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

Chemical degradation of poly(3-hydroxybutyrate) for efficient biopolymers recirculation

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ATHENS 2023

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Χημική αποικοδόμηση του πολύ(3-υδροξυβουτυρικού) εστέρα με σκοπό την αποτελεσματική ανακύκλωση βιοπολυμερών

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Department of Food Science & Human Nutrition
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Abstract

Plastics have become an omnipresent part of modern life with their mass production increasing continuously. However, their environmental impact has become extremely alarming during the last years. Plastic pollution is pervasive, with waste management lacking, and plastic waste accumulating in landfills, oceans, and ecosystems globally. These non-biodegradable materials take hundreds of years to decompose, releasing harmful chemicals, and posing a significant threat to the environment, wildlife, and even human health. Thus, the urgent need for the development and adopting of alternative biodegradable materials has never been clearer. Taking those into account, researchers have directed their attention towards sustainable development, by producing environmentally friendly products such as bioplastics, derived from renewable sources.

In the present study, the alkaline degradation of a promising biopolymer, named poly(3-hydroxybutyrate) (PHB), was initially investigated. The produced hydrolysate after the alkaline degradation of PHB was used as carbon source in shake flasks fermentations, using the bacterial strain *Parabulkhorderia sacchari* (DSM 17165), in order to evaluate the ability of the bacteria to consume efficiently the carbon source and accumulate PHB. Different specimens of PHB (PHB powder, solvent-casted PHB film, compounded PHB pellet and extrusion molded PHB-based cup) and different concentrations of alkaline solutions (NaOH and NH_4OH solutions) were used for this purpose. NaOH solutions in concentrations higher than 0.5M can totally degrade PHB, producing the monomers 3-hydroxybutiric acid (3HB) and crotonic acid (CA), with the ratio CA:3HB being 0.52-0.65. PHB can also be degraded successfully by using NH_4OH solutions in concentrations of 1M, 2M and 4M, but the produced hydrolysate contains one more monomer in addition to 3HB and CA, that is going to be determined in future work. The hydrolysate produced after the alkaline

degradation of PHB using 0.6 M NaOH solution, was consequently used as a carbon source for batch and fed-batch fermentations for circular PHB production from *P. sacchari* (DSM 17165). Batch fermentation resulted in 3 g/L total dry weight (TDW) and 50% PHB accumulation with 0.168 g/g yield, while the fed-batch fermentation led to 2.4 g/L TDW, with 75% PHB content and a yield of 0.136 g/g.

Scientific area: Bioprocess engineering

Keywords: Poly(3-hydroxybutyrate), chemical degradation, biopolymers, recirculation

Χημική αποικοδόμηση του πολύ(3-υδροξυβουτυρικού) εστέρα με σκοπό την αποτελεσματική ανακύκλωση βιοπολυμερών

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

Περίληψη

Τα πλαστικά αποτελούν αναπόσπαστο κομμάτι της σύγχρονης κοινωνίας, με την μαζική παραγωγή τους να αυξάνεται συνεχώς. Ωστόσο, ο αντίκτυπός τους στο περιβάλλον τα τελευταία χρόνια αξιολογείται ως εξαιρετικά ανησυχητικός. Η ρύπανση των πλαστικών εξαπλώνεται διαρκώς, ενώ παράλληλα υπάρχει έλλειψη διαχείρισης των αποβλήτων, με αποτέλεσμα τα απόβλητα να συσσωρεύονται σε χωματερές, ωκεανούς και οικοσυστήματα, παγκοσμίως. Αυτά τα μη διασπώμενα υλικά απαιτούν χρόνια έως και αιώνες για την αποικοδόμησή τους, απελευθερώνοντας επιβλαβή χημικά, και αποτελώντας έτσι έναν σημαντικό κίνδυνο για το περιβάλλον, την άγρια πανίδα καθώς και την ανθρώπινη υγεία. Όπως είναι φανερό, η ανάγκη για την άμεση ανάπτυξη και υιοθέτηση εναλλακτικών βιοαποικοδομήσιμων υλικών είναι πιο μεγάλη από ποτέ. Έχοντας υπόψιν τα παραπάνω, τα βλέμματα της επιστήμης έχουν στραφεί στην βιώσιμη ανάπτυξη παράγοντας προϊόντα φιλικά προς το περιβάλλον, όπως τα βιοπλαστικά, τα οποία μπορούν να παραχθούν από ανανεώσιμες πηγές ενέργειας.

Στην παρούσα μελέτη αρχικά έγινε διερεύνηση της χημικής αποικοδόμησης του πολύ-(3-υδροξυ-βουτυρικού) εστέρα (PHB), ένα πολλά υποσχόμενο βιοπολυμερές. Το παραγόμενο υδρόλυμα που προέκυψε από την αλκαλική αποικοδόμηση του PHB χρησιμοποιήθηκε στη συνέχεια ως πηγή άνθρακα για μικροβιακές ζυμώσεις σε κωνικές φιάλες χρησιμοποιώντας το βακτηριακό στέλεχος *Parabulkhorderia sacchari* (DSM 17165). Ο μικροοργανισμός αυτός αξιολογήθηκε ως προς την ικανότητά του να καταναλώνει την συγκεκριμένη πηγή άνθρακα και να συσσωρεύει ενδοκυτταρικά το PHB. Για το σκοπό αυτό χρησιμοποιήθηκαν διαφορετικές μορφές του PHB (PHB σε μορφή σκόνης, PHB φιλμ μετά από χύτευση με διαλύτη, πέλλετ PHB

και κύπελλο με βάση το PHB μετά από εξώθηση) και διαφορετικές συγκεντρώσεις αλκαλικών διαλυμάτων (διαλύματα NaOH και NH_4OH). Τα διαλύματα NaOH σε συγκεντρώσεις μεγαλύτερες από 0.5M αποικοδόμησαν αποτελεσματικά το PHB στα μονομερή του, 3-υδροξυβουτυρικό οξύ (3HB) και κροτονικό οξύ (CA), με την αναλογία CA:3HB να κυμαίνεται από 0.52 έως 0.65. Αποτελεσματική αποικοδόμηση του PHB επιτεύχθηκε και στην περίπτωση των διαλυμάτων NH_4OH σε συγκεντρώσεις 1M, 2M και 4M, με τη σύσταση του υδρολύματος να διαφέρει, εμφανίζοντας ένα ακόμη μονομερές πέραν του 3HB και του CA, το οποίο θα προσδιοριστεί στο μέλλον. Το παραγόμενο υδρόλυμα μετά την αποικοδόμηση με διάλυμα NaOH 0.6M χρησιμοποιήθηκε στη συνέχεια ως πηγή άνθρακα σε ασυνεχή (batch) και ημι-συνεχή (fed-batch) ζύμωση, για την κυκλική παραγωγή του PHB αξιοποιώντας τον μικροοργανισμό *P. sacchari* (DSM 17165). Στην περίπτωση της ασυνεχούς ζύμωσης η τιμή παραγωγής ξηρής βιομάζας που σημειώθηκε ήταν 3 g/L, με 50% συσσώρευση PHB και απόδοση 0.168 g/g. Η ημι-συνεχής ζύμωση είχε ως αποτέλεσμα 2.4 g/L ξηρής βιομάζας, 75% συσσώρευση PHB και 0.136 g/g απόδοση.

Επιστημονική περιοχή: Μηχανική βιοδιεργασιών

Λέξεις κλειδιά: Πολύ(3-υδροξυβουτυρικός) εστέρας, χημική αποικοδόμηση, βιοπολυμερή, ανακύκλωση

Acknowledgments

The present master's thesis took place in the Laboratory of Food Process Engineering of Agricultural University of Athens, within the M.Sc. in Food Science and Technology with specialization in Food Processing, Preservation and Biotechnological Processes – Bio-economy Products Development.

As I approach the end of my master's journey, I would like to express my sincere gratitude to my supervisor Associate Professor Apostolos Koutinas, for the edifying collaboration, the mentorship, and the trust he showed me throughout the research process. His invaluable contributions have not only shaped this thesis but have also enriched my overall academic experience. Moreover, I would like to thank Associate Professor Seraphim Papanikolaou and Assistant Professor Theofania Tsironi, for being members of my Examination Committee.

I would also like to acknowledge the PhD candidate Argeiti Chrysanthi. Her willingness to share her knowledge, to collaborate and offer me guidance in every step of my research, were instrumental in shaping this work. To the wonderful group of people that were part of the same laboratory, I extend my heartfelt thanks, for our shared experiences and discussions, the support, and the encouragement. During this journey, I have come to value not only our professional collaboration but also the meaningful friendships that have developed among us.

Lastly, I want to convey my gratitude to my family and loved ones for their unwavering support, understanding, and encouragement. Their belief in me and their patience during this journey have been a source of strength and motivation.

Με την άδειά μου, η παρούσα εργασία ελέγχθηκε από την Εξεταστική Επιτροπή μέσα από λογισμικό ανίχνευσης λογοκλοπής που διαθέτει το ΓΠΑ και διασταυρώθηκε η εγκυρότητα και η πρωτοτυπία της.

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Abbreviations

3HB	3-hydroxybutiric acid
ACP	Acyl Carrier Protein
ASTM	American Society for Testing of Materials
BL	β-butyrolactone
CA	Crotonic acid
FID	Flame Ionization Detector
GC	Gas Chromatography
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography
ISO	International Standards Organization
LCA	Life Cycle Assessment
NaOH	Sodium hydroxide
NH_4OH	Ammonia solution
P(3HHx)	Poly(3-hydroxyhexanoate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(3HV)	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
PCL	Polycaprolactones
PE	Polyethylene
PEA	Polyesteramides
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PLA	Polylactic acid

POP	Persistent Organic Pollutants
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinylchloride
ROP	Ring Opening Polymerization
TDW	Total Dry Weight
WOP	Waste Office Paper

1. Introduction

1.1 Plastics and environmental pollution

Polymers are large molecules, composed of distinct units called monomers, that are joined together to create long chains (Rao et al., 2014). Synthetic polymers, commonly known as plastics, are one of the most used materials worldwide with numerous uses including packaging, building and construction, sterile medical equipment, transportation, commercial and agricultural activities.

The first truly synthetic polymer was Bakelite, formulated in 1907, but the real mass production of plastics started in the 1950s (Chamas et al., 2020). Since then, the ubiquity of plastics in the environment has increased rapidly over the decades. In the 1950s, 1.5 million tons of plastics were produced globally, while this number was increased to 335 million tons in 2016 (P. Li et al., 2021). Figure 1 shows the annual main plastic production worldwide from 1950 to 2017 based on consuming sector.

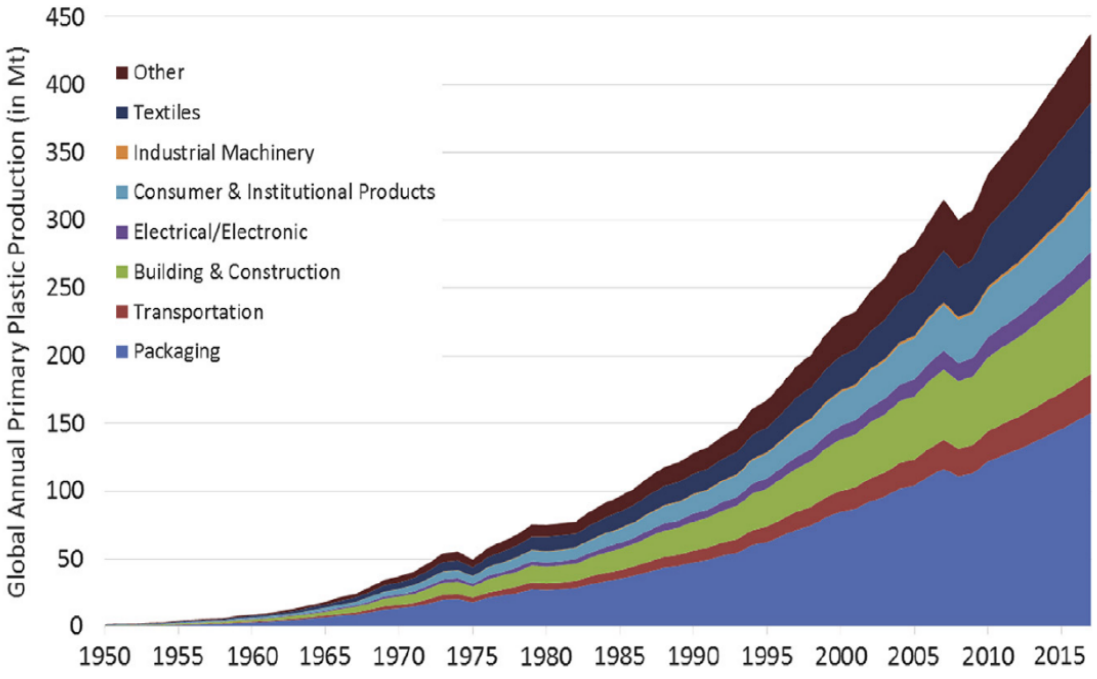


Figure 1 Global annually primary plastic production (in Mt) by consuming sector from 1950 to 2017 (Geyer, 2020).

