases. The results of the fermentations are presented in Table 4.

In all fermentations the medium used was the same as the preculture medium, which contains a high amount of meat and peptone. The total dry weight in all cases was very high with a very low PHB accumulation. These results can be attributed to the composition of the nutrient medium as it contains a high amount of nitrogen, thus an extra amount of carbon which can be consumed by the bacteria in order to produce biomass.

Lactic acid was used as carbon source in shake flasks fermentations with an initial concentration of 10.34 g/L. The carbon source was completely consumed until 11h of fermentation and the produced total dry weight was 13.85 g/L. The dry biomass production reached 13.85 g/L, but the percentage of PHB reached 17% of PHB to biomass ratio. The product yield factor reached 0.22 g/g.

According to Linko et al., (1993), corresponding fermentations were performed in a bioreactor with initial concentrations of 5 g/L, 10 g/L and 20 g/L with lactic acid. The highest amount of PHB recovered was 5.8 g/L, while in the corresponding experiments conducted in shake flasks, the amount of PHB was 3 g/L, which is the same as with the corresponding fermentations conducted in this study (2.864 g/L PHB). The fermentation time was around 25 h, while in this study the fermentations lasted 11h. The yield of the fermentation was 0.29 g/g

Succinic acid was used as carbon source in a concentration of 9.16 g/L. The carbon source was completely consumed at 20h, with a total dry weight of 9.4 g/L. The results shows that the consumption rate of SA using the bacterial strain *C. necator* is much slower than this of *P. sacchari*, while the PHB accumulation is also very low (8%).

Crotonic acid was also tested in a concentration of 7.72 g/L and was completely consumed until 15h. The dry biomass was around 8.2 g/L, with the PHB with a very low PHB content of 6.64%. It seems that CA is not a suitable carbon source for bacterial fermentations, as bacteria can't use it in order to accumulate intracellularly PHB.

Subsequently, fermentations with combinations of monomers were carried out. Initially, PHB hydrolysate, produced after the alkaline degradation of PHB using NaOH solution. The total initial of the hydrolysate was 8.47 g/L, with 60% 3HB acid and 40% crotonic acid. The carbon source was fully consumed at 20h with a produced biomass of 9.25 g/L. The PHB content (23.19%) was much higher than the previous experiments and the yield of the fermentation was 0.26 g/g.

Finally, a mixture of LA:3HB:CA was used as carbon source in a concentration of 11.07 g/L (50% LA, 30% 3HB and 20% CA). The carbon source was fully consumed at around 10-12h, with the

monomers being consumed in parallel. The dry biomass produced was 10 g/L with a PHB content of 17.05% and a yield of 0.17 g/g.

Table 4 PHB production efficiency using various organic acids as carbon sources in shake flask cultures of *C. necator* H16

Carbon source	Consumed carbon source (g/L) [1]	TDW (g/L)	PHB (g/L)	PHB content (%)	Yield (g/g)
Succinic acid	9.16	9.4	0.53	7.95	0.06
Lactic acid	10.34	13.85	2.35	16.9	0.22
Crotonic acid	7.72	8.2	0.54	6.64	0.07
3HBA+CA	8.47	9.25	2.15	23.19	0.26
LA+3HBA+CA	11.07	10	1.82	17.05	0.17

Rodríguez-Contreras et al. (2015), examined bioreactor fermentations using two different bacterial strains, *P. sacchari* and *Cupriavidus necator* H16, with glycerol as the primary carbon source. When glycerol was used as carbon source, a high PHB accumulation was achieved by *C. necator*, reaching up to 64.55%, while in the case *P. sacchari* the total PHB accumulation was 10.22%.