The most common plastics that are used nowadays are PE (polyethylene), PP (polypropylene), PVC (polyvinylchloride), PET (polyethylene terephthalate), PS (polystyrene) and PU

(polyurethane), which are divided in two categories: C-C backbone polymers and heteroatomic polymers. Figure 2 depicts these main plastics, their chemical type, and their dominance in the market, sorted by their chemical backbone structure. C-C backbone polymers (PVC, PS, PE, PP) comprise most of the market dominance with almost 80%, while the rest are C-O backbone polymers (PU and PET) (Ali et al., 2021).

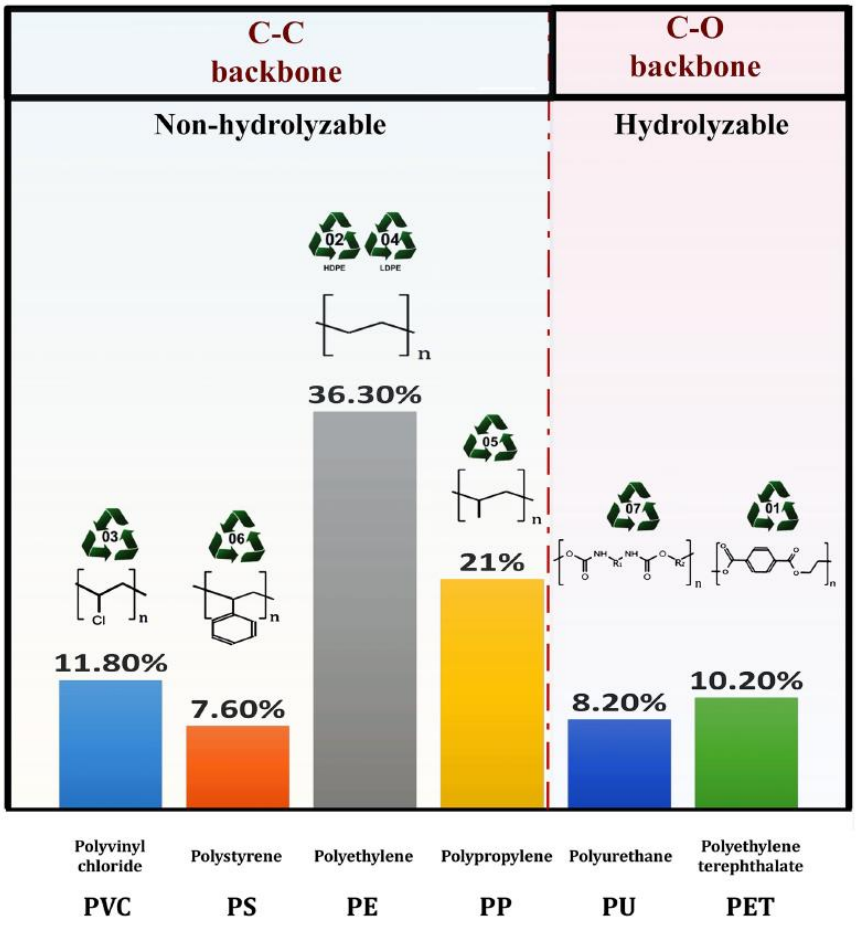


Figure 2 The main plastics, their chemical type and market dominance, sorted by their chemical backbone structure (Ali et al., 2021).

The greater portion of plastics produced, are composed of fossil oil, natural gas, and coil, namely petrochemicals (Chamas et al., 2020). Due to their composition, plastics have a great variety of properties such as resilience, low weight, transparency, and hydrophobicity (Bucci et al., 2020). They can easily be shaped into any form wanted and their durability, absorbency, pore structure, density and color can be adjusted to the desirable extent with ease and low cost of manufacturing (Chamas et al., 2020). However, the industrial attractive properties that have made plastics so

versatile for humans, are the same that have created a rising threat for the environment. (Bucci et al., 2020).

According to their size, plastics can be divided into macroplastics (plastics >5 mm), which exposure to sun and/or wave can lead to smaller plastics called microplastics (plastics <5 mm). Both macro- and micro- plastics are eventually released into the environment, ending up in the oceans, the lakes, and rivers, in soils and sediments, in the atmosphere and in animal biomass. Different processes, such as photodegradation, thermal oxidation, hydrolysis, and biodegradation are happening to plastics, right after their exposure to the environment. In the natural environment, the sequence of processes occurring to plastics is photodegradation, hydrolysis and finally thermal oxidation. Therefore, new compounds with low molecular weight are formed, that can be utilised by microorganisms. However, compared to other organic materials, it takes years even centuries for plastics to be completely degraded (Ali et al., 2021). Thus, their resistance to degradation, causes a major environmental issue.

Despite this rapid globally increasing of plastics' production, waste management is still ineffective in many cases (P. Li et al., 2021). Only a small fraction of 18% of total plastics are recycled worldwide, while the 24% of them are burnt. The rest 58% are disposed in landfills or accumulated in the environment (Chamas et al., 2020). Landfilling and burning are convenient and low-cost methods, but they cause huge environmental pollution and greenhouse gas emissions (Lau et al., n.d.). Plastic waste incinerating can lead to poisonous emissions such as carbon monoxide, chlorine, hydrochloric acid, dioxin, furans, amines, nitrides, styrene, benzene, 1, 3-butadiene, and acetaldehyde. Nevertheless, the contribution of plastics to greenhouse gas emissions and global warming starts from their manufacturing and handling (Mangaraj et al., 2019). Moreover, plastics can be used to transfer persistent organic pollutants (POP's), heavy metals and microorganisms in the environment. Furthermore, they can be considered as toxic pollutants, since it has proved that they can release chemicals such as oligomers, monomers and additives in the nature (P. Li et al., 2021).

If plastic production continues increasing with the same rate, and there is still lack of waste management systems, plastic waste is expected to be more than the double by 2050 (Figure 3), causing to a determined increase of plastic pollution (Lau et al., n.d.). Plastics' pollution can be defined according toLi et al. (2021), as the uncontrolled release of plastics into the environment, that may be threats to the environment, the organisms, or even the human health.

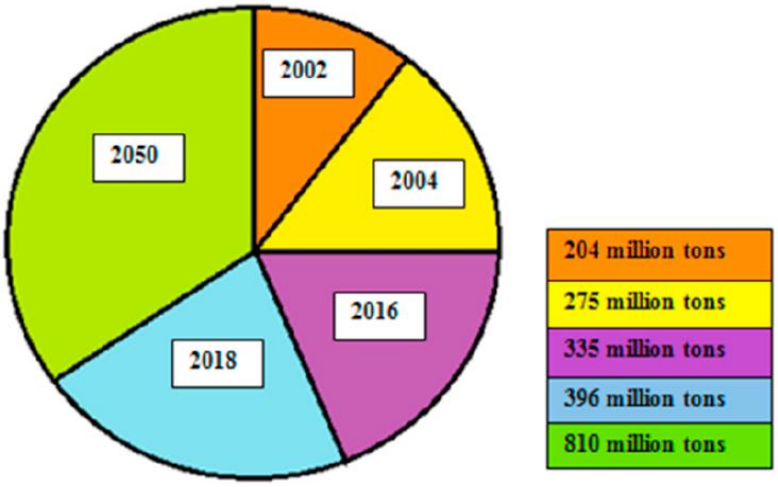


Figure 3 Estimated tons of plastic wastes from 2002 and predicted for 2050 (Sharma et al., 2021).

The unceasing production of plastics and their limited disposal is rising global concerns. Thus, extended research in new alternative materials is being conducted to alleviate this environmental impact.

1.2 Functions and synthesis of biopolymers

Biopolymers, also known as natural polymers, are the polymers that are synthesized naturally by metabolic processes within the cells of some living organisms (Rao et al., 2014). Lately, there is an increasing attention in biopolymers, recognising them as an attractive option instead of conventional polymers, due to their technological properties and their Life Cycle Assessment (LCA) (Lionetto & Esposito Corcione, 2021).

Bio-based polymers show similar properties to fossil oil-based polymers, such as strength and durability. The important difference is that biopolymers are produced from renewable sources, having a zero-carbon footprint, and their degradation can be accomplished by using microorganisms and/or enzymes (Brigham, 2017; Kabir et al., 2020).

Biopolymers can be divided into 5 big categories depending on their manufacturing way and sources: (i) derived from plants (e.g., starch, cellulose, zein, pectin and lipid); (ii) derived from animals and dairy products (e.g., casein, whey protein, gelatin, chitosan, and lipid); (iii) derived from fermenting products or by-products (e.g., poly(3-hydroxybutyrate) (PHB) and polyhydroxyalkanoates (PHA)); (iv) derived from chemical synthesis of naturally derived substances (e.g., polylactic acid (PLA)); and (v) derived from chemical synthesis of fossil resources (e.g., polycaprolactones (PCL) and polyesteramides (PEA)) (Figure 4) (Kabir et al., 2020).

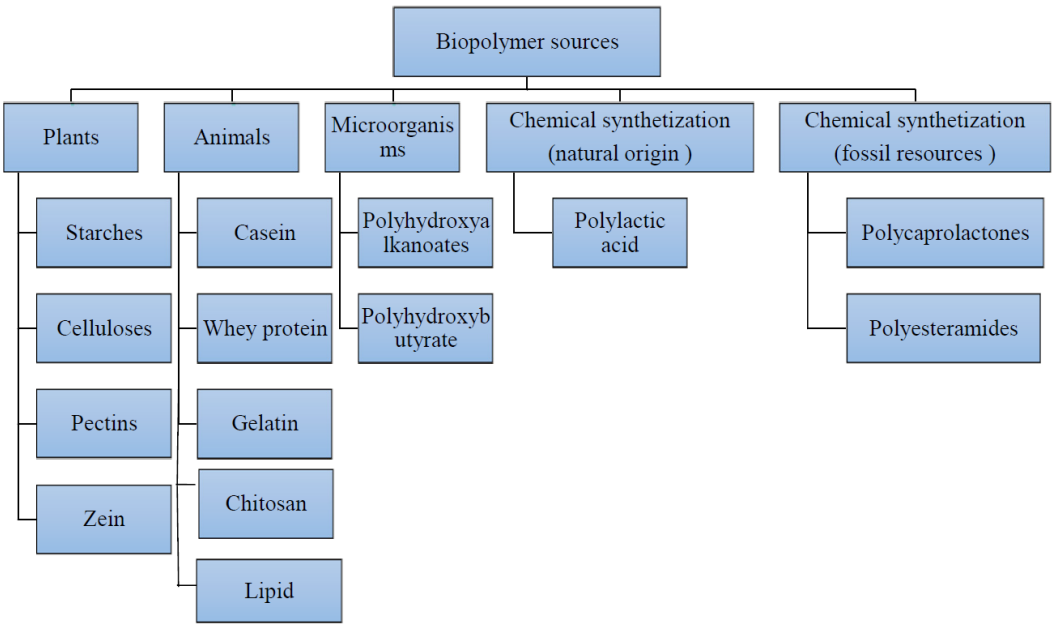


Figure 4 Categories of biopolymers based on their manufacturing way and sources (Kabir et al., 2020).