Haas et al. (2015), used different species of *Cupriavidus necator*, such as DSM 428 (H16), DSM 531 and DSM 545. Through these studies the PHB accumulation reached 46-78% and the total dry biomass varied between 3-14 g/L. Fatty acids derived from food industry wastes were also used as carbon source in by *Cupriavidus necator*. The highest percentage PHB content (63%) was achieved in the case that a mixture of cooking oils was used as carbon source. Ertan et al. (2021), used different sugars as a carbon source for microbial PHB production. The highest PHB yield was achieved by mixing acids, with the total dry biomass being 12 g/L and a PHB accumulation of 56%, demonstrating that the mixing of different sources and nutrients achieves this high productivity. Wheat hydrolysate has also been used for the production of PHB by Annamalai and Sivakumar (2016). The PHB accumulation achieved in *C. necator DSM 545* fermentations was 62.5%. Soto et al. (2019) have achieved a higher PHB accumulation (80%) using the bacterial strain *Cupriavidus necator DSM 545* with wheat hydrolysate.

4.4. Bioreactors fermentations for PHB production

Batch and fed-batch fermentations were conducted using the bacterial strain *P. sacchari* and *C.necator* H16, with PLA and a mixture of PLA:PHB hydrolysate respectively. Initially, a batch bioreactor fermentation using PLA hydrolysate, derived after the hydrothermal degradation of PLA, was conducted in order to study the ability of the bacterial strain *P. sacchari* to consume the carbon source and accumulate PHB. A fed-batch fermentation was followed using PLA hydrolysate as carbon

source and as feeding solution. The ability of the bacterial strain *C. necator* to consume the mixture of PLA and PHB hydrolysate, after the alkaline degradation of PHB with NaOH solution, was also studied. A fed-batch fermentation using a mixture of PLA and PHB hydrolysate as initial carbon source and as feeding solution, with pulse feeding strategy was conducted.

At the first set of experiments a batch fermentation was conducted using 8.55 g/L PLA hydrolysate with the bacterial strain *P. sacchari,* in order to study the ability of the microorganism to consume the carbon source. Lactic acid was totally consumed at 8h with parallel bacterial growth. A fed-batch fermentation was conducted using 18.15 g/L PLA hydrolysate as carbon source and then a feeding solution of concentrated PLA hydrolysate was added (Figure 15). A phosphorus limitation strategy was followed, with an initial concentration of inorganic phosphorus being approximately 600 mg/L. the PHB accumulation began at around 13h, when the inorganic phosphorus was significantly low. The produced total dry weight was 27.7 g/L with a PHB content of 71.6%. The yield of the fermentation was 0.25 g/g and the productivity was 0.5 g/L/h.

Figure 16 shows the efficiency of PHB production by the bacterial strain *C. necator* H16, using PLA and PHB hydrolysate as initial carbon source and as feeding solution. A fed-batch fermentation with pulses feeding strategy was conducted using 9.77 g/L initial carbon source. The composition of the hydrolysate was 70% LA, 30 g/L 3HB and 10 g/L CA. After 23h of fermentation the total dry weight was around 9.8 g/L with a PHB content of 50%. The yield of the fermentation was 0.25 g/g and the productivity was 0.21 g/L/h.