The synthesis of all biopolymers occurs by enzymatic processes happening in several different places depending on the biopolymer. Some of the biopolymers are synthesized in the cytoplasm, while others at the cytoplasmic membrane, the cells' surface or even outside of the cell.

In some cases, a biopolymer may start synthesizing in one part of the cell but continue synthesizing in another part (Rao et al., 2014).

There are several ways to make each biopolymer available for further use. Biopolymers such as agar and alginates are in abundance in nature and are extracted from plants and algae. A small category is extracted from extremely natural sources, like hyaluronic acid is extracted from the umbilical cords of infants. A common way of biopolymers' production is *in vitro* synthesis utilising isolated enzymes in cell-free systems. In industrial scale, biopolymers such as polysaccharides can be produced as a fermentation product, either intracellularly or extracellularly (Rao et al., 2014).

1.3 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) is a group of biopolymers produced naturally from several renewable carbon resources by microorganisms, gathered as intra-cellular granules, as shown in Figure 5 (Sharma et al., 2021).

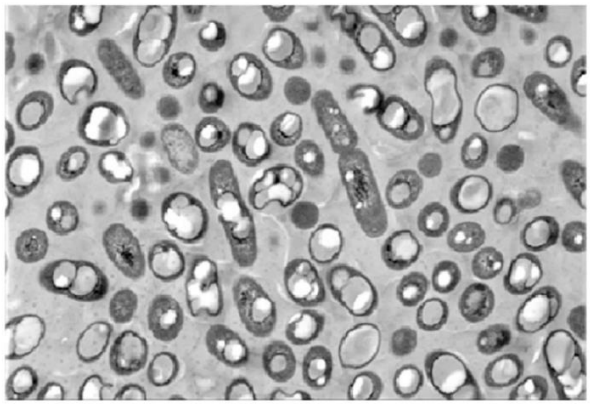
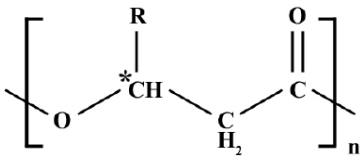


Figure 5 PHAs granules formed intracellularly (Sharma et al., 2021)

PHAs are more and more attracted by researchers due to their inherent properties such as biodegradability, biocompatibility, insolubility in water, UV rays resistant, thermoplasticity, and sustainability (Sharma et al., 2021). A PHAs' chemical structure consists of 600 up to 35,000 (R) – hydroxy fatty acid monomers. Each monomer has a side chain R-group, which most of the time is a saturated alkyl group, or unsaturated alkyl groups, branched alkyl groups, and rarely substituted alkyl groups. Their chemical structure is shown in Figure 6 (Tan et al., 2014).



Poly(3-hydroxyalkanoate)

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 6 Polyhydroxyalkanoates' chemical structure (Tan et al., 2014)

Based on the number of carbon atoms existing within the monomer's molecule, PHAs can be grouped into short chain length PHA (3 to 5 carbon atoms), such as poly(3-hydroxybutyrate) P(3HB), poly(4-hydroxybutyrate) P(4HB), and poly(3-hydroxyvalerate) P(3HV) or the copolymer P(3HB-co-3HV), medium chain length PHA (6 to 14 carbon atoms) such as poly(3-hydroxyhexanoate) P(3HHx), poly(3-hydroxyoctanoate) P(3HO), or copolymers such as P(3HHx-co-3HO), and long chain length PHA (15 or more carbon atoms) (Anjum et al., 2016; Tan et al., 2014).

1.3.1 Biosynthesis of PHAs

PHAs play a major role in preparing microorganisms for surviving under stress conditions. In the case that there is lack of nutrients in the environment, PHA can serve as carbon and energy source for both spore-forming and non-spore-forming bacteria, helping them survive for a long period (Tan et al., 2014). PHAs' biosynthesis occurs naturally in bacteria mainly by three different pathways, and the monomer composition is based on the carbon source used. These pathways are

linked with the bacterium's central metabolic pathways including Krebs cycle, glycolysis, and *β*-oxidation (Sharma et al., 2021). Among them, profound knowledge has been gained on the metabolic pathway used by the microorganism *Cupriavidus necator* for the biosynthesis of PHA. In this pathway (pathway I), two molecules of acetyl-CoA derived from tricarboxylic acid cycle (Krebs cycle) are linked together to form a molecule of acetoacetyl-CoA, helped by the enzyme β-ketothiolase. Following, acetoacetyl-CoA is transformed into 3-hydroxybutyryl-CoA by the enzyme NADPH-acetoacetyl-CoA reductase. In the final step, PHA synthase acts as a catalyst to form the ester bond in 3-hydroxybutyryl-CoA, which is finally converted into poly(3-hydroxybutyrate) (PHB) (Anjum et al., 2016; Sharma et al., 2021).

In pathway II the substrates are several fatty acids that can be converted into PHAs monomers through the *β*-oxidation pathway (Anjum et al., 2016). The primary function of the *β*-oxidation pathway is to break down fatty acids in order to generate reducing equivalents that can be utilized for energy production through the respiratory electron transport chain. With the help of an acyl-CoA synthase and ATP, fatty acids are converted into a precursor that will finally produce acetyl-CoA and will reduce the number of carbon atoms by two in every cycle (Lu et al., 2009). To produce PHA, several hydroxyalkanoate monomers are used. The latter are produced through the fatty acids metabolism by the action of enzymes such as (R)-enoyl-CoA hydratase, acyl-CoA oxidase, and 3-ketoacyl-CoA reductase. Finally, the polymerization is helped by the enzyme PHA-synthase (Sharma et al., 2021). *Pseudomonads* are mainly being used to produce PHA through *β*-oxidation pathway (Lu et al., 2009).

Pathway III is of great importance because it utilizes simple and cheap carbon sources such as glucose, sucrose, fructose, lactose etc., for the production of PHA monomers. The (R)-hydroxyacyl intermediates from the fatty acid metabolic pathway, are transformed from their acyl carrier protein (ACP) form to Co-A form catalyzed by acyl-ACP-CoA transacetylase and finally the polymerization of the monomers is helped by the enzyme PHA-polymerase (Anjum et al., 2016;

Sharma et al., 2021). Figure 7 describes the three different biosynthetic pathways used for PHA production.

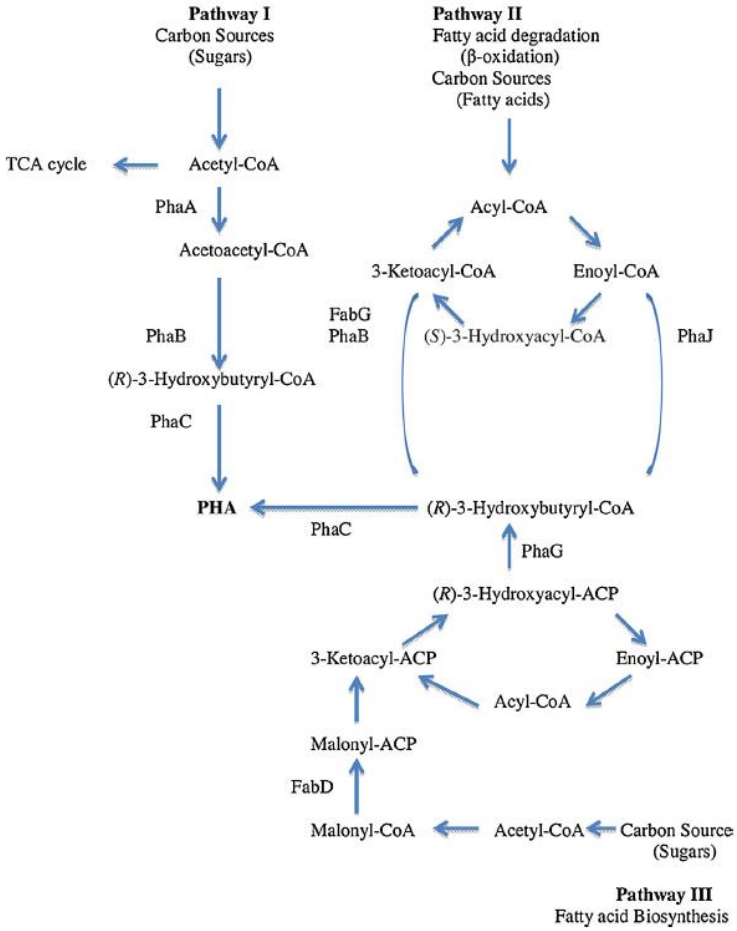


Figure 7 PHAs' biosynthetic metabolic pathways (Anjum et al., 2016)

1.4 Structure and biosynthesis of Poly(3-hydroxybutyrate)

1.4.1 Structure of Poly(3-hydroxybutyrate)

Poly(3-hydroxybutyrate) (PHB) is a member of the family of polyhydroxyalkanoates (PHAs), and it is the biopolymer with the most significant scientific interest. PHB was discovered in 1926 by Maurice Lemoige in France in the bacterium *Bacillus megaterium*, appeared in intra-cellular granules (Anjum et al., 2016). Although PHB was identified so early, the first studies demonstrating the similarities between the properties of PHB and polypropylene, as well as PHB's properties such as biodegradability and biocompatibility were conducted many years later, in 1982, by King and

Howells (Palmeiro-Sánchez et al., 2022). Since then, more and more research has been undertaken on PHB, making it today the most broadly studied and well-characterized member of PHA group.

PHB is a short chain homopolymer of 3-hydroxybutyrate with significant properties making it the most attractive alternative over conventional plastics (Rajan et al., 2018). The chemical structure of PHB is represented in Figure 8.

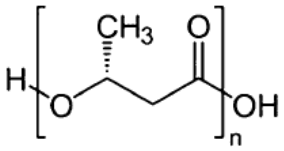


Figure 8 Chemical structure of poly(3-hydroxybutyrate) (PHB) (Rajan et al., 2018)

The properties that make PHB so enticing are its high melting point, the high crystallinity, and the low porosity of O_2, H_2O, and CO_2. Poly(3-hydroxybutyrate)'s molar weight can fluctuate from 2 to 4 x 10^3 KDa, depending on the way it is produced and extracted. Moreover, PHB shows thermoplastic properties and biodegradability, but also some other mechanical properties like polypropylene. Table 1 compares the main properties of PHB with those of polypropylene (Rajan et al., 2018). Another notable advantage of PHB, is that the raw materials used for its production can be a combination of renewable and environmentally friendly resources like: food waste (molasses, whey, glycerol), agricultural waste (cellulose, hemicellulose, lignin), or hydrolyzed polysaccharides like starch, cellulose, or sucrose (McAdam et al., 2020a; Sirohi et al., 2020).

Table 1 Main properties of PHB and PP (Rajan et al., 2018)

Parameters	PP	PHB
Percentage of crystallinity (%)	50-70	60
Melting temperature	176	177
Glass transition temperature (^{o}C)	-10	2
Tensile strength (MPa)	38	40
Elongation at break (%)	400	6
Density (g/cm3)	0.905	1.25
Tensile modulus MPa	1900	3500
UV resistance	Poor	Good

According to Harding et al. (2007), PHB production is more beneficial than polyolefins' production, in terms of environmental impact, requiring lower energy consumption and emitting lower carbon dioxide concentrations at the same time (Harding et al., 2007). The first trial for bacterial production of PHB, through fermentation using sucrose as carbon source in industrial scale, took place around 1990 by Chemie Linz GmbH in Austria. However, commercial success has not been achieved so far (Akiyama et al., 2003). Despite all the favorable properties mentioned, there are some factors that inhibit PHB from expanding and progressing in the marketplace. Concerning large-scale capacity, PHB requires high costs and technologically complex processes to be manufactured, while the yield of the whole process is low and the final product is vulnerable to degradation (McAdam et al., 2020a).

1.4.2 Biosynthesis of Poly(3-hydroxybutyrate)

Poly(3-hydroxybutyrate) is an intracellular product (McAdam et al., 2020a), produced through secondary metabolism of several microbes mainly under nutrient stress conditions, when carbon

sources are in excess while other essential nutrients are limited (Ganapathy et al., 2018). Based on reports, PHB can be formed by 75 different bacteria species, both gram-positive and gram-negative. PHB accumulation is a result of a naturally occurring approach used by bacteria in order to save carbon and energy. There are three main methods for forming PHB: through ring opening polymerization (ROP) of *β*-butyrolactone (BL), through utilising natural/genetically modified plants, and through bacterial fermentation. When the parametrs of the bacterial fermentation are optimal, a very high percentage of the cells' dry weight (over 90%) can cosist of PHB, and that is the main reason why the third method is widely used for PHB accumulation (McAdam et al., 2020a). The biochemical pathway used for this approach is as described above (Figure 7, pathway 1), starting from two molecules of acetyl-CoA from Krebs cycle (Ganapathy et al., 2018).

1.4.3 Bacterial strains producing PHB

PHB can be produced by some bacterias, also characterized as PHB producers. According to bibliography, more than 300 different bacterial strains have been identified for this characteristic. The most widely researched strains used for this aim are *Ralstonia eutropha* (also known as *Cupriavidus necator*), *Alcaligenes spp.*, *Azotobacter spp.*, *Bacillus spp.*, *Nocardia spp.*, *Pseudomonas spp.*, and *Rhizobium spp.*, with *Ralstonia eutropha* being the most extensively studied (McAdam et al., 2020b). Among the strains mentioned, only a small number has been successfully utilised for PHB production at the industrial level so far, mainly *Cupriavidus necator*, *Alcaligenes latus* (also known as *Azohydromonas lata*) and *R. eutropha* (López et al., 2012; Oliveira-Filho et al., 2021). There are also some microorganisms and modified strains, capable to produce PHA co- and ter-polymers like *Cupriavidus spp., Pseudomonas spp., Bacillus spp., Azotobacter spp., Halomonas spp., E. coli spp., Saccharomyces spp.* (Raposo et al., 2017).

Lately, in the 1990's, a Gram-negative bacterium named *Burkholderia sacchari* or *Paraburkholderia sacchari* (strain IPT101), was isolated from sugarcane crops in Brasil. This strain was proved to be a good poly(3-hydroxybutyrate) producer from sugarcane carbohydrates

(*Burkholderia Sacchari Sp. Nov., a*, n.d.; Oliveira-Filho et al., 2021). Since then, more and more experiments have been conducted using *Paraburkholderia sacchari* for PHB production, using different carbon sources.

Al-Battashi et al. (2019), proved that *P. sacchari* DSM 17165 can be successfully utilized for PHB production using hydrogen peroxide pretreated waste office paper (WOP) hydrolysate as substrate, and they optimised the conditions under the fermentation was conducted. M. Li & Wilkins (2021) in their work, used *Paraburkholderia sacchari* DSM 17165 in fed-batch mode and three sugar mixtures (glucose:xylose:arabinose = 4:2:1, 2:2:1, 1:2:1) as substrate, to produce PHB. All sugars were consumed at the same time, but at different rates: glucose was consumed first, xylose was following and arabinose was the last consumed. Mixed sugars derived from fruit waste can also be consumed by *Paraburkholderia sacchari* in fed-batch bioreactor fermentations, to efficiently produce PHB, as Psaki et al. proved in their work (Psaki et al., 2023). Pradella et al. in their study suggested airlift bioreactors as a potential alternative way to produce PHB at high-cell density, utilizing *P. sacchari* IPT 189 for the production of PHB using sucrose syrup as carbon source, achieving yield 0.22 g/g (Pradella et al., 2010). Successful PHB biosynthesis in bioreactor has also been reported with glycerol as the only carbon source using *P. sacchari* DSM 17165 (Rodríguez-Contreras et al., 2015).

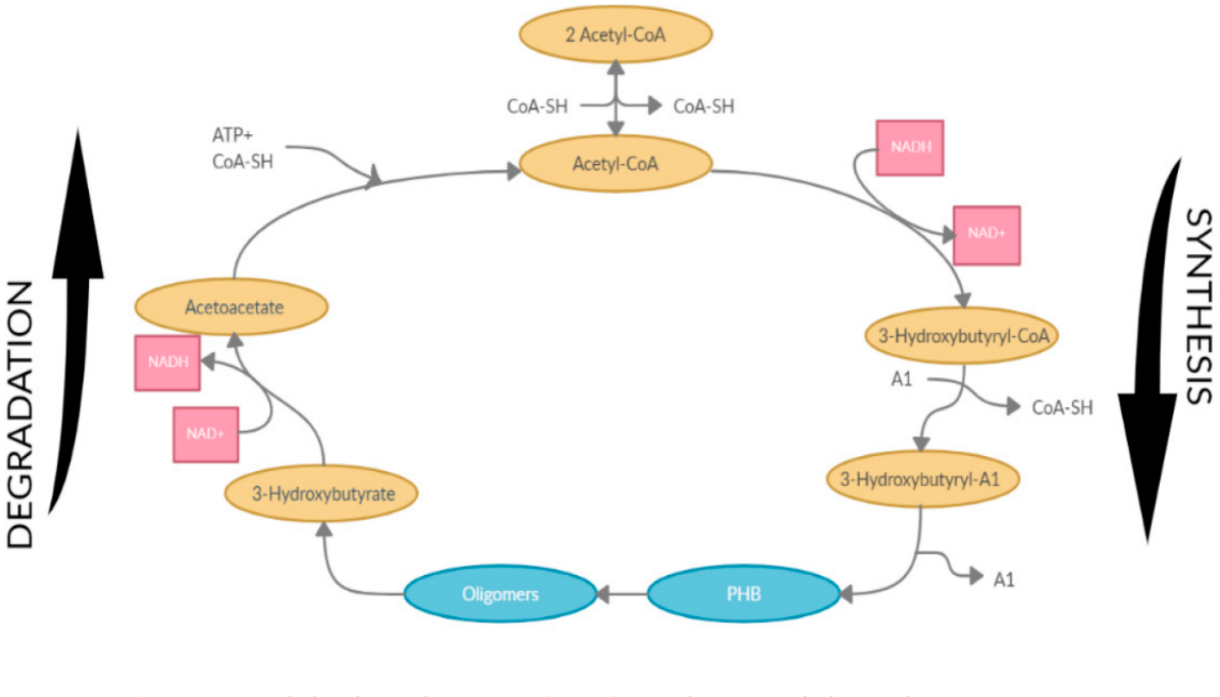


Figure 9 PHB synthesis and degradation process (McAdam et al., 2020)

1.5 Biodegradability of polymers

A definition for degradable plastics has been given by the the American Society for Testing of Materials (ASTM) and the International Standards Organization (ISO) as those that their chemical composition changes significantly under environmental circumstances. The degradation of biodegradable plastics occurs naturally by microorganisms like bacteria, fungi, and algae. The

possibility of a material to be biodegraded depends on its chemical composition. Table 2 shows the degradation conditions for different natural and synthetic polymers (Gowthaman et al., 2021).

Table 2 Degradation of natural and synthetic polymers (Gowthaman et al., 2021)

Polymer	Chemical nature	Degradation time	Degradation mechanism
Collagen types I, II, III	Triple helix with the tripeptide sequence Gly–X–Y	12 h	Enzymatic: collagenase
Crosslinked collagen	Crosslinked with 1,4-butanediol diglycidyl ether	>6 weeks	Enzymatic: collagenase
Alginate	Polysaccharide with (1,4)-linked β-D-mannuronic and α-L-guluronic acid repeat units	About 80 days	Hydrolytic disintegration
Crosslinked chitosan	Polysaccharide with (1,4)-linked 2-acetamido-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose units	>20 weeks	Enzymatic: chitosanase and lysosome
Hyaluronan films	Polysaccharide with (1,4)-linked disaccharide units of negatively charged 1,3-linked monosaccharides glucuronic acid and *N*-acetyl glucosamine	1 week –4 months	Enzymatic: hyaluronidase
Braided silk	Protein consisting of poly-Gly-Ala repeat units	6 weeks	Proteolysis
Polycaprolactone	Polyester consisting of hexanoate repeat units	>24 months	Hydrolytic
PLA	Polyester with lactide repeat units	>24 months	Hydrolytic
PHA/PHB	Polyester consisting of hydroxyalkanoate repeat units	>24 months	Bacterial fermentation

PHA/PHB, *Polyhydroxyalkanoate/polyhydroxybutyrate;* PLA, *polylactic acid*.

As mentioned before, petrochemical polymers are not biodegradable, since after their disposal, they cannot be degraded by the microorganisms that live in the soil. On the other hand, biopolymers are totally biodegradable, due to their natural sources (Gowthaman et al., 2021). Thus, microorganisms can break them down, under aerobic or anaerobic conditions, converting the polymer into soluble by changing its durability and color. These soluble compounds can be consumed by the microorganisms giving mainly carbon dioxide, water, and biomass, but also CH_4 and inorganic compounds in some cases (Figure 12) (Kabir et al., 2020). The ideal biodegradable biopolymer manufacturing consists of their disposal in a biowaste collection, followed by composting. In this way, the only by-products produced are carbon dioxide and water, both environmentally friendly (Gowthaman et al., 2021).

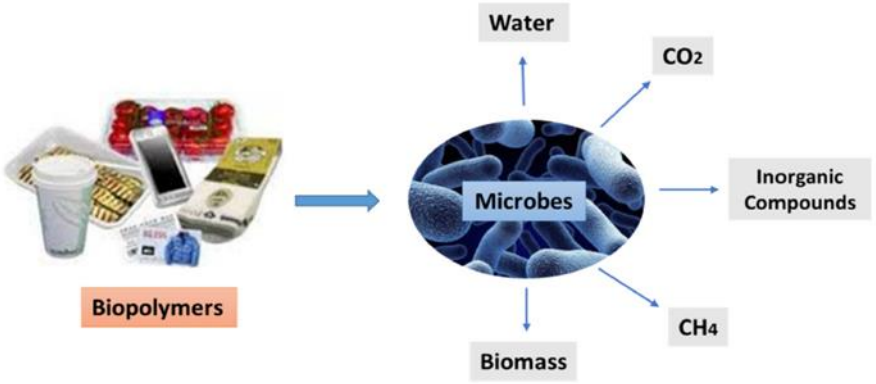


Figure 10 Microbial degradation of biopolymers (Kabir et al., 2020)

Not all biopolymers are biodegradable. The degradation extent is based on their chemical structure and properties. Apart from microbial degradation, other degradation mechanisms of biopolymers are hydrolysis and photodegradation.

During hydrolysis, because of the water that is present in the environment, the biopolymer breaks down into soluble monomeric by-products through two different ways: bulk and surface erosion. In bulk erosion, aquatic molecules disperse instantly into the non-crystalline regions, leading to a significant decrease of stability of the biopolymer. During the surface erosion, the degradation of the biopolymer starts from the outside, while the inner part is degraded last, as it can be seen in Figure 13 (Kabir et al., 2020).

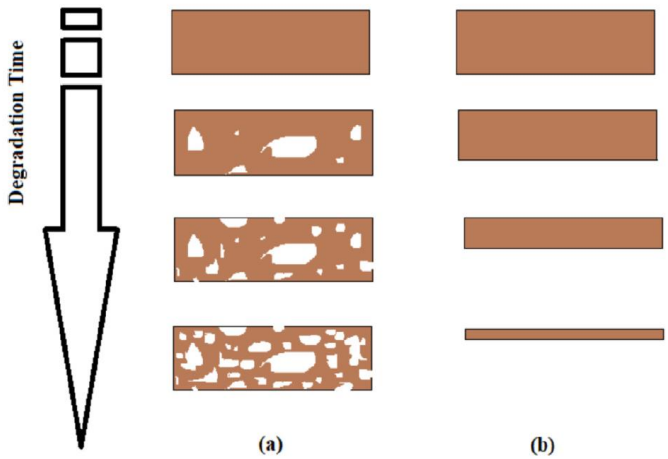


Figure 11 Hydrolysis of biopolymers: (a) Bulk erosion and (b) Surface erosion (Kabir et al., 2020)

During photodegradation, the photolysis is catalysed by additives such as TiO_2 and ZnO that are added to the biopolymer, combined with UV light from 200-700 nm wavelength, as described in Figure 14 (Kabir et al., 2020).