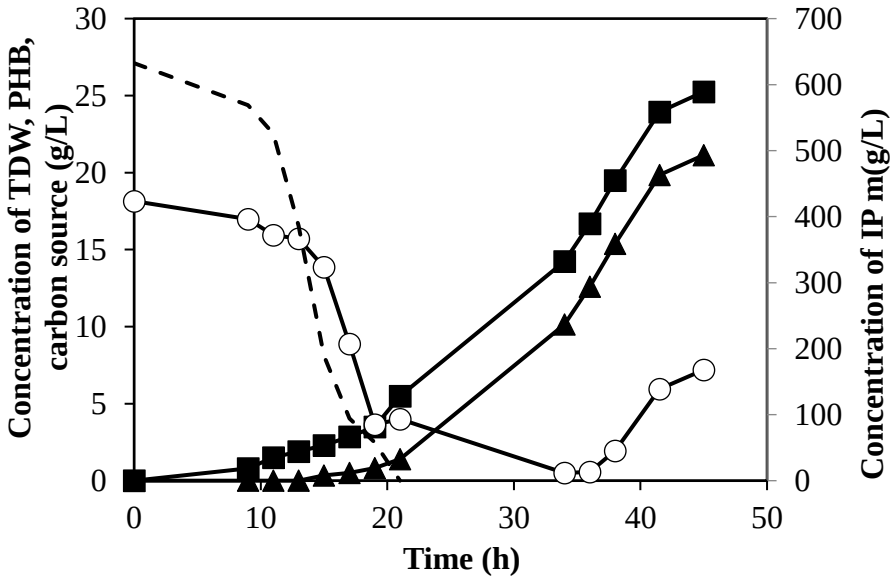


Figure 16 Concentration of LA (○), TDW (■), PHB (▲) and IP (▬) in fed-batch bioreactor fermentation using PLA hydrolysate as carbon source, with the bacterial strain *P.sacchari*.

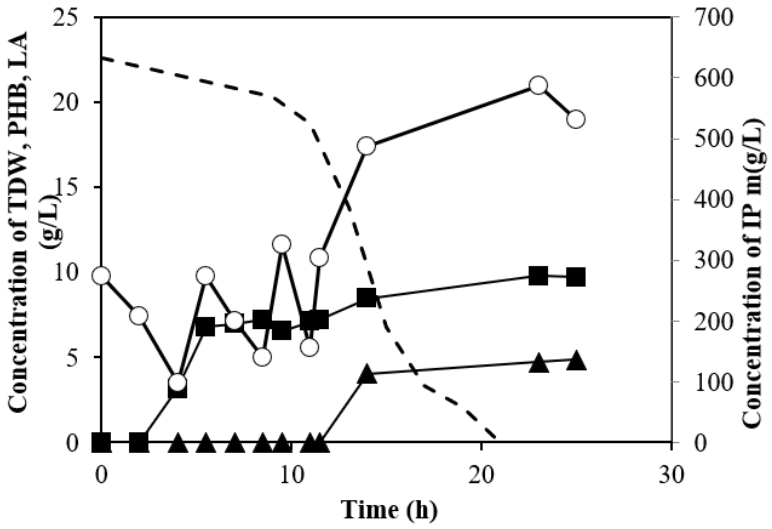


Figure 17 Concentration of total carbon source (○), TDW (■), PHB (▲) and IP (▬) in fed-batch bioreactor fermentation using PLA and PHB hydrolysate as carbon source, with the bacterial strain *C. necator*

Table 5 Results of fed-batch bioreactor fermentations using different bacterial strains and different carbon sources

	Bacterial strain	TDW (g/L)	PHB (g/L)	PHB content (%)	Yield (g/g)	Productivity (g/L/h)
PLA hydrolysate	*P. sacchari*	27.7	23.59	71.63	0.25	0.50
PLA and PHB hydrolysate	*C. necator* H16	9.8	3.82	39.00	0.25	0.21

Psaki et al. (2023), has also studied the cultivation of the bacterial strain *P. sacchari* in fed-batch bioreactor fermentations, using mixed sugars as carbon and nutrient source. Different initial sugars concentrations were tested and in the case of 65 g/L, the highest PHB production was achieved. The resulted PHB content was 57.4%, while the highest total dry weight was obtained in the case of 40 g/L initial sugars concentration, reaching up to 149.1 g/L. The yield of the fermentation was 0.32 g/g and 0.31 g/g respectively.

Nygaard et al. (2021) performed fed batch fermentations in bioreactors with the bacterial strain *Burkholderia sacchari* DSM 17165. Glycerol was used as carbon coagulant reaching productivity of 0.08 g/L h. Fermentations were also performed with combinations of carbon sources: 1) glucose with glycerol and 2) glycerol with xylose. In the first case, the total consumption of the carbon source

reached 4.89 g/L, while PHB reached 0.63 g/g glucose and 0.094 g/g glycerol. In the second fermentation, the efficiency reached 0.22 g/g with the PHB reaching 62%.