Figure 12 Photodegradation of biopolymers (Kabir et al., 2020)

1.6 Degradation of poly(3-hydroxybutyrate)

As it is already mentioned, many bacteria can produce biopolymers under stress conditions. Specifically, many bacterial strains can produce PHB in order to adapt to a high carbon and low nitrogen, phosphate, or oxygen environment. However, based on Müller-Santos et al. (2021), after a certain point and under specific conditions (thermal and oxidative shock), bacteria change from PHB accumulation to mobilization, producing 3-hydroxybutyric acid (3HB) and its oligomers, as a protection against protein compilation and cells' wreckage.

Another procedure to degrade a polymer is its direct depolymerization using microorganisms. PHB depolymerases are extracellular carboxylesterases, able to degrade PHB when exist outside of the cell. Not every microorganism able to produce PHB has the ability to degrade it. Bacteria like *Aspergillus fumigatus* Pdf1, *Aureobacterium saperdae*, *Thermus thermophiles* HB8, *Streptomyces bangladeshensis* 77T-4, *Penicillium simplicissimum* LAR13, *Acidovorax sp.* TP4, and *Streptomyces* KJ-72 acquire this enzyme, while others like *Pseudomonas putida* KT2440 don't. The final product of the depolymerization is the corresponding hydroxyalcanoic acid, that can possibly be used as raw material for *β*-oxidation by microbes (Jendrossek & Handrick, 2002). PHB depolymerases from *A. faecalis* and *C. testosterone* give a mixture of monomer and dimer, while those from *P. pickettii* give 3-hydroxybutyrate as final product (Mukai et al., n.d.) .

On the other hand, Alejandra et al. (2012), studied the enzymatic degradation of PHB using a commercial lipase. The results showed that Lipopan Conc BG, a triglyceride synthetic lipase produced by *C. necator*., is able to depolymerize PHB produced.

Alcoholysis is another approach that can be used for the degradation of poly(3-hydroxybutyrate). Špitalský et al. (2006),used two different alcohols (ethylene glycol and glycerol) for the alcoholysis of PHB catalyzed by p-toluene sulfonic acid. The reaction can be seen in Figure 10. As a result, various oligomers with available terminal hydroxyl groups were produced, which can be utilized for further reactions like crosslinking and chain-extension. Acidic alcoholysis with hydrochloric acid as catalyst was also used by Lee et al. (2000) to produce 3-hydroxybutyrate as final product from the degradation of PHB.

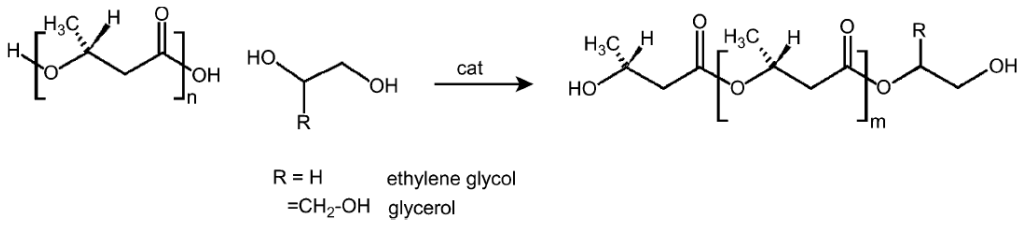


Figure 13 Degradation reaction of PHB with ethylene glycol and glycerol (Špitalský et al., 2006)

An alternative technique to decrease PHB's molecular weight is thermal degradation. Ariffin et al. (2010) successfully applied thermal degradation to PHB material, catalyzed by MgO and $Mg(OH)_2$, for the production of crotonic acid. The crotonic acid derived, pure or as copolymer, can have potential uses as dental, make-up or hair-styling products.

1.6.1 Alkaline PHB degradation

Depending on parameters like temperature, polymer form and concentration, abiotic degradation of PHB under alkaline conditions can be very effective. During the reaction, random polymers' ester bonds break down, forming soluble and insoluble oligomers, ending up in the production of the final degradation products. Although it is widely examined by researchers, there is still not a clear picture of the mechanism of PHB degradation (Yu et al., 2005). Based on Yu et al. (2005),

PHB hydrolysis can give the soluble monomers 3-hydroxybutyric acid (3HB) and crotonic acid (CA) as final products, a reaction that follows the 0th-order kinetics.

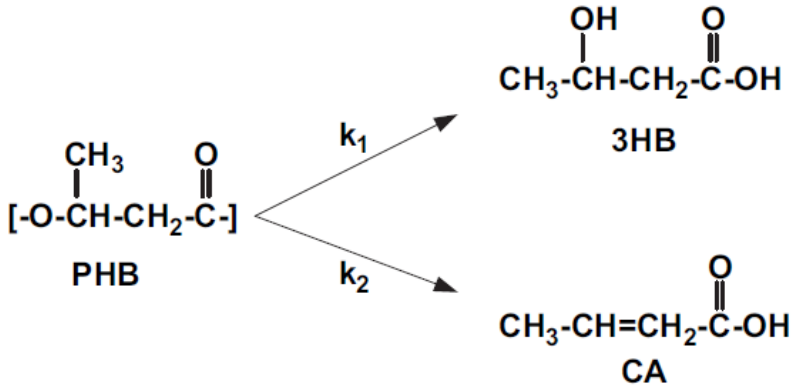


Figure 14 Alkaline hydrolysis of PHB and parallel production of 3HB and CA (Yu et al., 2005)

1.7 Applications of biopolymers

Over the last few decades there has been a growing demand for eco- friendly alternative materials, that could mitigate the huge environmental issue caused by fossil oil-based polymers. Biopolymers appear as a promising solution to the problems posed by plastics, by covering already a substantial fraction of todays' products in the fields of medicine, agriculture, and packaging (George et al., 2020).

Among their applications, biopolymers are mainly utilized in the field of biomedicine, where they are being used as medical implants for orthopedic and circulatory transplantation objectives and mainly as stitches, enhancing the integration of impaired tissues. Poly- (glycolic acid), poly- (L-lactic acid) and their copolymers, are commonly used as stitches, since they can be easily sterilized and provide strength, tolerance, and functionality during the adaption period of the tissue until they are either removed or absorbed from the body. Biopolymers are suitable for osteosynthesis transplants, by supporting the bones to be restored effectively and avoiding extra surgery for removal, since they can be completely dissolved afterwards. Biopolymers with poly – (hydroxy acid) and poly- (ortho ester) groups in their chemical structure are being commonly utilized for medication release and uptake, because they show greater compatibility with the blood

tissues than the synthetic polymers. The high level of compatibility, combined with strength and adaptability, are the reasons why polyurethanes are broadly used for making blood vessels artificially (George et al., 2020).

Biopolymers seem to be a reliable solution to the extended use of plastics in the agricultural sector also, by taking advantage of the facts that they can be degraded naturally and enrich the soil quality (George et al., 2020). Specifically, starch and chitosan have been used, alone or mixed with other organic elements, for making stable, biodegradable films that can be applied as composts. These films can be mainly used for vegetable and flower crops to limit the use of fertilizers, chemicals, water, and energy (Gamage et al., 2022). Plastic films used for covering silage are difficult to recycle, since they are contaminated with soil, sand, and other organic materials. Their replacement with biodegradable plastic films can be a potential solution to overcome this difficulty, as they can equally provide the essential mechanical properties and allow low concentration of oxygen to enter the bales, as needed (Gamage et al., 2022). Mixing different biopolymers, and especially mixing them with polyvinyl chloride (PVC), could lead to the formation of completely biodegradable plastic film that can be used as packaging material for groceries or compost bags, but also as more stiff packaging like containers, and one-use cups, plates, knives, forks, and spoons (Gamage et al., 2022; George et al., 2020).

2. Purpose of this work

The purpose of this study is the chemical and biological recycling of post-consumer biobased packaging. Specifically, the alkaline degradation of different forms of poly(3-hydroxybutyrate) (PHB), and the ability of the bacterial strain *Paraburkholderia sacchari* to grow and accumulate PHB in shake flask fermentations using the PHB hydrolysate as carbon source, were investigated.

The alkaline degradation was carried out in 4 different forms of PHB (PHB powder, solvent-casted PHB film, compounded PHB pellet and extrusion molded PHB-based cup) and in different PHB concentrations (20 g/L, 40 g/L, and 60 g/L), using various concentrations of NaOH solutions (0.1M, 0.3M, 0.5M, 2M, 4M). Moreover, several concentrations of NH_4OH solutions (1M, 2M, 4M) were evaluated for their ability to degrade the different forms of PHB. The produced PHB hydrolysate after alkaline degradation using NaOH solution, was used as carbon source for batch and fed-batch shake flask fermentations, aiming to achieve *P. sacchari* growth and PHB accumulation.

3. Materials and Methods

3.1 Chemicals

Ammonia solution (NH_4OH), Crotonic acid (CA), 3-Hydroxybutiric acid (3HB), and Sodium hydroxide (NaOH), were purchased by Sigma Aldrich.

3.2 Alkaline degradation of Poly(3-hydroxybutyrate)

Four different specimens of PHB (PHB powder, solvent-casted PHB film, compounded PHB pellet and extrusion molded PHB-based cup) were evaluated, regarding their ability to be degraded during alkaline treatment. All the samples were purchased by Biomer (Schwalbach,Germany).

Alkaline degradation of PHB was carried out, using different concentrations of NaOH (0.1M, 0.3M, 0.5M, 1M, 2M, 4M) and NH_4OH (1M, 2M, 4M) solutions. In both cases 20 mg of PHB in different forms were added in 1 mL aqueous solution of NaOH or NH_4OH. In the case of alkaline degradation using NaOH, 40 mg and 60 mg of PHB powder were also tested. The degradation of PHB was performed in McCartney glass bottles at the temperature of 70 ºC (where NaOH and NH_4OH solutions were used) and 90 ºC (where NH_4OH solutions were used). Samples were taken at regular intervals and analysed for soluble degradation products. The solution was diluted to 25 mL with distilled and deionized water and filtered with a 0.2 μm membrane filter. The filtrate kept at 4 ºC for later HPLC analysis. In the case of NaOH treatment, the membrane filters, after the filtration, were kept at 30 ºC and then weighed to measure the residual PHB mass.

3.3 Microorganism and inoculum preparation

The bacterial strain that was utilized in all fermentations was *Paraburkholderia sacchari* (DSM 17165). The preculture medium for *P.sacchari* contained (per liter): $(NH_4)_2SO_4$, 1.0 g; $Na_2HPO_4 \cdot 2H_2O$, 4.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; yeast extract, 1 g; trace elements, 1 mL; glucose, 20 g as described by Cesário et al. (Cesário et al., 2014). Solution of 2M HCl was used to adjust the pH of the medium to 6.8. For the incubation of the preculture were used 500 mL baffled flasks, containing 100 mL medium, incubating at 30 ºC and 250 rpm for 14–16 h. The

optical density was measured at 600 nm until the value was around 8-10 and then 10% of the total volume was transferred in the shake flask fermentations.

3.4 Shake flask fermentations for PHB production

PHB hydrolysate produced after alkaline degradation, was used as carbon source for the production of new PHB. For this reason, 20 g of PHB powder was added in Duran glass bottles with 1L of 0.6M NaOH solution, remaining in water bath at 90ºC for 28 hours.

Batch and fed-batch fermentations were carried out in 500 mL baffled flasks containing 100 mL medium at 30ºC, pH 6.8 and 250 rpm. Batch fermentations were carried out with 6.21 g/L initial 3HB concentration and 3.37 g/L CA concentration. Fed-batch fermentations started with 5.85 g/L initial 3HB concentration and 3.12 g/L CA. During the fermentations pH was adjusted to 6.8 using 2 M HCl and 5 M NaOH. Carbon source consumption, biomass and PHB accumulation were monitored by taking samples at regular intervals (usually every 2-3 h) during the fermentations, based on the carbon source consumption, the bacterial cell growth and PHB accumulation. In both cases fermentations were carried out in duplicate. Hydrolysate used as substrate for the fermentation, was sterilized separately using filter with pores' diameter 0.2 μm (Polycap 36AS, Whatman), in order to avoid the thermal processing that could lead to a possible degradation of its components.

3.5 Analytical methods

Fermentation samples (1 mL) were centrifuged (9000 *g*, 10 min, 4 ºC). The supernatant was stored at 20 ºC for further analysis. The precipitate was washed twice with distilled water and once with acetone to remove impurities. Then it was transferred into pre-weighted McCartney vials. The precipitate was dried at 50 ºC and left in a desiccator until constant weight was achieved, in order to determine the total dry weight (TDW).

PHB was determined via propanolysis of the precipitate from the fermentation samples. For this aim, 2 mL of 1,2-dichloroethane were mixed with 2 mL of HCl-propanol (25 % v/v) and 200

μL of benzoic acid, well-vortexed in between stages and boiled at 100°C for 2 h in a water bath. Once cooled to room temperature, 4 mL of distilled water were added, resulting the creation of two immiscible liquid phases. The organic phase was collected and run through a gas chromatographic analyzer (Shimadzu, Nexis GC 2030) composed of an autosampler (AOC-20i plus), a Flame Ionization Detector (FID) and a Mega-Wax column (30 m × 0.25 mm, film thickness 0.25 μm) for quantitative determination of PHB. Helium was used as carrier gas at a flow rate of 1 mL/min. Oven initial temperature was set at 100 ºC for 1 min, followed by a temperature ramp of 25 ºC/min at 160 ºC, held constant for 1 min, and then increased to 188 ºC at a rate of 10 ºC/min. The temperature was further increased to 250 ºC at 25 ºC/min with a final isothermal period of 5 min. The injector and detector temperatures were 230 ºC and 250 ºC, respectively. The standard curve was determined using commercial PHB (Sigma-Aldrich).

CA and 3HB were determined using a Shimadzu HPLC system equipped with a Shimadzu RI detector and a Rezex ROA-Organic acid H^+ column. The temperature of the column was 65 ºC and the mobile phase was 10 mM H_2SO_4 aqueous solution at 0.6 mL/min flow rate.

4. Results and Discussion

4.1 Alkaline degradation of PHB using NaOH solutions

The alkaline degradation of the different PHB specimens (PHB powder, solvent-casted PHB film, compounded PHB pellet and extrusion molded PHB-based cup) was carried out using different NaOH solutions, as it is described in 3.2.

Initially, alkaline degradation of 20 g/L PHB powder was conducted using 4 M NaOH solution, based on the experiments of Yu et al. (2005), to determine the approximate duration of the hydrolysis and detect the degradation monomers produced. Subsequently, more experiments with the same initial amount of PHB (20 g/L) were carried out, using different concentrations of NaOH solutions (0.1 M, 0.3 M, 0.5 M, and 2 M) and the results were compared. Figure 15 shows the kinetic of 3-hydroxybutiric acid (3HB), crotonic acid (CA) and total PHB recovery after the alkaline degradation of 20 g/L PHB powder using different concentrations of NaOH solutions. Figure 15 shows the kinetic of PHB (Fig. 15a), 3HB (Fig. 15b) and CA (Fig. 15c) recovery, after the degradation of 20 g/L powder PHB solution using different concentrations of NaOH solutions (0.1M, 0.3M, 0.5M, 2M, 4M). It can be clearly seen that the concentration of the alkaline solution affects not only the duration of the hydrolysis, but also the fraction of PHB that can be degraded and thus, the fraction of the monomers 3HB and CA produced.

In the case where 0.1 M NaOH solution was used, the fraction of PHB degradation was 29.96%, achieving a significant low recovery. The alkaline solutions with a higher concentration led to a complete degradation of PHB, while the duration of the degradation in the case of 4M and 2M NaOH solution was significantly faster (4h), compared to 0.5M and 0.3M (18h and 28h respectively).

Moreover, a parallel increase in the concentration of the two monomers was observed through time, with the ratio of CA:3HB in the end of the degradation process being similar in all

cases CA:3HB = 0.56-0.65. These ratios found seem to be close to the ratio of CA:3HB found by Yu et al., 2005, (CA:3HB=0.42-0.68).

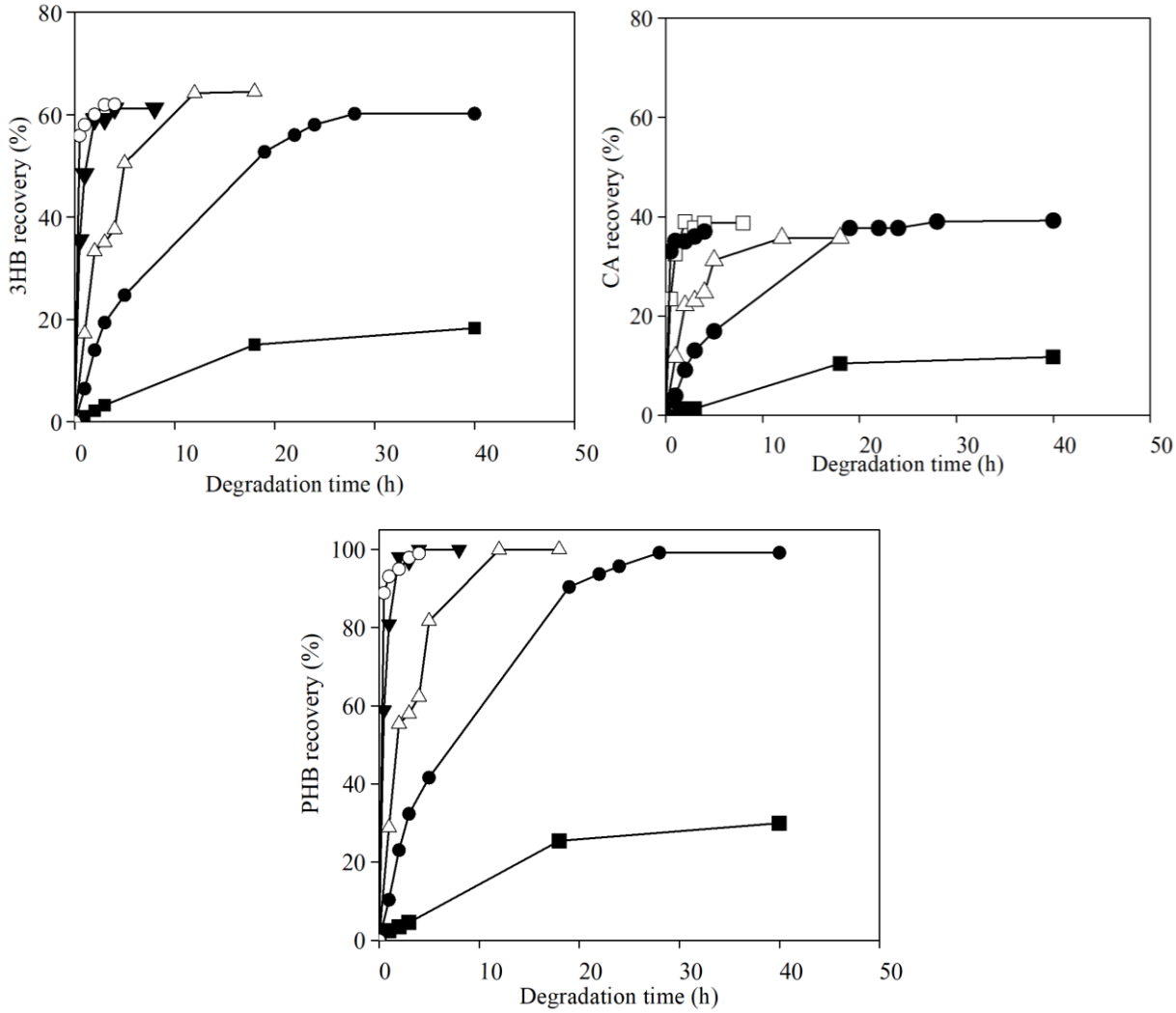


Figure 15 Kinetic of 3-hydroxybutiric acid (3HB), crotonic acid (CA) and total PHB recovery after alkaline degradation of 20 g/L PHB powder using 0.1M (■), 0.3M (●), 0.5M (△),2M (▼) and 4M (○) NaOH solutions, at 70ºC.

At the next set of experiments different concentrations of PHB (40 g/L and 60 g/L) were degraded using NaOH solutions (Table 3). In the case of 40 g/L PHB solution, 0.3M and 0.6M NaOH solutions were used. In the case of 0.3 M only half of the PHB amount was degraded (50.23%), while in the case of 0.6 M almost total degradation was achieved (93.45%), with the fractions of the monomers 3HB and CA being 56.40% and 37.05% respectively.

One more experiment was conducted, using a higher PHB concentration of 60 g/L powder. The results showed that NaOH solution in the concentration of 0.9 can efficiently degrade the 60g/L of PHB with a recovery reaching up to 94.2, while in the case of 0.6 M NaOH solutions the recovery was 66.54%. The ratio of CA:3HB in the final hydrolysate was approximately 0.66 in all cases.

Table 3 Akaline degradation of 40 g/L and 60 g/L PHB powder using different concentrations of NaOH solutions

PHB (g/L)	Concentration (M)	Reaction duration (h)	PHB degradation (%)	3HB (%)	CA (%)	Ratio CA:3HB
40	0.3	28	50.23	30.08	20.15	0.67
	0.6	24	93.45	56.40	37.05	0.66
60	0.6	26	66.54	40.11	26.43	0.66
	0.9	26	94.20	56.94	37.26	0.65

At the next set of experiments the degradation of solvent-casted PHB film was conducted, using different concentrations of NaOH solutions. For this aim, 20 g/L of solvent-casted PHB were used in every experiment, following the same method as above. Five different concentrations of NaOH solutions (0.3 M, 0.5 M, 1 M, 2 M, and 4 M) were tested, and the results are shown in Table 4.

It can be clearly seen that the degradation of solvent-casted PHB using NaOH solution, is not as efficient as in the case of PHB powder. From the different concentrations of NaOH solutions examined, only 2M and 4M NaOH solutions could achieve a high recovery of the solvent-casted PHB degradation (92.21% and 90.74% respectively). Degradation using 1M NaOH could lead to a relatively high fraction of PHB recovery (76.10%), while 0.3M and 0.5M NaOH resulted in a low PHB recovery (16.17% and 19.62% respectively). Moreover, concerning the duration of the degradation process, a great difference was observed between the different specimens of PHB. In the case of 20 g/L solvent-casted PHB using 4M NaOH, the duration of the degradation was around 25h, while in the case of PHB powder it lasted only 4h.

During the next experiments, the degradation of compounded PHB pellet and extrusion molded PHB-based cup was conducted, using different concentrations of NaOH solutions. The initial concentration of PHB solutions was 20 g/L and the concentrations of NaOH solutions were used were 0.5 M, 1 M, 2 M, and 4 M. The concentrations of NaOH solutions, were selected based on the results of the previous experiments. The results are depicted in Table 4. It is obvious that the degradation was successful in all cases. In the case of the NaOH 0.5 M solution, a relatively high fraction of PHB degradation was noticed in both PHB forms (75.58 % for pellet and 71.86 % for cup). In the rest cases, PHB was fully hydrolyzed to the two monomers 3HB and CA, with the fractions of the monomers being almost the same in all cases. It is noticeable that even though the hydrolyses were successful, the durations of the reactions of compounded PHB pellet and extrusion molded PHB-based cup were much longer than the respective of the PHB powder.

All the relevant data from the different PHB specimens investigated for alkaline degradation using different concentrations of NaOH solutions, are compiled in Table 4.