Nygaard et al. (2021), also carried out fermentations in bioreactors with the bacterial strain *Cupriavidus necator* ATCC 17697. Fructose was used as carbon source and a small concentration of ammonium sulfate as nitrogen source. Initial concentration in one case was 20 g/L fructose with 1.5 g/L ammonium sulfate. The fermentation was completed at 31 h with dry biomass reaching 7.9 g/L and the produced PHB was 3.4 g/L (42%). A batch fermentation was also carried out using higher initial fructose concentration (40 g/L), with the dry biomass reaching 14.4 g/L and PHB 9.9 g/L (69%).

5. Conclusions

In the present study, the bacterial strains *Paraburkholderia sacchari* DSM 17165 and *Cupriavidus necator* DSM 428 (H16) were evaluated regarding their ability to grow and accumulate the biopolymer PHB, using as carbon source different commercial organic acids (lactic acid, succinic acid, acetic acid, crotonic acid, 3-hydroxybutiric acid with carbon source) as well as monomers derived after the hydrothermal and alkaline degradation of PLA and PHB respectively. Fermentations using mixtures of these monomers were also performed to evaluate the ability of the different bacterial strains to efficiently consume these mixtures and produce PHB.

Based on the experiments conducted, the following conclusions were drawn:

- The bacterial strain *P. sacchari* can efficiently consume all the organic acids tested as carbon source in shake flasks fermentations and produce PHB.
- Mixtures such as LA:3HB:CA and SA:3HB:CA couldn't be consumed by *P. sacchari*, hindering the growth of the microorganism.
- When CA was used combined with other organic acids as carbon source, the efficiency of PHB fermentations using *P. sacchari* was significantly lower than the fermentations where the organic acids were used alone.
- When 20.51 g/L lactic acid were used as carbon source, the highest PHB accumulation by *P. sacchari* was achieved (71.18%), with a PHB production of 5.55 g/L and a total dry weight of 7.80 g/L.
- The consumption of 20.66 g/L PLA hydrolysate by the *P. sacchari* resulted in the production of 9.58 g/L total dry weight and 5.04 g/L PHB.
- The nutrient medium used in this study for shake flasks cultivation of *Cupriavidus necator* (H16) is not suitable for the efficient production of PHB.
- The different organic acids used alone or as mixtures, can be consumed by *C. necator* H16 in shake flasks fermentations.
- PHB accumulation in shake flasks fermentations using *C. necator* H16, was much lower than the respective shake flasks fermentations using *P. sacchari*.
- Fed-batch bioreactor fermentation using PLA hydrolysate as carbon source with the bacterial strain *P. sacchari*, resulted in 27.7 g/L total dry weight (TDW) with a PHB content of 71.63% and a yield of 0.25 g/g.
- Fed-batch bioreactor fermentation using a mixture of PLA and PHB hydrolysate as carbon source, with the bacterial strain *C. necator* H16, resulted in a TDW of 9.8 g/L with a PHB content of 39% and a yield of 0.25%.

6. Future work

The results from this work have created new questions that need to be studied further. Initially, a gradual adaptation of the bacterial strain *C. necator* H16 to a synthetic medium should be carried out, in order to improve the ability of consuming the different organic acids in combination with PHB accumulation. As a next step more organic acids alone or as mixtures should be tested as carbon sources for both bacterial strains and compare the efficiency of the fermentations. More monomers derived from the degradation of different biopolymers, like 1,4-butanediol derived after the degradation of PBS, should be studied as carbon sources in microbial fermentations. Moreover, the optimization of fermentations using both bacterial strains should be conducted, in order to improve the efficiency of PHB production. More bacterial strains should be also studied, regarding their ability of consuming the different monomers derived from the degradation of different biopolymers, in order to achieve a sustainable and efficient recirculation of post-consumer biopolymers.

7. Literature

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