Table 4 Alkaline degradation of different PHB specimens (20 g/L) using different concentrations of NaOH solutions

	Concentration (M)	Reaction duration (h)	PHB degradation (%)	3HB (%)	CA (%)	Ratio 3HB:CA
PHB Powder	0.1	40	29.96	18.26	11.7	0.64
	0.3	28	99.17	60.17	39	0.65
	0.5	18	100	64.3	35.7	0.56
	2	4	100	61.3	38.7	0.63
	4	4	100	63	37	0.59
Solvent-casted PHB film	0.3	25	16.17	9.67	6.5	0.67
	0.5	25	19.62	11.82	7.8	0.66
	1	30	76.10	46.2	29.9	0.65
	2	27	90.74	56.94	33.80	0.59
	4	25	92.21	62.31	29.90	0.48
Compounded PHB pellet	0.5	50	75.58	47.09	28.49	0.61
	1	31	100	63.20	36.80	0.58
	2	30	100	65.16	34.84	0.53
	4	7	100	65.92	34.08	0.52
Extrusion molded PHB-based cup	0.5	50	71.86	43.37	28.49	0.66
	1	28	100	62.20	37.80	0.61
	2	25	100	63.44	36.56	0.58
	4	7	100	64.50	35.50	0.55

4.2 Alkaline degrdation of PHB using NH_4OH solutions

Different concentrations of NH_4OH solutions were investigated as an alternative base for PHB degradation. To illustrate the reason for using NH_4OH solutions for PHB degradation, it should be mentioned that NaOH solutions need to be neutralized after hydrolysis using HCl, to adjust the pH of the hydrolysate so that it can be used later as a substrate for fermentation. In case

of NH_4OH, in contrast, there is no need for neutralization, saving chemicals in this way, and the solution can be used as nitrogen source for the microorganism directly.

For this reason, alkaline degradation experiments were conducted starting with 20 g/L of PHB powder and different NH_4OH solutions at 70ºC. The concentrations of NH_4OH solutions investigated were based on the results of the NaOH solutions experiments, and the more effective concentrations were chosen (1M, 2M and 4M).

During the HPLC analysis the concentration of the produced 3HB and CA was determined. In the case of the degradation of PHB using NH_4OH solutions, an unknown peak was detected in the chromatogram during HPLC analysis that was increasing through the time, in all experiments, but it couldn't be determined the type and the concentration of the produced monomer. An LCMS analysis is going to be conducted in order to determine the concentration and the type of the produced monomer. In this study the concentration and the ratio of the produced 3HB and CA are going to be discussed.

Figure 16 shows the kinetic of 3-hydroxybutiric acid (3HB), crotonic acid (CA) and total PHB recovery after the alkaline degradation of 20 g/L PHB powder using different concentrations of NH_4OH solutions at 70ºC. The duration of the reactions was almost the same in all cases (48 hours for 1M and 51 hours for 2M and 4M), but it was much longer than the respective NaOH solutions. The highest fraction of PHB degradation was noticed in the case of 4M solution (66,44%), followed by 2M solution (46.88%), and finally 1M solution with 17.64% PHB degradation. The CA fractions were very low in every case with 5.20% (4M), 3.90% (2M), and 2.60% (1M).

What was remarkable during these experiments was that even though PHB degradation fractions were relatively low compared to NaOH solutions experiments, the fractions of 3HB produced were as high as in the experiments of PHB powder using NaOH solutions with the respective concentration. On the other hand, CA fractions were much lower, but the number was

almost the same in every case. However, in the solutions after the hydrolysis, PHB powder seemed to be fully dissolved optically.

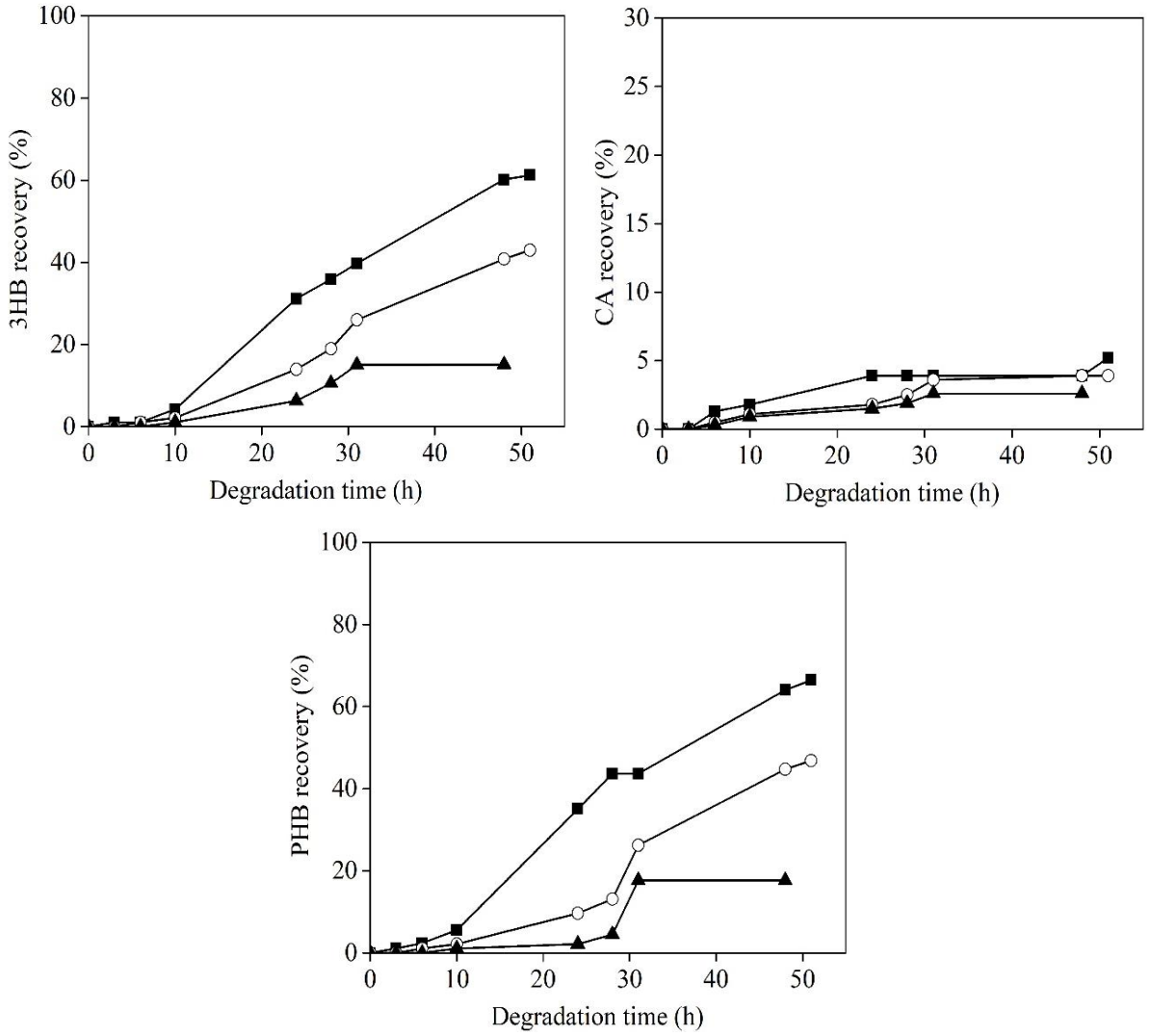


Figure 16 Kinetic of 3-hydroxybutiric acid (3HB), crotonic acid (CA) and total PHB recovery after alkaline degradation of 20 g/L PHB powder using 1M (▲), 2M (○) and 4M (■) NH_4OH solutions, at 70ºC.

Following, the same set of experiments was repeated, increasing this time the temperature from 70ºC to 90ºC, to see if temperature can affect the degradation of PHB. Table 3 shows the results of degradation of different PHB specimens using different concentrations of NH_4OH

solutions at 90^{o}C. A significant difference in the results was noticed. The duration of the hydrolysis was decreased to 30 hours for 1M solution, and 38 hours for 2M and 4M solutions. The highest degradation fraction (68.59%) was observed in the case that 4M solution was used, and the lowest degradation fraction (51.40%) was in the case that 1M solution. The produced 3HB fractions were much higher in all experiments compared to the experiments conducted at 70ºC. However, CA fractions were remained very low in all cases.

The next set of experiments was carried out using different concentrations of NH_4OH solutions (1M, 2M, 4M) for the degradation of solvent-casted PHB. The 3HB fractions were very similar to the powder's, with the highest being 67.69% (2M solution) and the lowest 59.09% (4M). The CA fractions were fluctuating from 3.90% (4M) to 9.10% (1M), being significant low in all cases. The duration of the hydrolyses was varying from 22 hours (1M NH_4OH) to 27 hours (2M NH_4OH and 4M NH_4OH).

The hydrolysis of compounded PHB pellet was also conducted using the same concentrations of NH_4OH solutions, with a duration of around 30 hours in all experiments. The maximum fraction of PHB degradation (64.96%) was noticed in the case where 4M NH_4OH solution was used, and the lowest in the case of 1M NH_4OH solution (33%). The ratio of the 3HB and CA monomers was similar to the previous experiments where different specimens of PHB were used.

The degradation of the extrusion molded PHB-based cup was the one that lasted the longest, 47 and 51 hours. The concentrations of NH_4OH solutions used, were the same as in the previous experiments. 1M NH_4OH led to the highest fraction of degradation (40.53%), and 4M solution led to the lowest (35.61%). Same as in every degradation conducted using NH_4OH, CA fractions were very low. Thus, the ratio of the 3HB and CA monomers was low as well.

Table 5 Alkaline degradation of different PHB specimens using different concentrations of NH4OH solutions at 90°C

	Concentration (M)	Reaction duration (h)	Total recovery of 3HB and CA(%)	3HB (%)	CA (%)	Ratio 3HB:CA
Powder	1	30	51.40	46.20	5.20	0.11
	2	38	67.51	62.31	5.20	0.08
	4	38	68.59	63.39	5.20	0.08
Solvent-casted PHB film	1	22	73.56	64.46	9.10	0.14
	2	27	74.19	67.69	6.50	0.08
	4	27	62.99	59.09	3.90	0.07
Compounded PHB pellet	1	30	33.00	28.50	4.50	0.16
	2	30	50.61	44.61	6.00	0.13
	4	30	64.96	61.96	3.00	0.05
Extrusion molded PHB-based cup	1	51	40.53	36.93	3.60	0.10
	2	47	36.69	33.09	3.60	0.11
	4	51	35.61	29.62	6.00	0.20

The degradation of different forms of PHB and under several alkaline conditions has been investigated in literature before. Yu et al., (2005) conducted alkaline hydrolysis in PHB precipitates, native granules and solvent-casted films using 0.1M, 1M, 2M and 4M NaOH solutions at 70ºC. The hydrolysis lasted 4 hours in every case. The smallest fraction of PHB recovery was noticed in the case that PHB precipitate was degraded with 0.1M solution, less than 5%, while the biggest (more than 70%) was achieved in the case of granules and precipitate by using 4M NaOH solution. In the case of solvent-casted films, the biggest fraction of PHB decomposition, around 45%, was achieved after 4 hours of hydrolysis using 4M NaOH solution, a fraction smaller than the one found in this study. However, the mass ratio CA:3HB indicated in every case was 0.42-0.68, which comes to an agreement with the results of this work.

Tapadiya & Vasanthan, (2017) investigated the hydrolysis of solvent-casted PHB films using 1M, 1.5M and 2M NaOH solutions, at 30ºC, 35ºC and 40ºC. The duration of the hydrolyses was 90-100 hours. After 90h at 30ºC, 20%, 26% and 35% of PHB film was degraded using NaOH solution 1M, 1.5M and 2M respectively. The concentration of the base affects the extent of the hydrolysis. Thus, their next experiments were conducted using 2M base solution. From their results, the higher the temperature, the biggest the fraction of PHB degradation that was achieved, starting from 35% degradation at 30ºC, reaching up to 90% at 40ºC, within 100 hours. The last fraction was bigger than the respective found in this work, but within the triple time.

PHB degradation experiments were carried out also by Myung et al., (2014), by base-catalyzed pyrolysis. Firstly, hydrolysis of the copolymer PHBV (Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid)) was conducted in aqueous base (pH 13) at 60ºC, resulting to the production of 51.7% 3HB, 37.8% CA, and other by-products (3HV (3-hydroxyvalerate) ,2P (2-pentenoate), 3P (3-pentenoate)), achieving total 98.1% decomposition within 18 hours. Moreover, thermal decomposition of commercial PHB was studied using two different temperature profiles, resulted in 55% and 69% PHB recovery.

Table 6 Results from alkaline degradation of PHB reported in literature-cited publications .

Conditions	Reaction Duration (h)	Recovery (%)	3HB (%)	CA (%)	Others (%)	Reference
PHB precipitate, 0.1M NaOH, 70ºC	4	<5	<5	<5	-	(Yu et al., 2005)
PHB precipitate, 1M NaOH, 70ºC	4	<15	<10	<5	-	(Yu et al., 2005)
PHB precipitate, NaOH 2M, 70ºC	4	<35	<20	<20	-	(Yu et al., 2005)
PHB precipitate, 4M NaOH, 70ºC	4	>70	<50	<30	-	(Yu et al., 2005)
Solvent-casted film, 2M NaOH, 40ºC	100	90	nk	nk	nk	(Tapadiya & Vasanthan, 2017)
PHBV, 60ºC, pH 13	18	98.1	51.7	37.8	3HV:4.9 2P:3.6 3P: 0.1	(Myung et al., 2014)
Commercial PHB, Pyrolysis	nk	55	nk	nk	nk	(Myung et al., 2014)
Commercial PHB, Pyrolysis	nk	69	nk	nk	nk	(Myung et al., 2014)

4.2 Shake flask fermentations for PHB production

Batch and fed-batch shake flask fermentations were carried out using the PHB hydrolysate produced after the alkaline degradation of PHB as carbon source. The composition of PHB hydrolysate after the degradation of PHB using 0.6M NaOH was 61% 3HB and 39% CA. The bacterial strain *Paraburkholderia sacchari* was employed in both fermentations. Figure 17 a and b,

show the concentration of total carbon source, the production of dry cell weight and the accumulation of PHB during the batch and fed-batch fermentation respectively.

Initially, a batch fermentation was carried out with an initial carbon source concentration of 9.58 g/L. The accumulation of PHB begins at around 5 hours simultaneously with the growth of bacteria. The dry cell weight at 28h was 3 g/L with a PHB content of 50%. The yield of the fermentations was 0.17 g/g (Table 5).

In the case of fed-batch fermentation, the initial concentration of the carbon source was 8.97 g/L, adding 4.29 g/L extra carbon source at around 15 hours. The total dry weight after 30 hours was around 2.4 g/L with a PHB content of 75%. The accumulation of PHB is much higher than the previous case, while the yield of the fermentation is 0.136 g/g (Table 5).

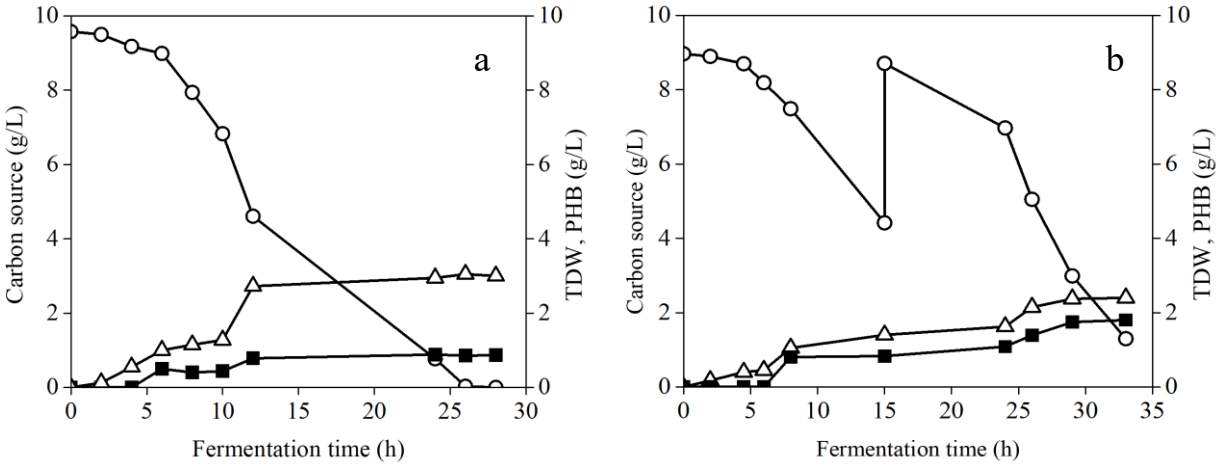


Figure 17 Total carbon source (3HB+CA) (○), TDW (△) and PHB (■) in batch (a) and fed-batch (b) shake flasks fermentations for the production of PHB by *P. sacchari* using PHB hydrolysate after alkaline degradation with 0.6M NaOH solution as carbon source

Several studies have been conducted investigating the ability of different strains of *P. sacchari* to accumulate PHB using various carbon sources. Zoghbi et al., (2023) in their work carried out batch and fed-batch shake flasks fermentations with *P. sacchari* (DSM 17165) using, among others, glucose and 3HB as carbon source. From their results, 3HB was consumed as

efficiently as glucose by the microorganism. In batch fermentation, starting with 10.8 g/L glucose, 2.2 g/L PHB were produced, with TDW 6.3 g/L and yield 0.21 g/g. In the case that 9.8 g/L of 3HB were used as sole carbon source, a bit lower PHB production (1.9 g/L) and TDW (4.9 g/L) were noticed. The fed-batch fermentation starting with 5.6 g/L 3HB and adding 5.5 g/L 3HB, resulted in 2.5 g/L PHB production, 6.5 g/L TDW and 0.26 g/g yield, higher than in the previous cases.

The same strain of *P. sacchari* (DSM 17165) was also utilized by Cesário et al., (2014), for growing and PHB production using glucose, xylose, and their mixture as carbon source. Batch fermentations starting with 10 g/L of glucose and 10 g/L of xylose resulted in similar PHB production (2.2 g/L and 2.4 g/L respectively) and yield (0.26 g/g and 0.24 g/g respectively). When the mixture of glucose and xylose (10 g/L + 10 g/L) was used, glucose was firstly consumed and a delay of xylose consumption was noticed, achieving 4.4 g/L of PHB production with a similar yield like before (0.25 g/g).

Xylose was also used as sole carbon source in shake flask fermentation for PHB production by a different strain of *P. sacchari* (IPT101) from Lopes et al., (2009). Batch fermentation was conducted starting with 15 g/L xylose, obtaining a bit lower value than Cesário et al., (2014), with 2.99 g/L PHB and 5.48 g/L TDW. The yield was the same 0.24 g/g.

Table 7 Shake flask fermentations for PHB production efficiency using 3HB and CA as carbon source in shake flask cultures of *P. sacchari* reported in literature-cited publications.

Carbon source	Consumed carbon source (g/L)	TDW (g/L)	PHB (g/L)	PHB content (%)	Yield (g/g)	Reference
PHB hydrolysate	9.58	3	1.6	50.0	0.168	This study
PHB hydrolysate	8.97 + 4.29	2.4	1.8	75.0	0.136	This study
		Literature-cited results				
Glucose	10.8	6.3	2.2	35.2	0.21	Zoghbi et al., 2023
Commercial 3HB	9.8	4.9	1.9	38.0	0.20	Zoghbi et al., 2023
Commercial 3HB	5.6 + 5.5	6.5	2.5	39.0	0.26	Zoghbi et al., 2023
Glucose	10	5.0	2.2	44.0	0.26	Cesário et al., 2014
Xylose	10	5.2	2.4	46.7	0.24	Cesário et al., 2014
Glucose + Xylose	10 + 10	7.4	4.4	58.9	0.25	Cesário et al., 2014

Xylose	15	5.48	2.99	54.61	0.24	Lopes et al., 2009

5. Conclusion

This study demonstrated the alkaline degradation of specimens of PHB (PHB powder, solvent-casted PHB film, compounded PHB pellet and extrusion molded PHB-based cup) using various concentrations of NaOH and NH_4OH solutions, for the production of the monomers 3HB and CA. The hydrolysate derived from the alkaline degradation of PHB powder using 0.6M NaOH solution, was utilized as carbon source for *P. sacchari* (DSM 17165) cultivation, and the microorganism was evaluated for its ability to consume the carbon source and accumulate PHB in batch and fed-batch shake flask fermentations, aiming to circular PHB production.

From the experiments that took place, the following conclusions were drawn:

- The degradation of PHB in any form by NaOH solutions, can efficiently degrade PHB, producing the monomers 3HB and CA.
- NaOH solutions in concentrations higher than 0.3M can totally degrade 20 g/L of PHB powder with the ratio of the produced monomers (CA:3HB) varying from 0.5 to 0.63.
- Higher concentrations of PHB solutions need higher concentrations of NaOH solutions in order to increase the recovery during the PHB degradation.
- In the case of 40 g/L PHB the highest recovery (93.45%) was achieved by using 0.6M NaOH solution with the ratio CA:3HB being 0.66, while in the case of 60 g/L PHB powder the total recovery was 94.20%, using 0.9M NaOH solution and the ratio CA:3HB was 0.65.
- The degradation of solvent-casted PHB using NaOH solutions was not as efficient as in the case of the PHB powder. Higher PHB recovery was achieved only when using 2M (92.21%) and 4M (90.74%) NaOH solutions.
- A total degradation of 20 g/L compounded PHB pellet and extrusion molded PHB-based cup was achieved, by using 1M, 2M and 4M NaOH solutions, with the ratio of the produced monomers (CA:3HB) varying from 0.52 to 0.58 and 0.55 to 0.61 respectively.

- During the degradation of PHB by NH_4OH solutions a fraction of an unknown monomer is produced in addition to the 3HB and CA monomers. The concentration of the unknown monomer seems to increase during the degradation.
- The hydrolysate derived from the degradation of PHB powder using NaOH solution, was effectively consumed by *P. sacchari* (DSM 17165) in batch shake flask fermentation resulted in 3 g/L total dry weight (TDW) and 50% PHB accumulation with 0.168 g/g yield.
- *P. sacchari* (DSM 17165) can efficiently consume the PHB hydrolysate produced after NaOH degradation as carbon source, resulting in 2.4 g/L TDW, 75% PHB content and 0.136 g/g yield.

In conclusion, NaOH solutions in concentrations higher than 0.3M could effectively degrade every form of PHB resulting in the production of the monomers 3HB and CA with a ratio CA:3HB=0.52-0.65. In addition, NH_4OH solutions can be successfully used as an alternative for the alkaline degradation of PHB. The produced hydrolysate contains expect from the 3HB and CA monomers, an unknown for now monomer which should be determined by LCMS analysis. Finally, *P. sacchari* (DSM 17165) can efficiently consume the PHB hydrolysate produced after alkaline degradation using NaOH solution, with more than 50% PHB accumulation.

6. Future work

In future research, there are several avenues to explore in the field of PHB and its utilization. The identification and the characterization of the unknown monomer produced during NH_4OH degradation of PHB should be a priority in the next experiments. The study of this unknown monomer presents a unique challenge and an opportunity to expand our understanding of PHB degradation pathways. Furthermore, the use of the PHB hydrolysate produced after the degradation of the rest forms of PHB, as potential carbon sources in microbial fermentations holds promise. This could shed light on the suitability of these hydrolysates for sustainable bioprocesses and their potential as renewable feedstocks for biotechnology applications. Finally, it is imperative to investigate alternative methods of PHB degradation, like enzymatic degradation or other chemical processes. Exploring novel enzymatic approaches can lead to efficient and eco-friendly strategies for PHB recycling and biodegradation. These future research directions not only contribute to our knowledge of PHB and its applications but also have the potential to drive innovation in the fields of biotechnology and sustainable materials.

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